

Determination of non polar metabolites in human plasma using innovative sample preparation strategies coupled with gas chromatography and mass spectrometry

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Overview:

- The main objective of this work is to develop simple, fast and selective extraction methods to isolate non polar metabolites or contaminants from human plasma.
- Solid phase extraction using a novel adsorbant (MonoTrap™) was tested for direct extraction from diluted plasma samples or from the corresponding headspace.
- Both direct extraction and headspace extraction with MonoTrap™ revealed the presence of terphenyls and partially hydrogenated terphenyls in 39 human plasma samples.
- Dispersive liquid-liquid microextraction (DLLME) of plasma samples was tested using CCl₄ or using heptane. Conventional DLLME procedure was adapted to allow the use of non halogenated solvents.
- A new method of simultaneous derivatisation, extraction and concentration of plasma fatty acids is proposed. This method couples direct transesterification of plasma samples with methanolic KOH and homogeneous liquid-liquid microextraction (HLLME) with halogenated or non halogenated solvents. All steps take place in a single vial with an extraction time of 15 min or less.

Introduction:

- Metabolomics addresses the screening of compounds with medium to low molecular weight, with biological relevance as disease or exposure biomarkers [1].
- Sample preparation is a key step of the process, because biological fluids are complex samples often available at limited amounts (1-2 ml in the case of blood plasma).
- The metabolic profile of plasma samples includes free aminoacids, organic acids, amines, sugar, steroids, nucleic acid bases and other substances [2].
- The analysis of these metabolites by GC-MS requires the use of appropriated derivatization reactions.
- Non polar metabolites like aliphatic hydrocarbons have been determined in biological samples (breath samples) and related to lipid peroxydation processes occurring in cells of cancer patients or of smokers [3,4].

References

- [1] Harin Kanani, Panagiotis K. Chrysanthopoulos, Maria I. Klapa "Standardizing GC-MS metabolomics" Journal of Chromatography B, 871 (2008) 191-201
- [2] Kishore K. Pasikanti, P.C. Ho, E.C.Y. Chan "Gas chromatography/mass spectrometry in metabolic profiling of biological fluids" Journal of Chromatography B, 871 (2008) 202-211
- [3] Leiliane Coelho A. Amorim, Zenilda de L. Cardeal "Breath air analysis and its use as a biomarker in biological monitoring of occupational and environmental exposure to chemical agents" Journal of Chromatography B, 853 (2007) 1-9
- [4] Michael Phillips, Renee N. Cataneo, Andrew R.C. Cummin, Anthony J., Gagliardi, Kevin Gleeson, Joel Greenberg, Roger A. Maxfield, William N. Rom "Detection of Lung Cancer With Volatile Markers in the Breath" Chest 123 (2003) 2115-2123

Methods :

- Blood samples from 40 volunteers (20 smokers and 20 non smokers) were collected and the corresponding plasma was isolated and kept at -5°C until analysis.
- Plasma deproteinization was performed adding methanol and separating the precipitates by centrifugation.

- Solid phase extraction with MonoTrap™ DSC18 disks (GL Sciences, Japan)



Plasma samples (500µL) were diluted with distilled water and extracted with MonoTrap™ DSC18 disks using:

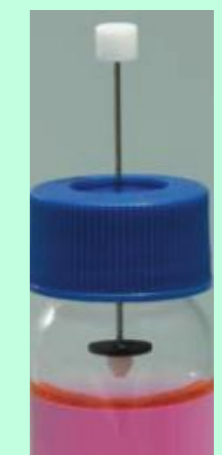
a) Direct extraction (DE)

MonoTrap™ disks are placed in direct contact with the plasma sample, at 60 °C, for 1h, under stirring.



b) Headspace extraction (HE)

