Improved HPTLC separation of lipids by using Automated Multiple Development (AMD) and Identification with TLC-MS Interface

HPTLC in Basel, Juli 2011

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Aim

- **Development of optimized chromatographic separation method**

  of various lipid substances of Stratum corneum and of lipids in cosmetics - AMD in the focus

- **Optimization of existing methods based on HPTLC/ AMD**
  - **shorter analysis time**
  - **less solvent consuming**

    existing methods are very time consuming and cost intensive concerning the amounts of solvents

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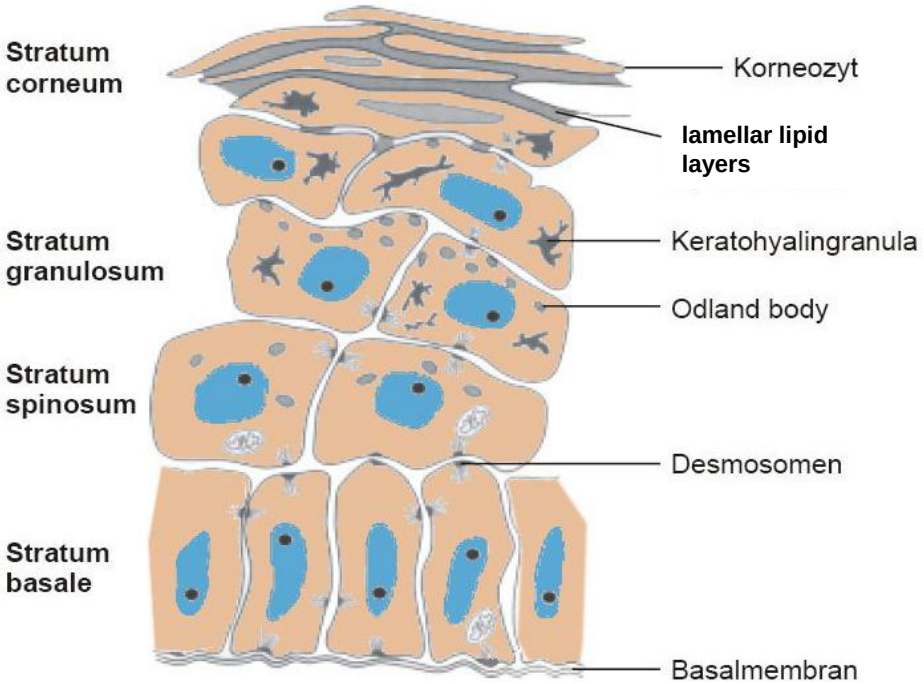
Skin lipids

The Stratum Corneum

- represents outer skin layer
- structured like a brick wall
- has unique lipid composition



Constitution of epidermis

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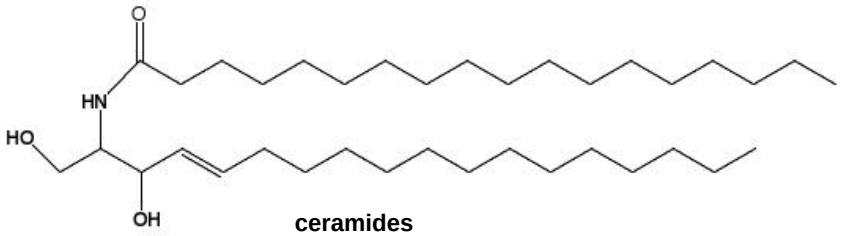
Skin lipids

Lipids	Percentage of SC-lipides
Ceramides	41 %
Cholesterol	27 %
Cholesterol ester	10 %
Fatty acids	9 %
Cholesterol sulfate	2 %
other	11 %

Composition of SC-lipids, [Wertz et al, 1991]

Ceramides

- sphingolipids consisting of sphingoid bases
- amide-linked to fatty acids with long chained hydroxylated sphingoid bases
- play a key role in maintaining the barrier function of the skin
- protecting the skin against excessive water loss
- various skin deseases (atopic dermatitis, psoriasis) have been reportet to be related to impairments in the ceramide profile



ceramides

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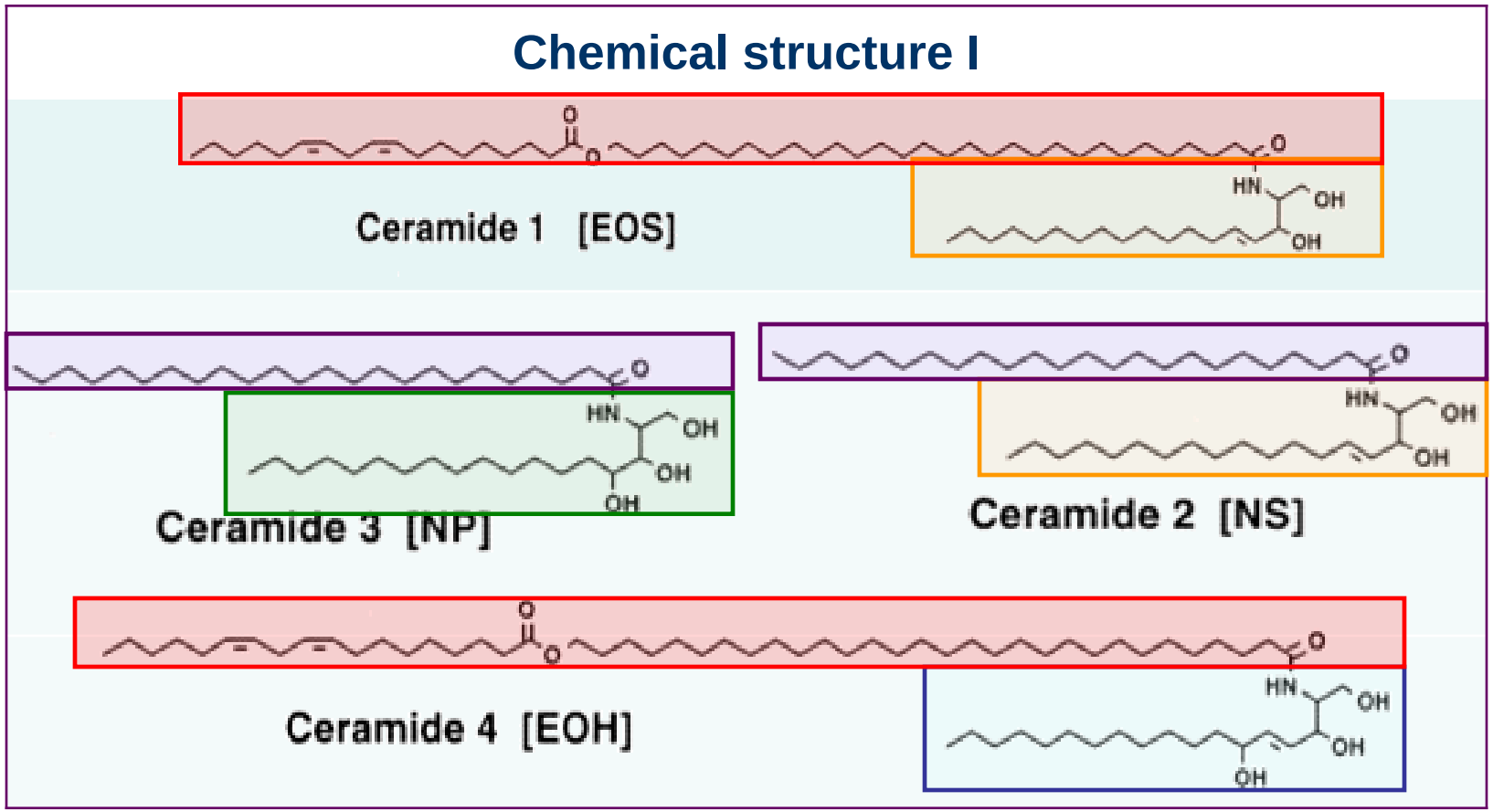
Ceramide classes

description of ceramides
from Motta et al. [1993]
and Robson et al. [1994]

Abbr.	Meaning
S	Sphingoid base = Sphingosin
P	Sphingoid base = Phytosphingosin
H	Sphingoid base = 6-Hydroxy-Sphingosin
A	**amide-like bounded FA** in α-position hydroxylated
N	… in α-position NOT hydroxylated
O	… in ω-position hydroxylated
E	… an ω-position ester-like bounded with other FA



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description of ceramides from Motta et al. [1993] and Robson et al. [1994]