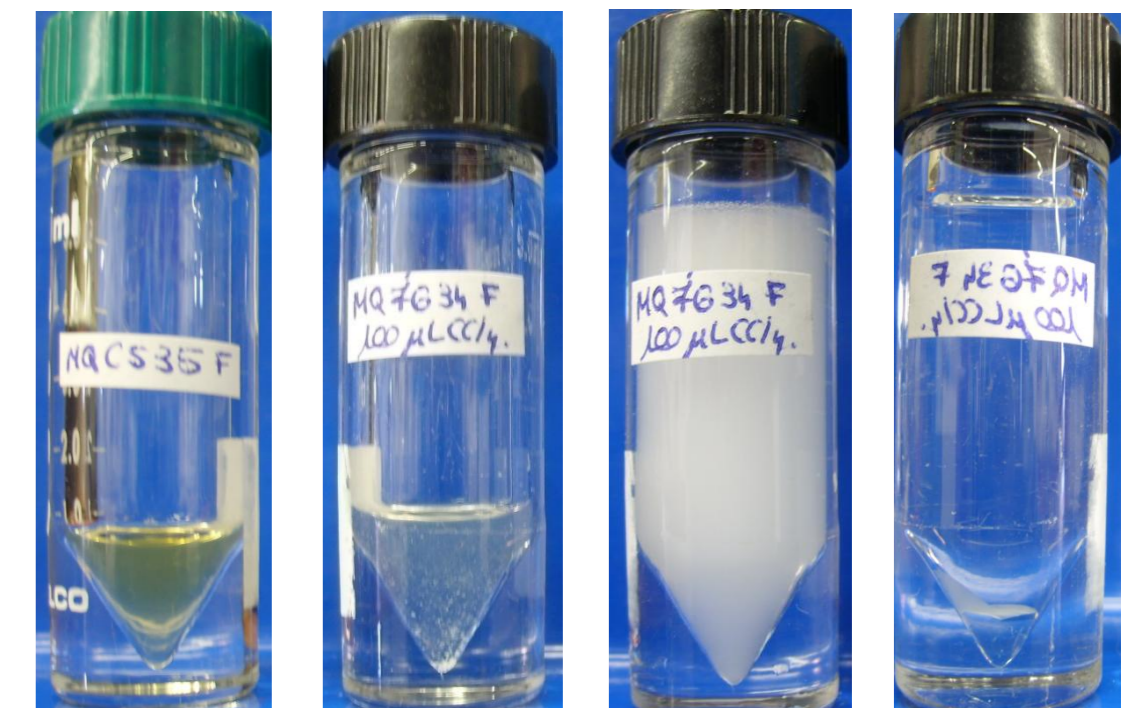
MonoTrap™ disks suspended in the headspace over the plasma sample, at 60 °C, for 1h, under stirring.



The SPE disks were extracted with dichloromethane, in an ultrasonic bath.

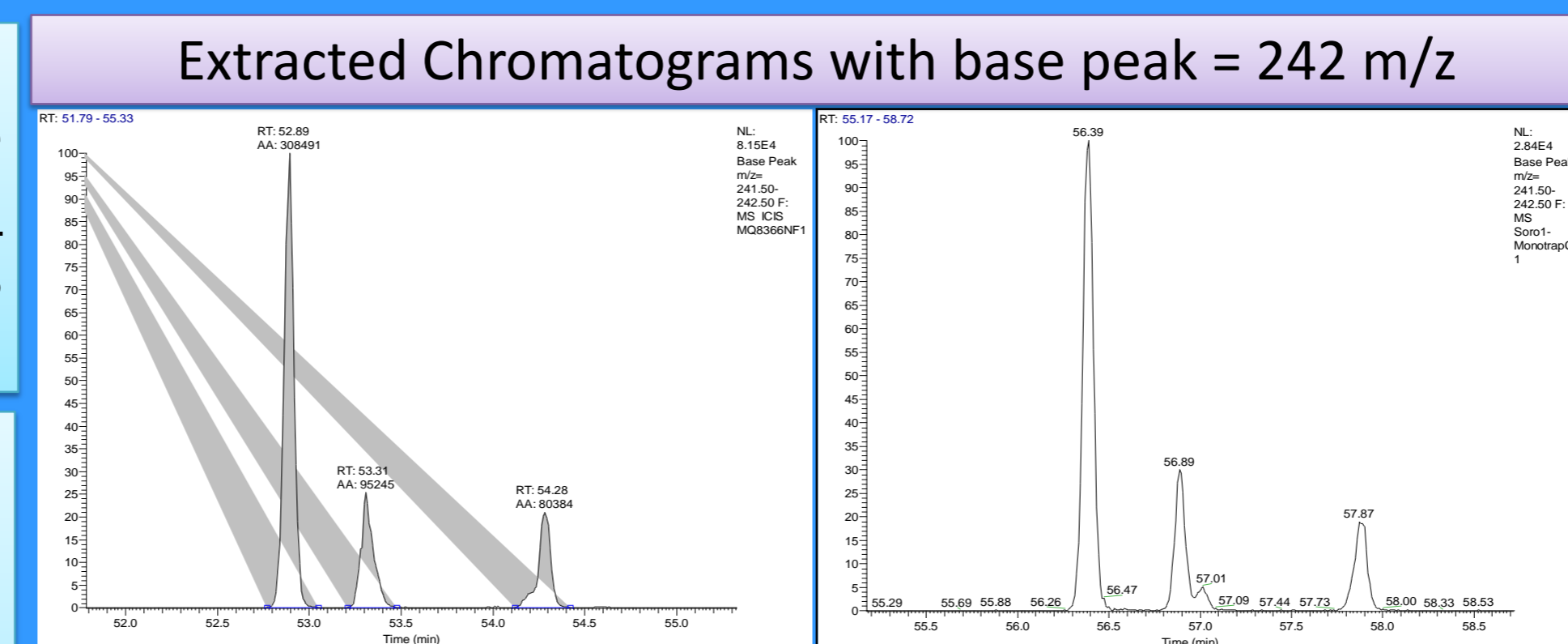
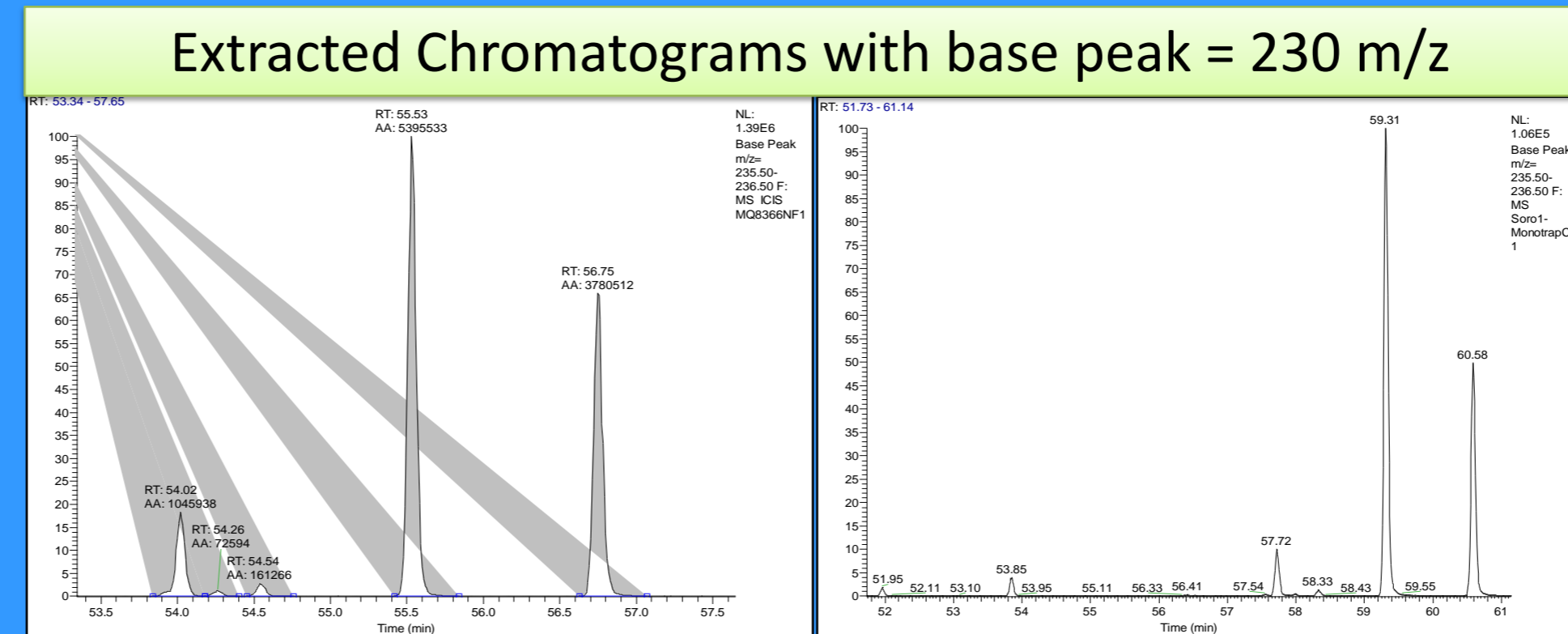