Ceramide classes

Overview shows: very few differences between the various ceramides in polarity

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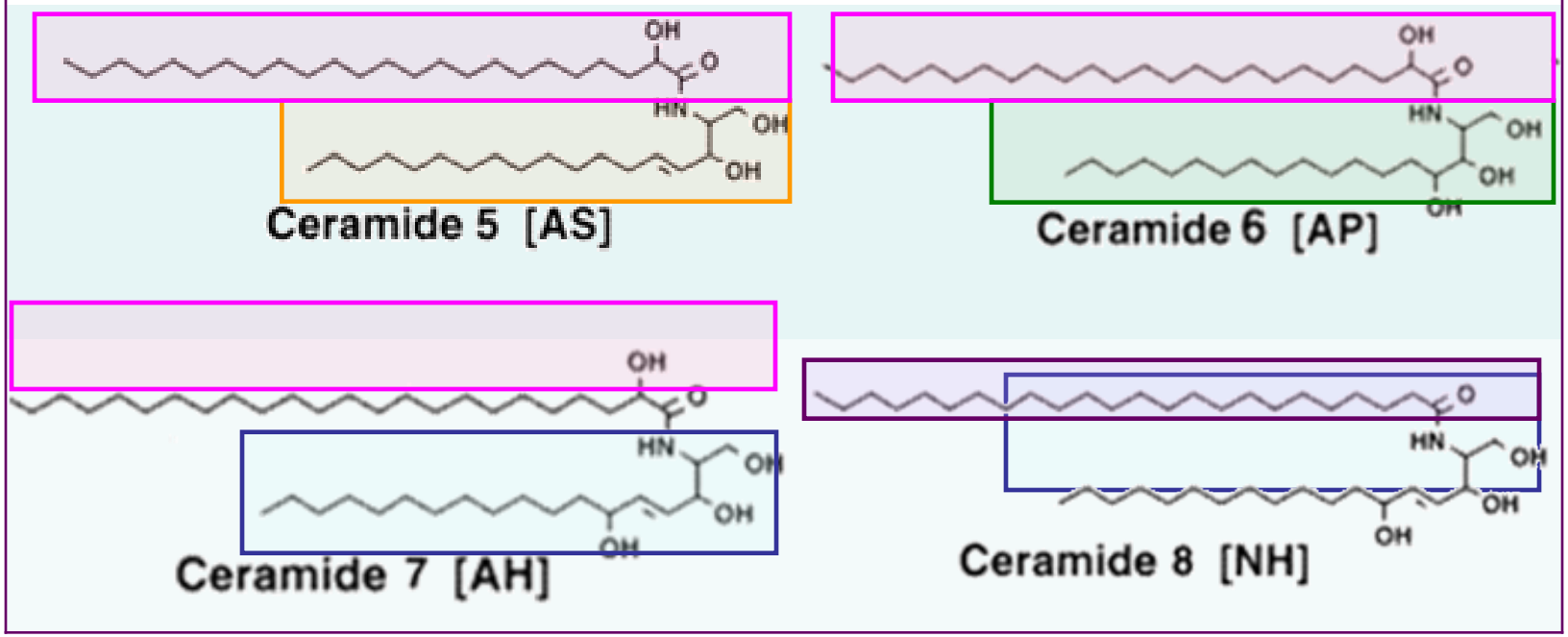
Chemical structures II



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HPTLC - AMD

- Necessity of high resolution of chromatography
  - because of low polarity differences
- Automated multiple development (AMD)
  - higher separation performance
  - better separation → higher resolution

- Identifying by using TLC-MS Interface

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Working stages and equipment in HPTLC

Sample preparation

Pre-washing/conditioning of stationary phase twice with chloroform:methanol (2:1)

Sample application

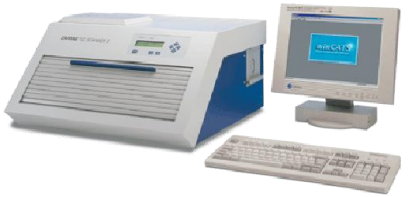
ATS 4

Chromatograph. development

mobile phase

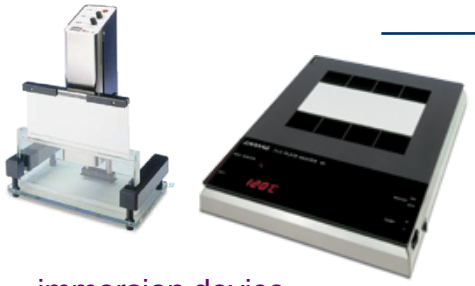
Derivatization

Detection



TLC-Scanner 3

Documentation



immersion device
and plate heater III

Reprostar 3

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Working stages in HPTLC

Sample preparation

Pre-washing/conditioning of stationary phase

Sample application

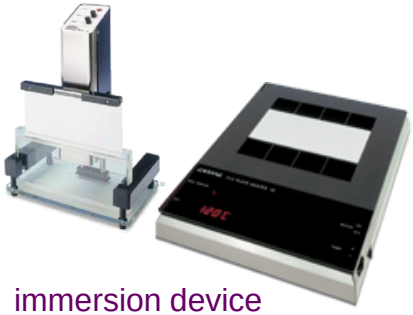
ATS 4

Automated Multiple Development

Detection

TLC-Scanner 3

Documentation



immersion device
and plate heater III

Reprostar 3

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AMD2 – developing chamber

- **Using this method thin layer is developed with a reproducible stepwise elution gradient from polar to unpolar (e.g. 8 to 30 steps)**

→ eich run extends over a longer solvent migration distance than the one before

→ from step to step the solvent front increases about 1- 5 mm

→ over a distance of 80 mm, up to 40 components can be completely resolved

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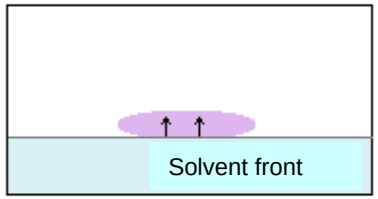

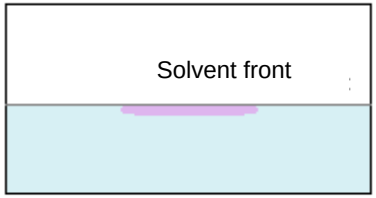

Advantages of AMD:

- **exact focussing effect**
- **separation over a wide range of polarity**
- **separation of substances with few differences in polarity**
- **software controlled gradient ensures a high reproducibility**

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Investigations

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Standard substances used

Lipid standard substances			
cholesterol	C	Ceramide NS	NS
glyceryl trioleate	GT	Ceramide NP	NP
cholesteryl oleate	CO	Ceramide AS	AS
oleic acid	OA	Ceramide AP	AP
squalene	SQ	cholesteryl-3-sulfate	C3S

- ceramidescNP and AP are recemic mixtures, resulting from synthesis of D- and L-forms of these compounds

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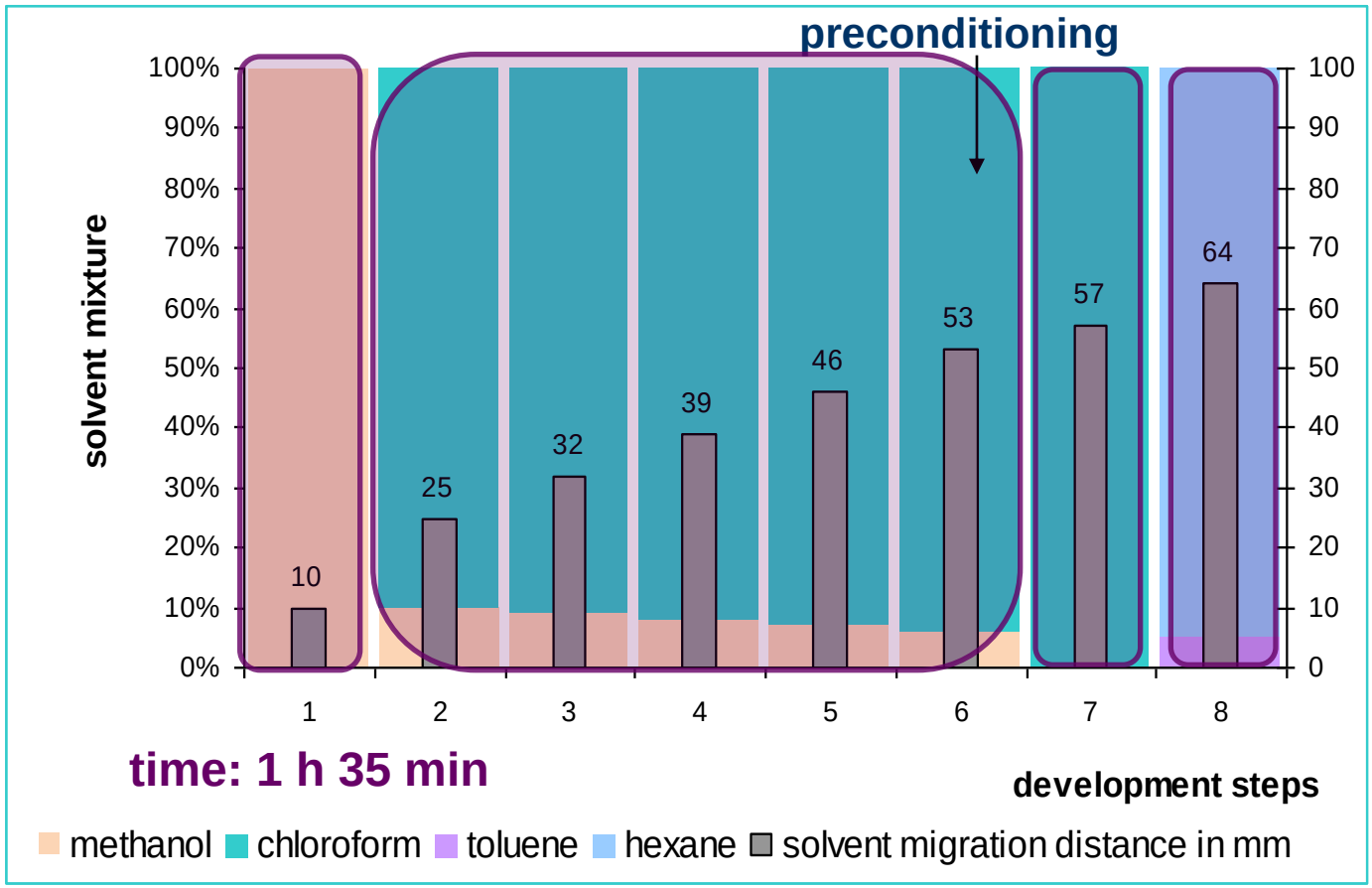
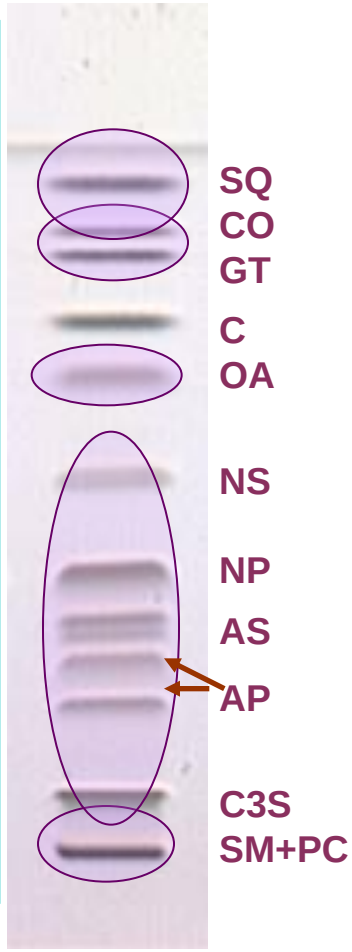
conditions of chromatography