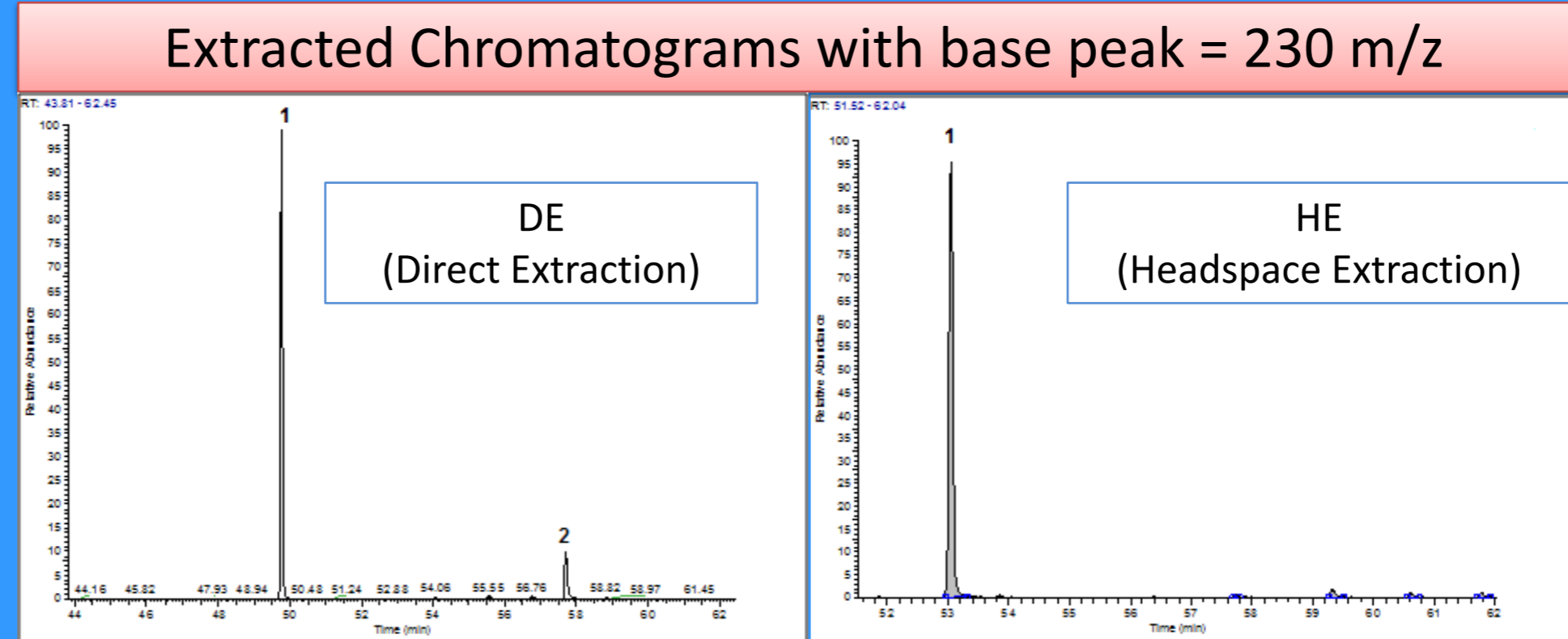
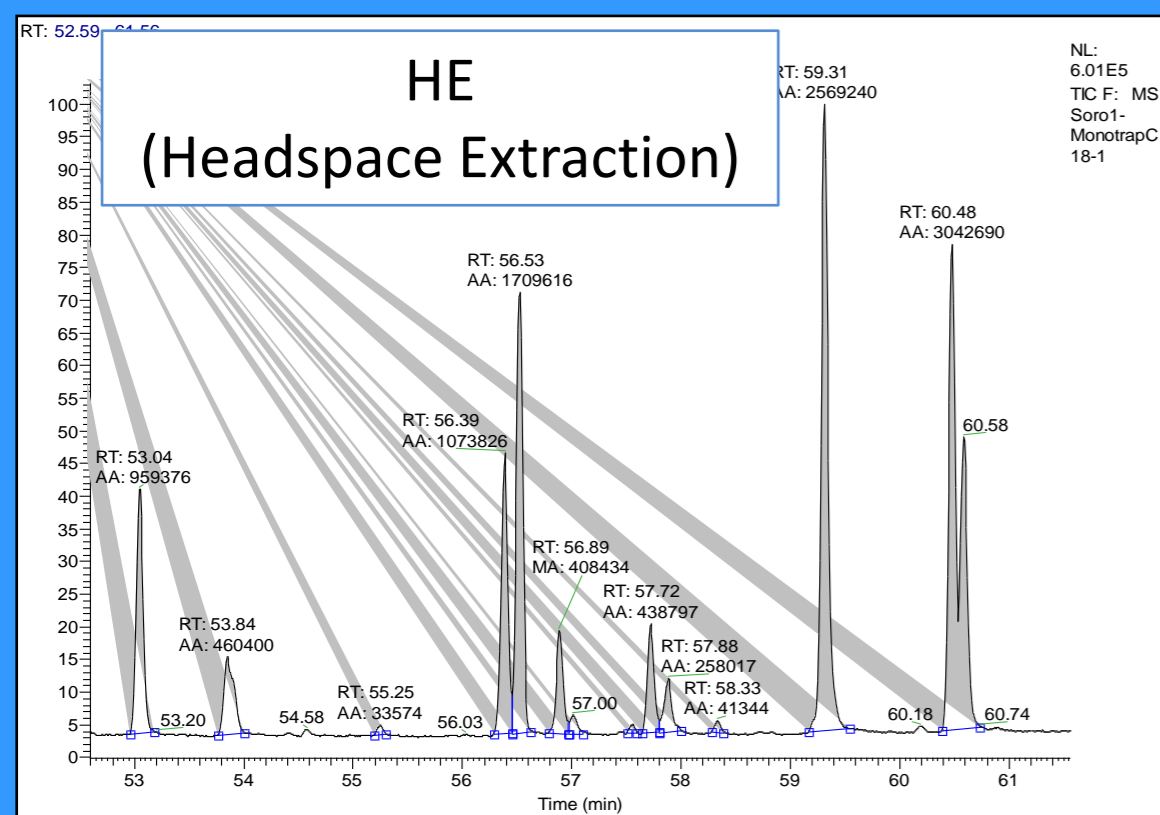
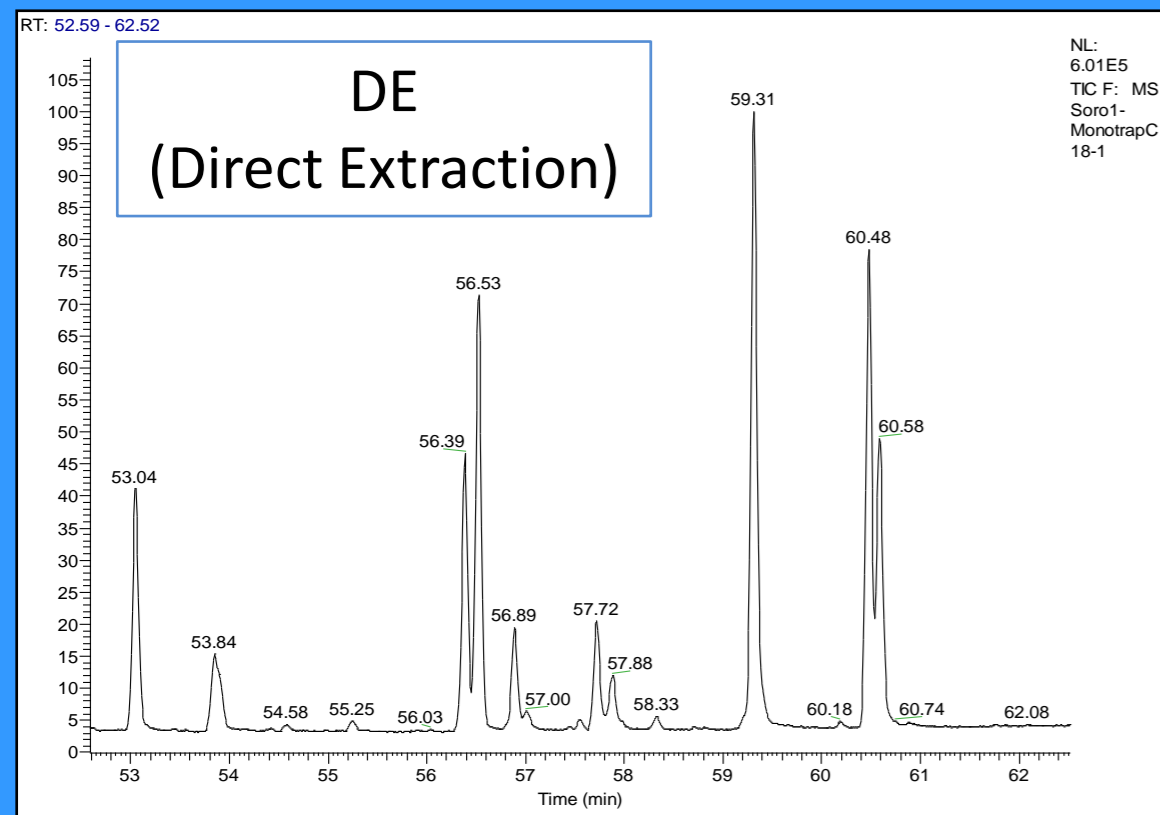
The extract was dried, concentrated to 200 µL and analysed by GC-MS.