- stationary phase: HPTLC glass plates 20 x 10 cm (silica gel 60 F_{254}, 0.1 mm, Merck)
- pre-washed twice with chloroform : methanol (2:1; v/v) and activated for 30 minutes at 120 °C in a drying oven
- application: strip wise (8 mm bands), 5 µl of single standards and standard lipid mixture using automatic TLC sampler
- derivatization: using a TLC chromatogram immersion device with a mixture of 10 % cooper sulfate in 8 % phosphoric acid with subsequent drying (170^0C for 8 min.) on a TLC plate heater
- detection: scanner and video system

Gradient with 8 steps and 4 solvents (IBAS)

8-steps gradient and chromatogram





SQ	squalene	CO	cholesteryl oleate	GT	glyceryl trioleate
PA	palmitic acid	C	cholesterol	OA	oleic acid
NS	Ceramide NS	NP	Ceramide NP	AS	Ceramide AS
AP	Ceramide AP	C3S	cholesterol-3-sulfate	SM	sphingomyeline
PC	phosphatidylcholine				

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Gradient with 8 steps and 4 solvents

Densitogram and chromatogram for the 8-step gradient

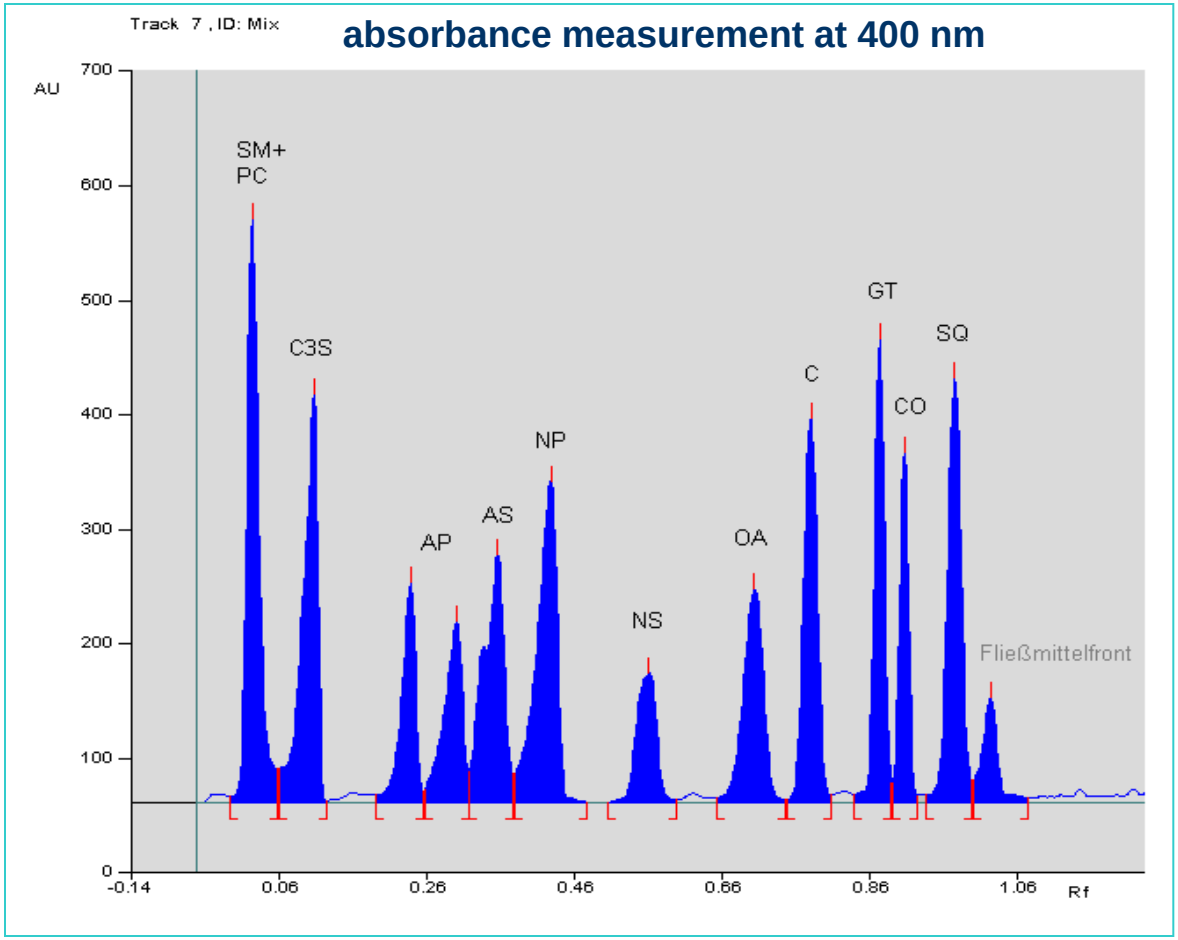

Image of plate under white light

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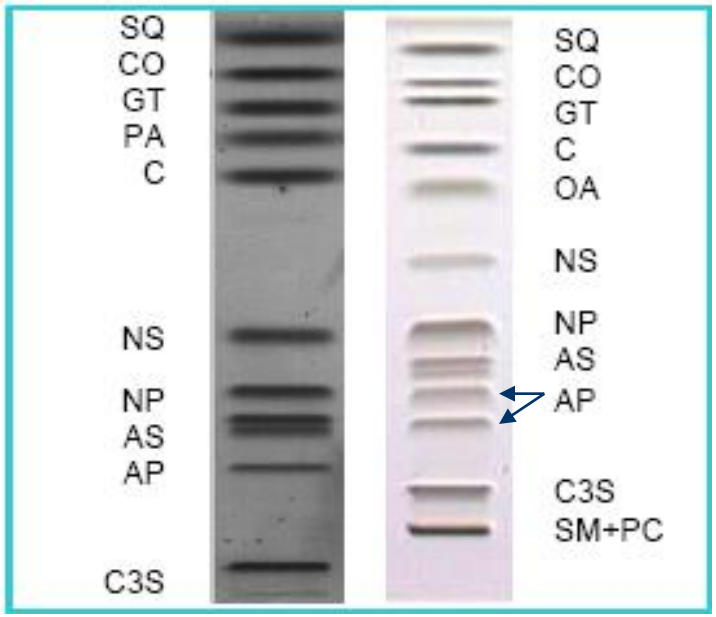
Comparison of chromatograms by Farwanah [2002] and by Schellenberg et al. [2008]

Farwanah [2002]:
- 17 steps
- 5 solvents
- solvent migration distance: 68 mm
- 2 h 30 min

Derivatisation:
150 °C, 20 min



Schellenberg [2008]:
- 8 steps
- 4 solvents
- solvent migration distance: 64 mm
- 1 h 35 min

Derivatisation:
170 °C, 8 min

SQ	squalene	CO	cholesteryl oleate	GT	glyceryl trioleate
PA	palmitic acid	C	cholesterol	OA	oleic acid
NS	Ceramide NS	NP	Ceramide NP	AS	Ceramide AS
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PC	phosphatidylcholine				

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Method validation

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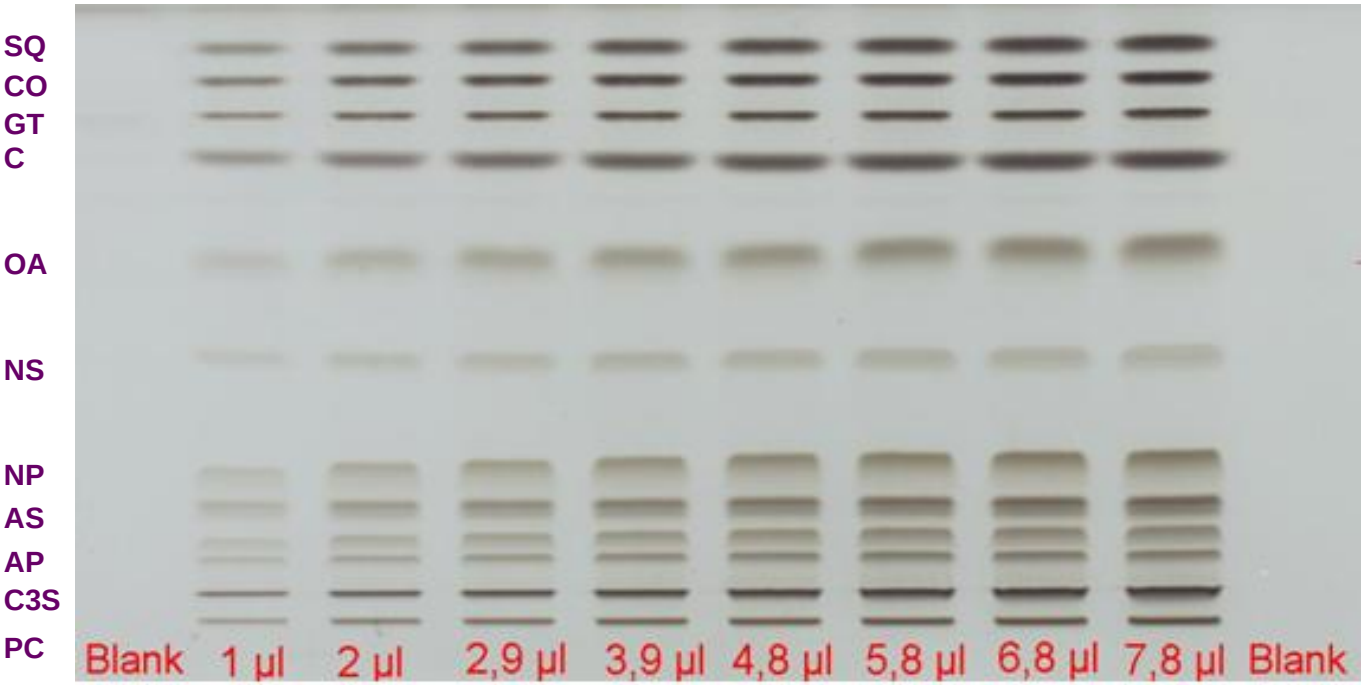
Method validation

level		1	2	3	4	5	6	7	8
sample	blank	standard mix							
application volume	5 µl	1 µl	2 µl	2,9 µl	3,9 µl	4,8 µl	5,8 µl	6,8 µl	7,8 µl
concentration [µg/µl]	0	0,515	1,03	1,49	2,01	2,47	2,98	3,5	4,01



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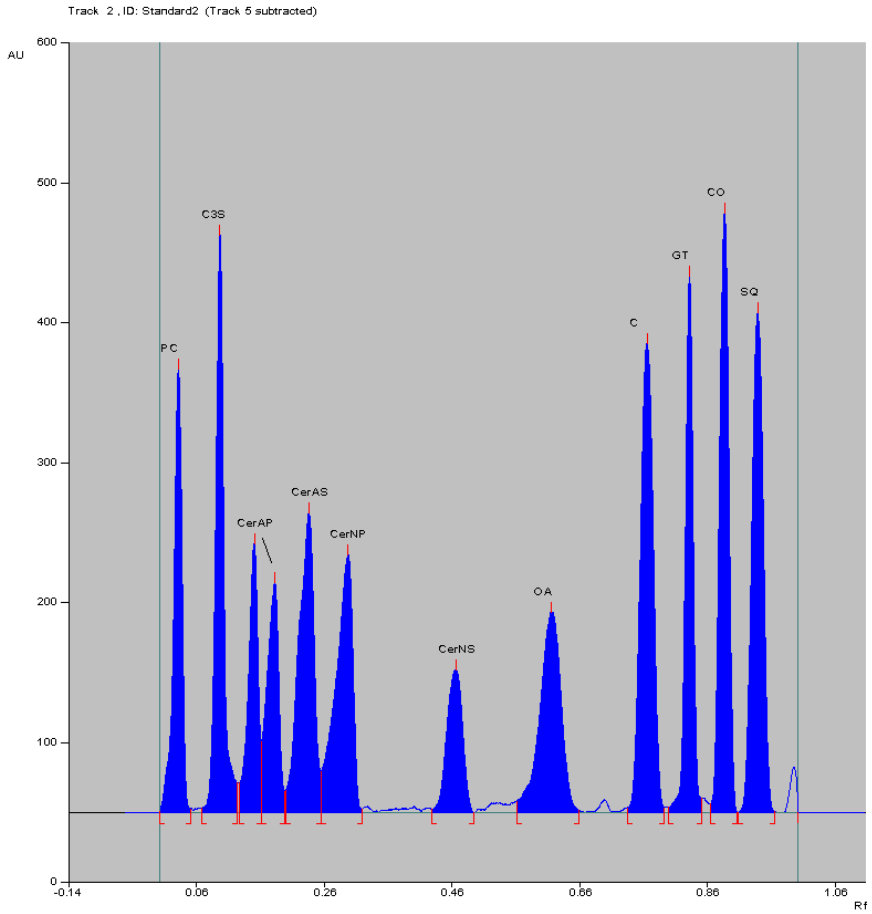
Method validation

- after development and derivatisation the absorption of lipids was measured at 400 nm
- blank tracks were subtracted
- resulting peak areas were processed by using statistical software for quality asscurance
- all regression equations were quadratically
- estimation of LOD and LOQ according to DIN 32634

  (DIN= German Institution of standardization)



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Example: Calibration function of Cholesterol

lipid	concentration in ng	peak area from 10 plates for 8 concentration level										AV	COV
		1	2	3	4	5	6	7	8	9	10		
C	140	9784	12354	10075	11233	11919	12451	13110	12906	11497	12690	11801,90	9,76
	280	14693	18143	15007	16998	17232	16907	18060	18027	17460	19077	17160,40	8,03
	406	18651	22231	18624	21594	20847	21355	22004	22292	21409	23723	21273,00	7,46
	546	21363	25512	20907	24434	24282	24061	24975	26103	24711	27387	24373,50	8,09
	672	24147	27051	23404	26190	26359	26391	28107	28639	27705	30367	26836,00	7,66
	812	26657	29796	25724	26873	28067	27805	29958	31439	30111	33336	28976,60	8,18
	952	28607	31995	27617	28127	29114	29810	32223	33964	32134	36081	30967,20	8,93
	1092	30710	33046	28808	29565	30756	31585	33792	36011	34049	36905	32522,70	8,29

determination of outliers by using Grubb‘s test

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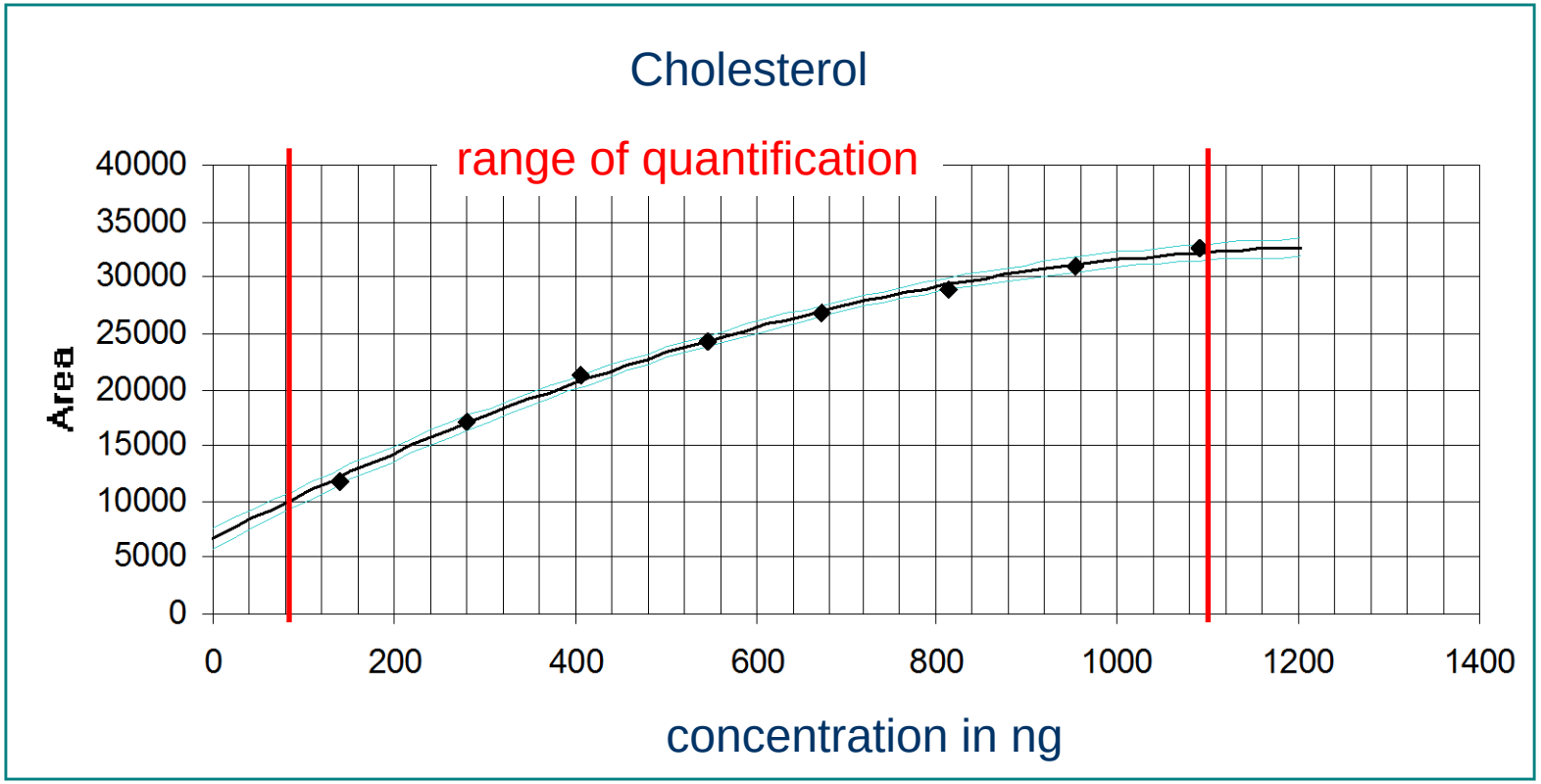
Example: Calibration function of Cholesterol



LOD: 25,58 ng **LOQ: 76,74 ng**

range of quantification for Cholesterol: from 76,74 ng to 1092 ng

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Method validation

- correlation coefficient of calibration functions:
  0,97014 – 0,99977

- LOQ:
  from 8,65 ng (Cer NP)
  to 366,01 ng (Cer AP)

lipid	range of quantification in ng	
	from	to
C	76,74	1092
CO	46,35	780
GT	86,42	1170
OA	15,41	702
PC	48,25	780
SQ	64,48	1560
C3S	212,03	1560
CerAS	28,76	936
CerNS	125,10	2730
CerAP	366,01	2730
CerNP	8,65	936

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PA	palmitic acid	C	cholesterol	OA	oleic acid
NS	Ceramide NS	NP	Ceramide NP	AS	Ceramide AS
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PC	phosphatidylcholine				