- Homogeneous liquid-liquid microextraction coupled with direct transesterification (HLLME-DT): Plasma samples (500µL) were diluted with methanolic KOH; a solution of organic solvent CCl₄ or heptane (50 - 150 µL) in acetone was added to the reaction mixture and an homogeneous solution was obtained. Phase separation was induced by adding 2 ml of water. After centrifugation the organic phase was recovered and injected in a GC-MS-MS (Focus GC, Polaris Q, Thermounicam).

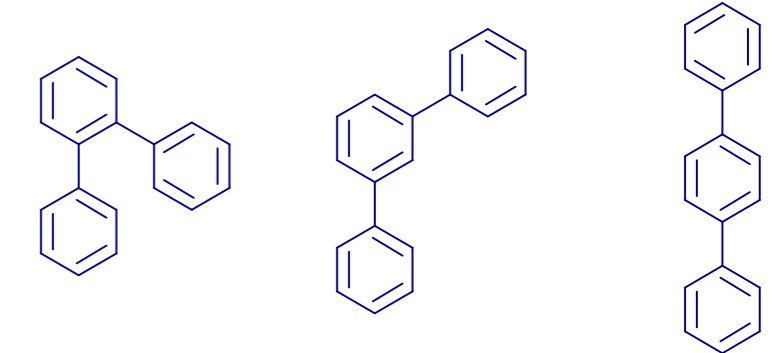


Results : Detection of terphenyls and hydrogenated terphenyls in human plasma

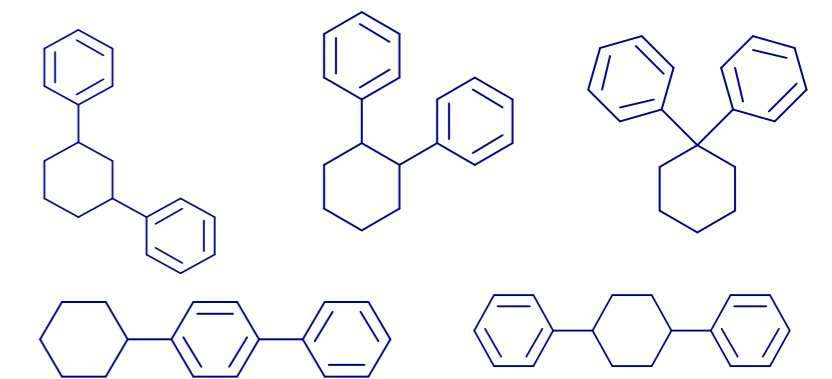
SPE with MonoTrap DSC18 disks (DE and HE) + GC-MS