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Extraction of skin lipids

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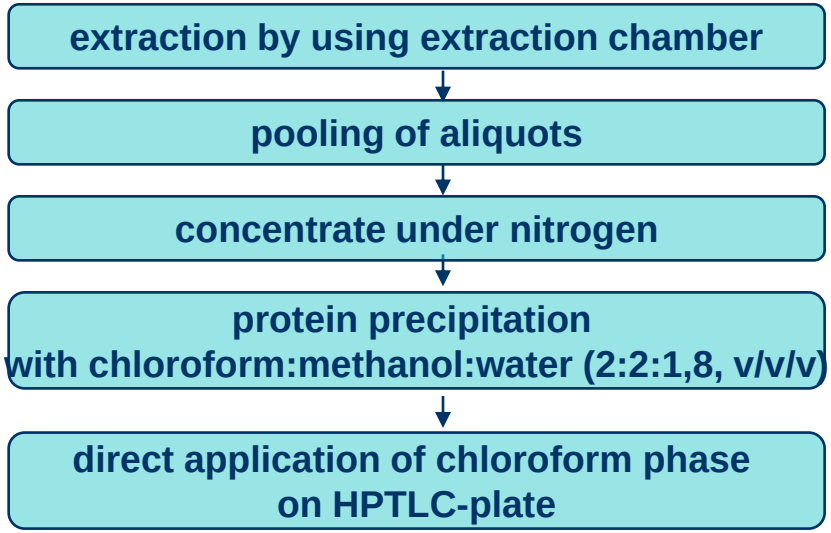
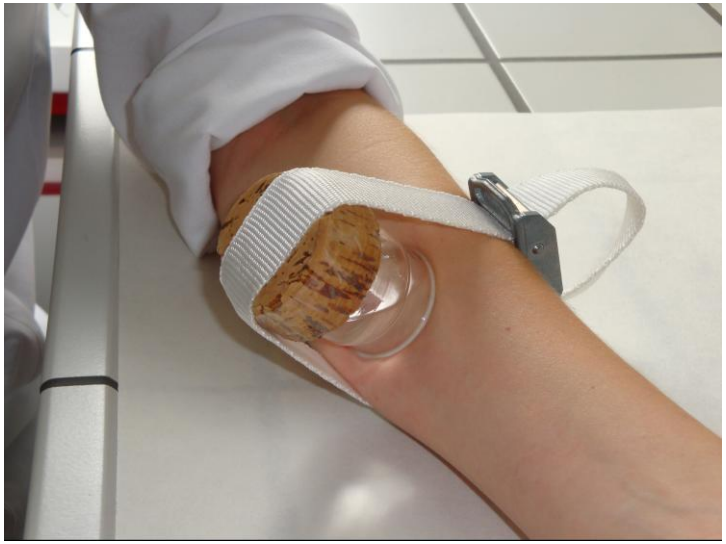
Extraction of skin lipids

- **test persons:** 13 healthy people (34 ± 12,8 years), 1 person with psoriasis
- **extraction solvent:** n-hexane:ethanol (2:1, v/v)
- **skin area:** healthy skin area from forearm (sampling twice from both forearms results in 4 aliquots (test portions)





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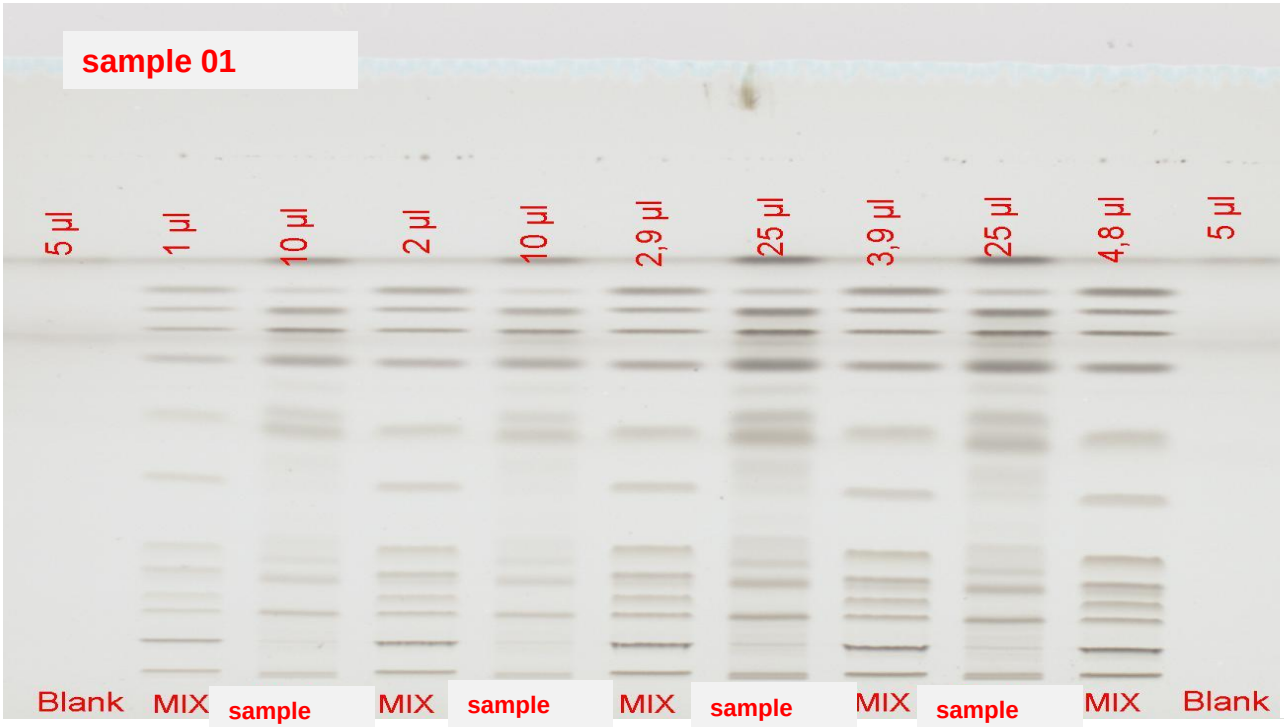
Application of real skin samples

- tracks: 2 blanks
  5 calibration levels (1 µl – 4,8 µl)
  skin sample with different application volumes



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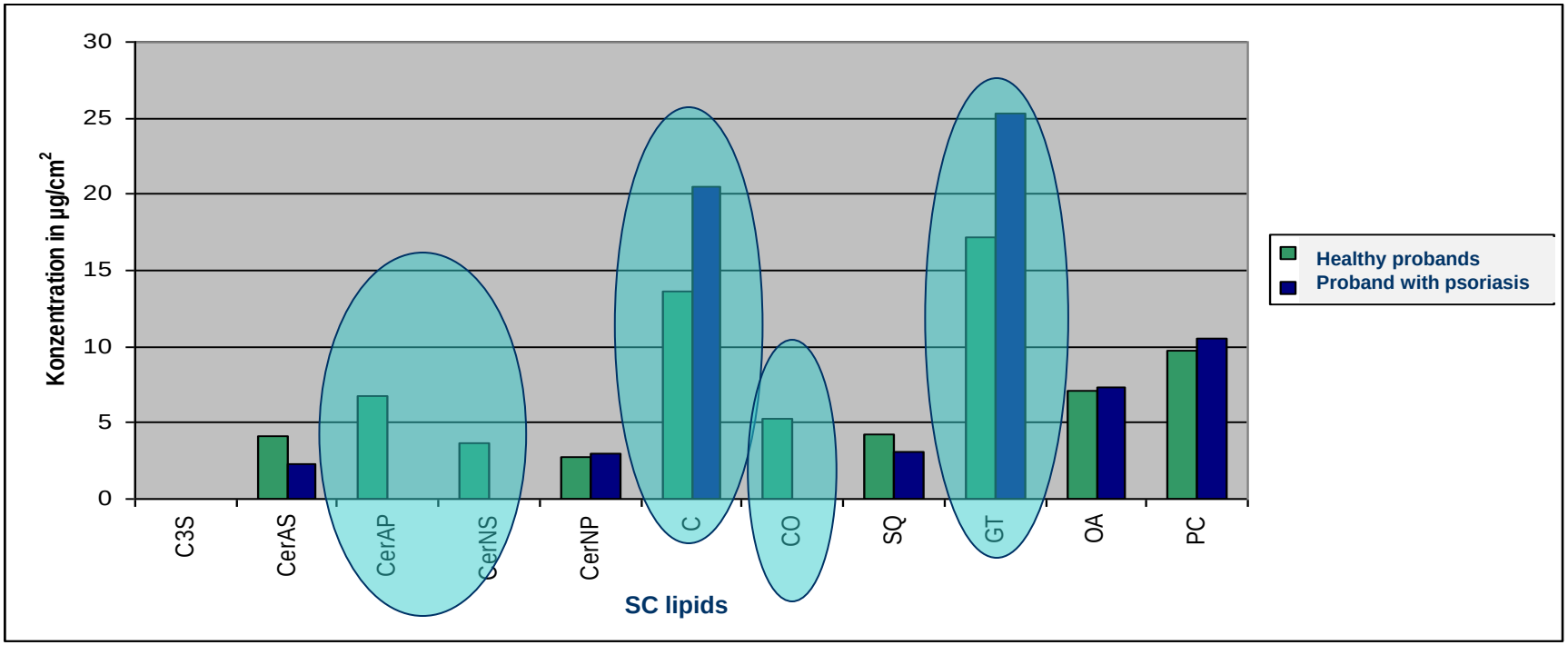
Results



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Identification of lipids by using TLC-MS Interface

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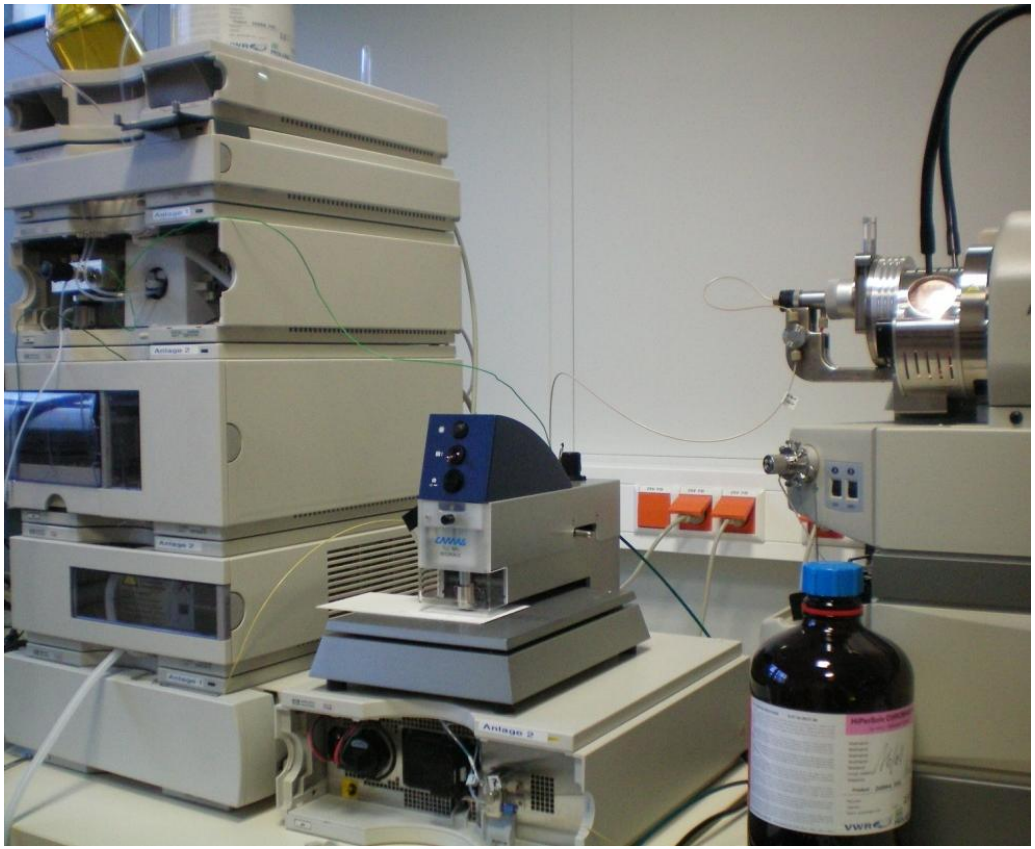
HPTLC-MS coupling by using TLC-MS Interface



Pump: Agilent, Serie 1100
solvent: methanol (2 mmol ammonium acetate)
flow rate: 0,1 ml/min

TLC/MS-Interface, CAMAG

MS: API 2000 with ESI source Applied Biosystems

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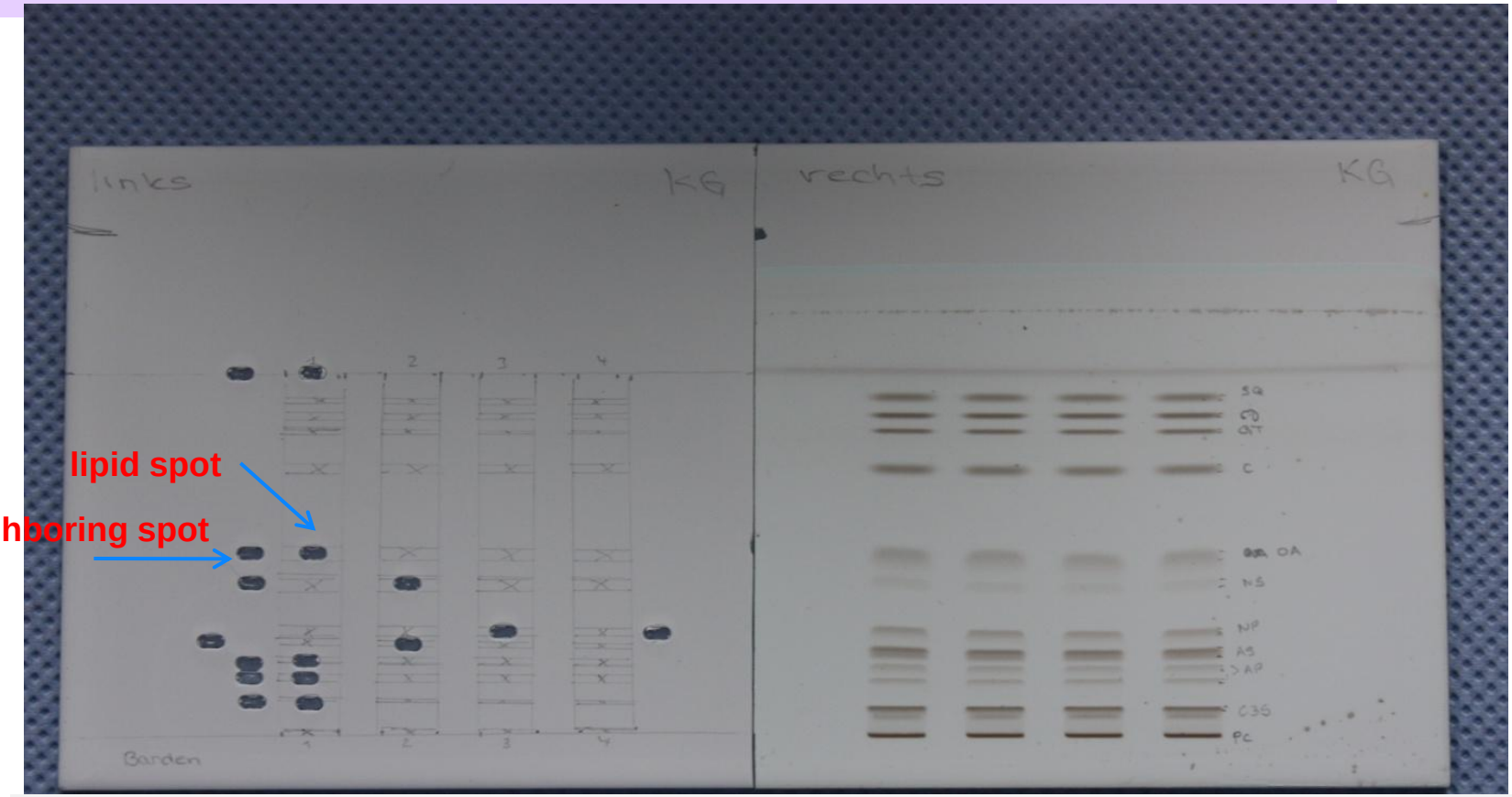
HPTLC-MS coupling by using TLC-MS Interface



after development plate was broken in two parts
right part → derivatisation
left part → transfer of substance positions from right side → mass spectra
important !! subtraction of mass spectra of neighboring spot

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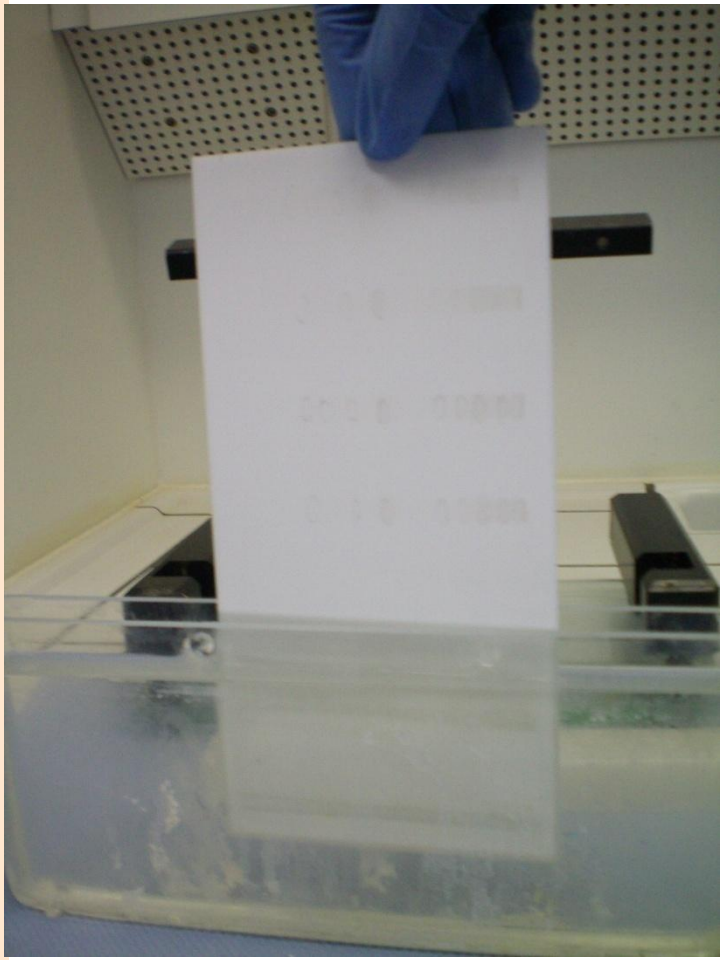
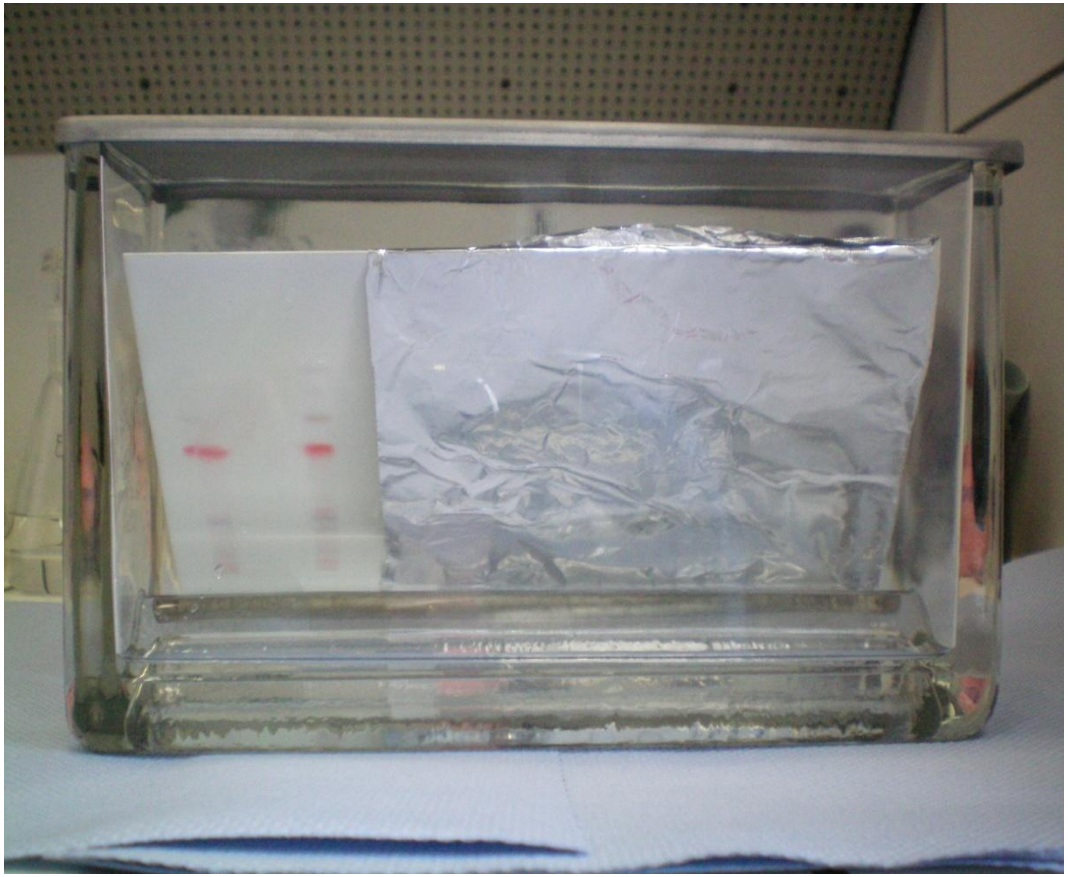
Derivatisation





derivatisation

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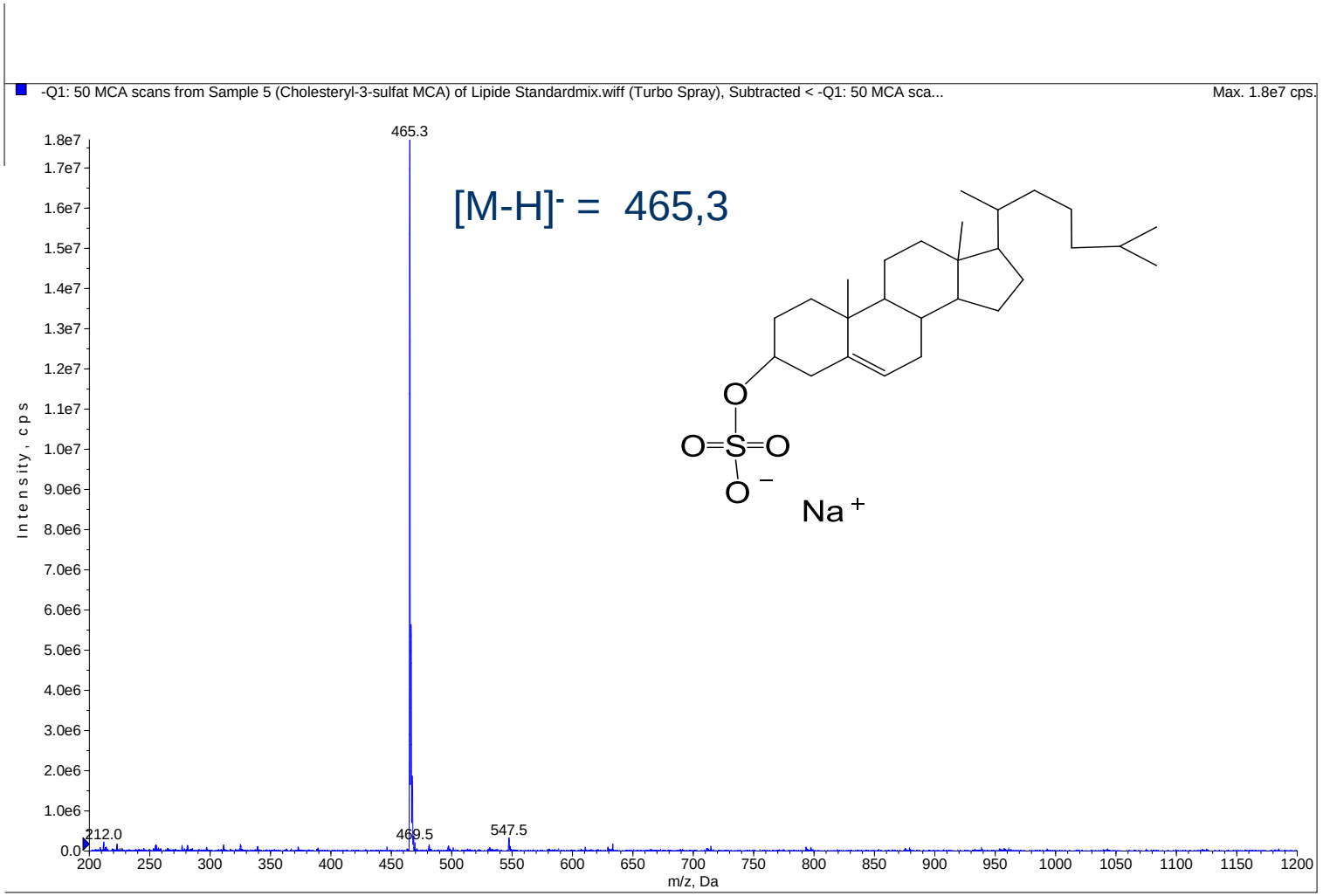

mass spectra of cholesterol -3-sulfate

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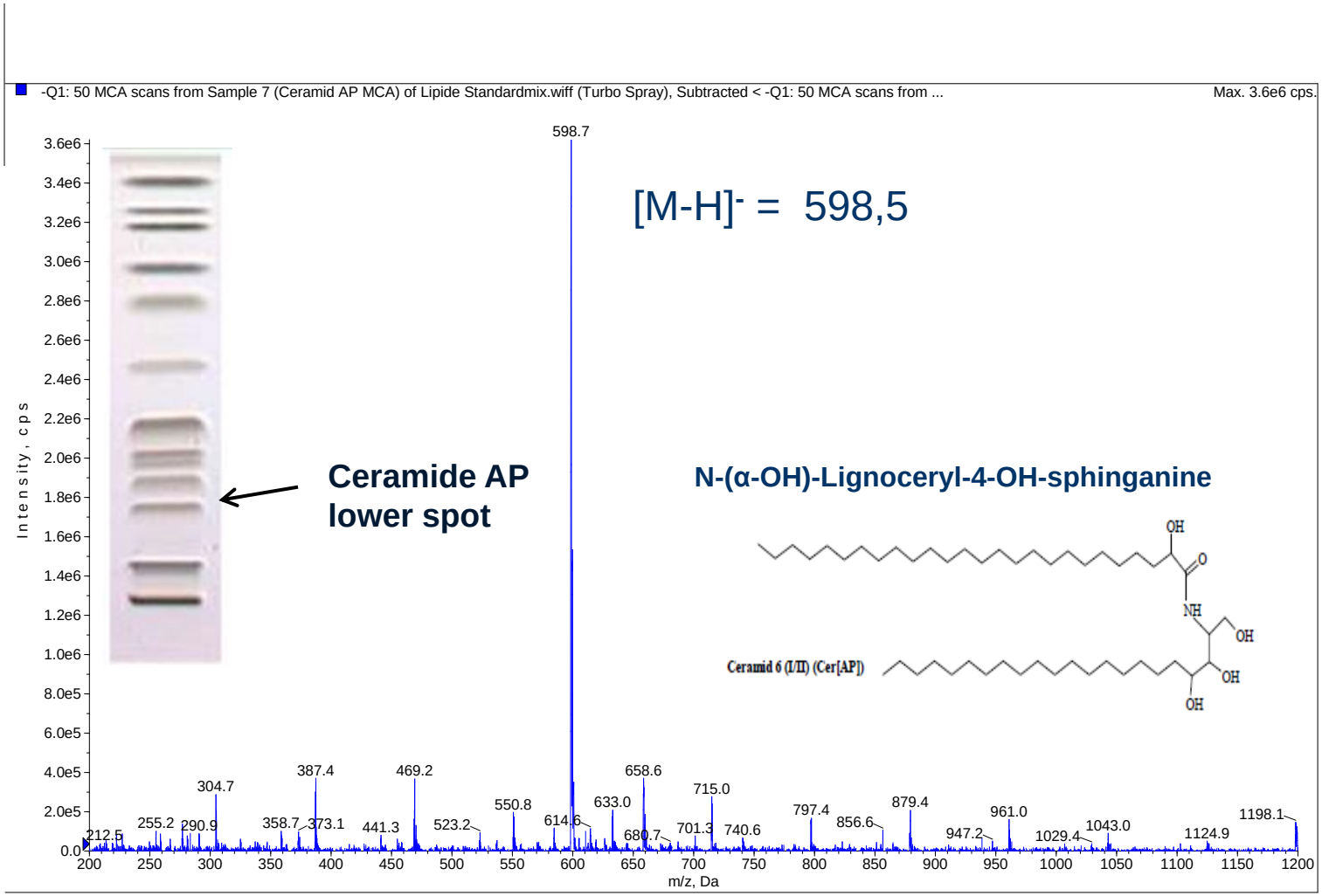

mass spectra of Ceramide AP: lower spot

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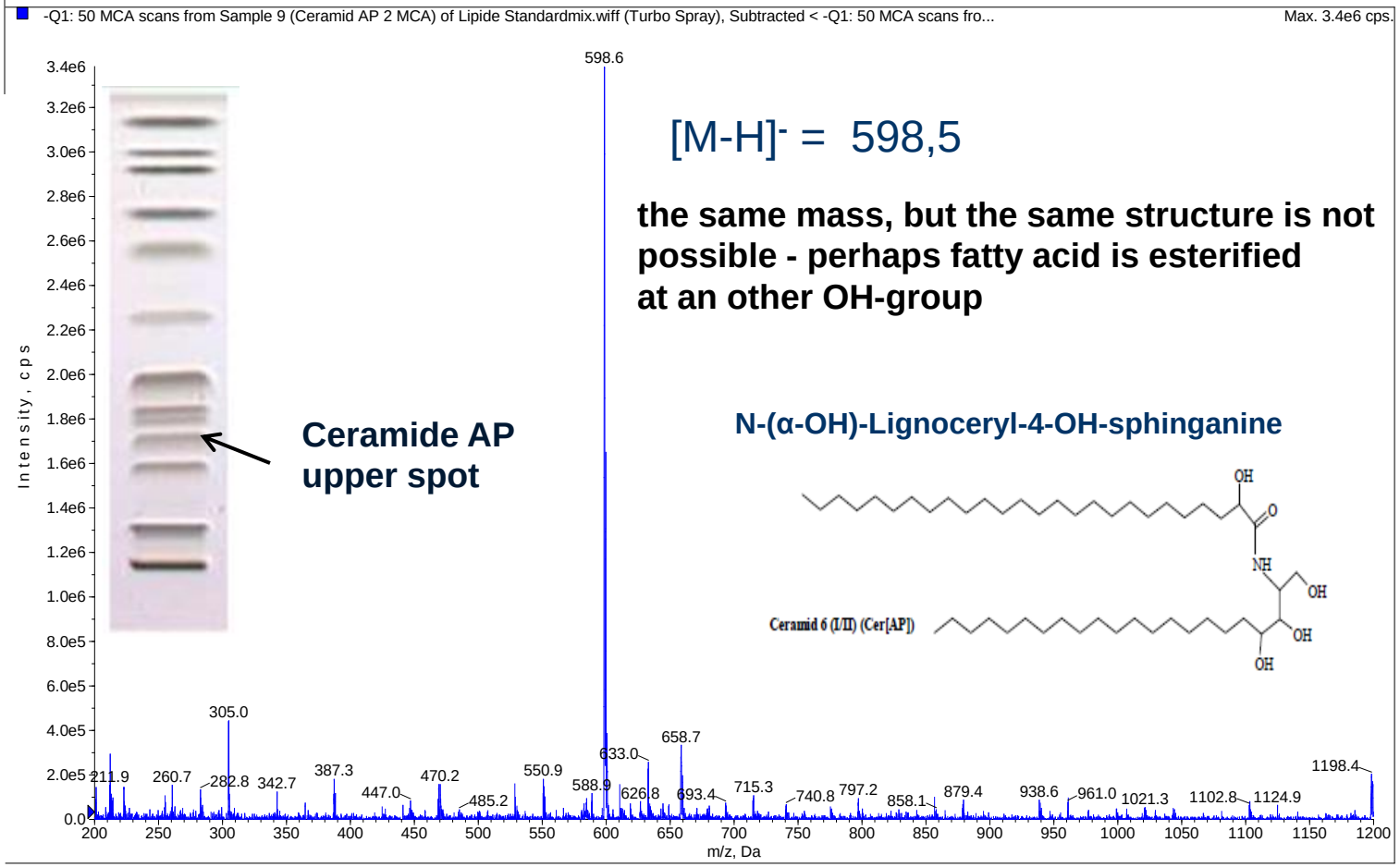
Identification of lipids by using TLC-MS Interface



mass spectra of Ceramide AP: upper spot

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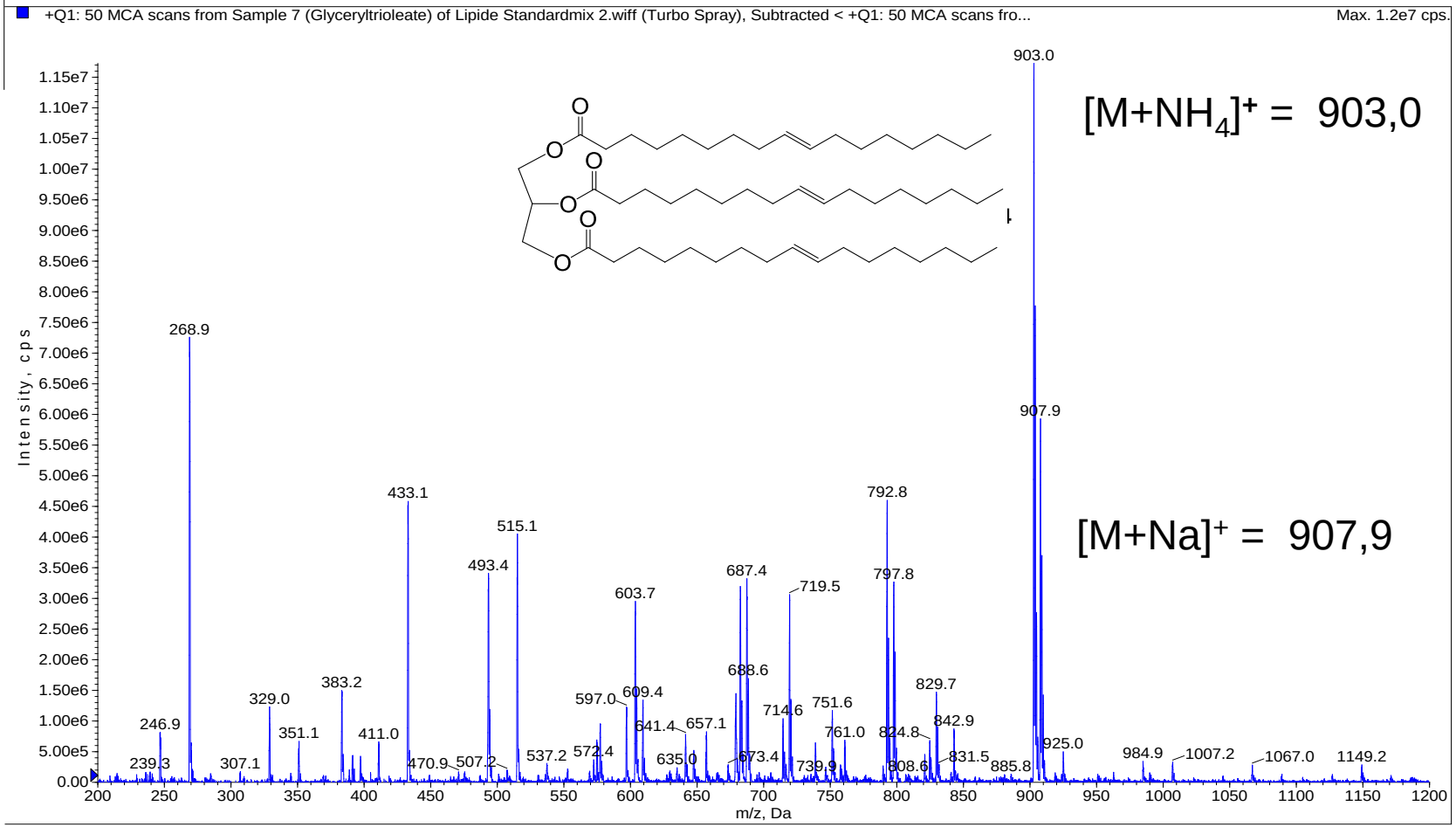

mass spectra of glyceryltrioleate

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Identification of lipids by using TLC-MS Interface

abbr.	name	pseudo molecular ion negative ionisation m/z	identified as
NS	Ceramide NS	$[M-H]^-$ = 566,7	N-Stearoyl-sphinganine
NP	Ceramide NP	$[M-H]^-$ = 470,5 $[M-H]^-$ = 580,7	N-Acyl—4-OH-sphinganine acyl-residue 1: oleic acid (C18:1) acyl-Residue 2: capric acid (C10:0)
AS	Ceramide AS	$[M-H]^-$ = 664,6	N-(α-OH)-Lignoceryl-sphingosine
AP	Ceramide AP	$[M-H]^-$ = 598,5	N-(α-OH)-Lignoceryl-4-OH-sphinganine
C3S	Cholesterol-3-sulfate	$[M-H]^-$ = 465,3	
OA	Oleic acid	$[M-H]^-$ = 281,2	

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Identification of lipids by using TLC-MS Interface

abbr.	name	pseudo molecular ion positive ionisation m/z identified as:
CO	Cholesteryl - oleate	$[M+Na]^+$ = 673,6
GT	Glyceryl - trioleate	$[M+NH_4]^+$ = 903,0 $[M+Na]^+$ = 907,9
PC	Phosphatidyl-choline	mixture: PC with different acyl residues for example $[M+Na]^+$ = 782

- cholesterol and squalene could be identified by using APCI

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Prediction for the future

- proof of practicability of developed method as diagnostic tool for clinical investigation
- proof of practicability in investigation of cosmetic formulations

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