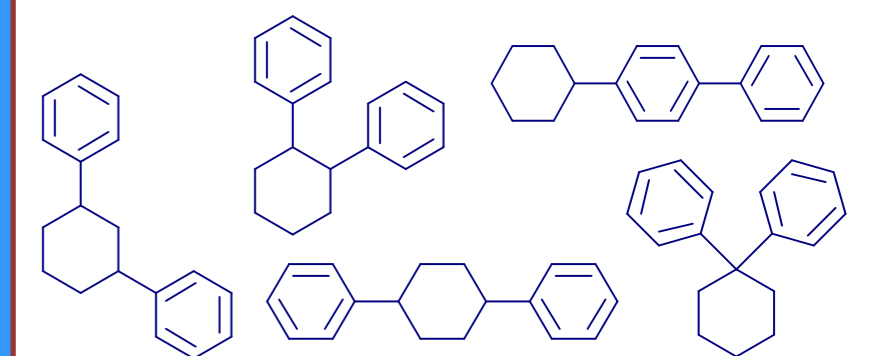
Terphenyls



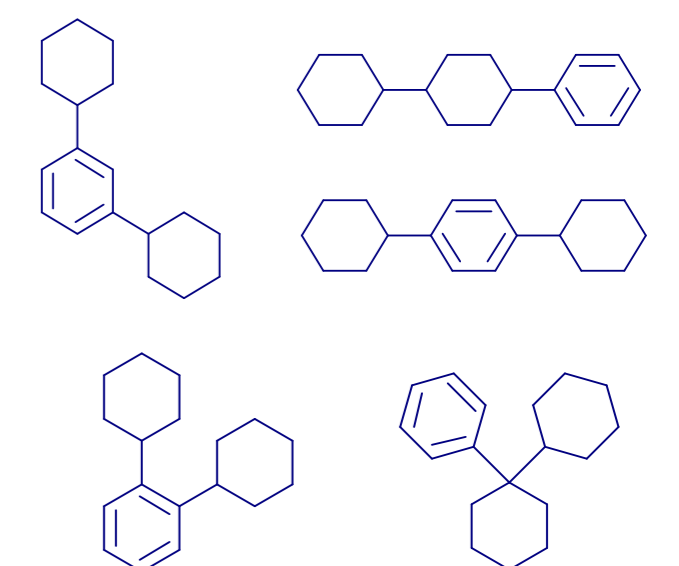
Diphenylcyclohexanes



Cyclohexylbiphenyls and diphenylcyclohexanes



Dicyclohexylbenzenes

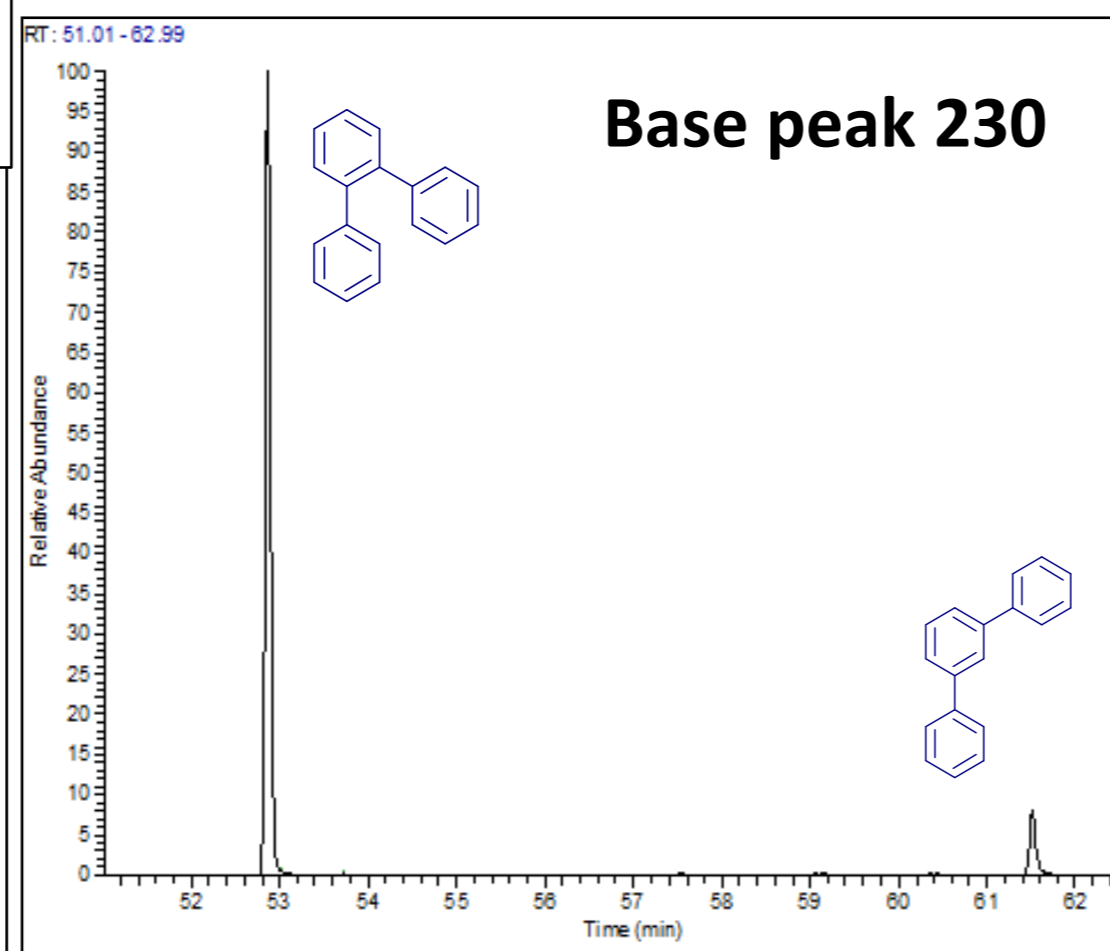
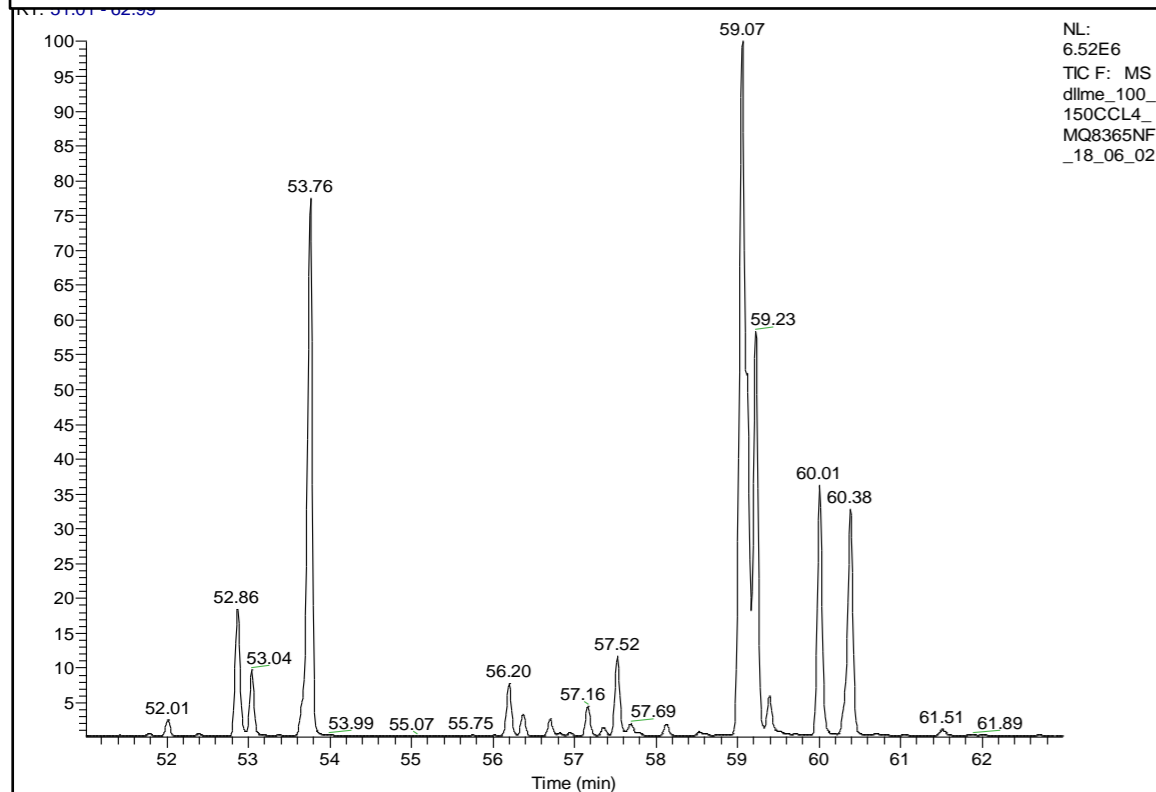


Partially hydrogenated terphenyls are produced in large amounts for industrial use – they are included in the HPV (high production volume) chemicals list by US-EPA. They are mainly used as heat transfer fluids and polymer plasticizers.

These compounds have been found in recycled paper and cardboard, as well as in food products packed with these recycled materials. Our results show the presence of these contaminants in human plasma samples of 39 volunteers, both smokers and non smokers.

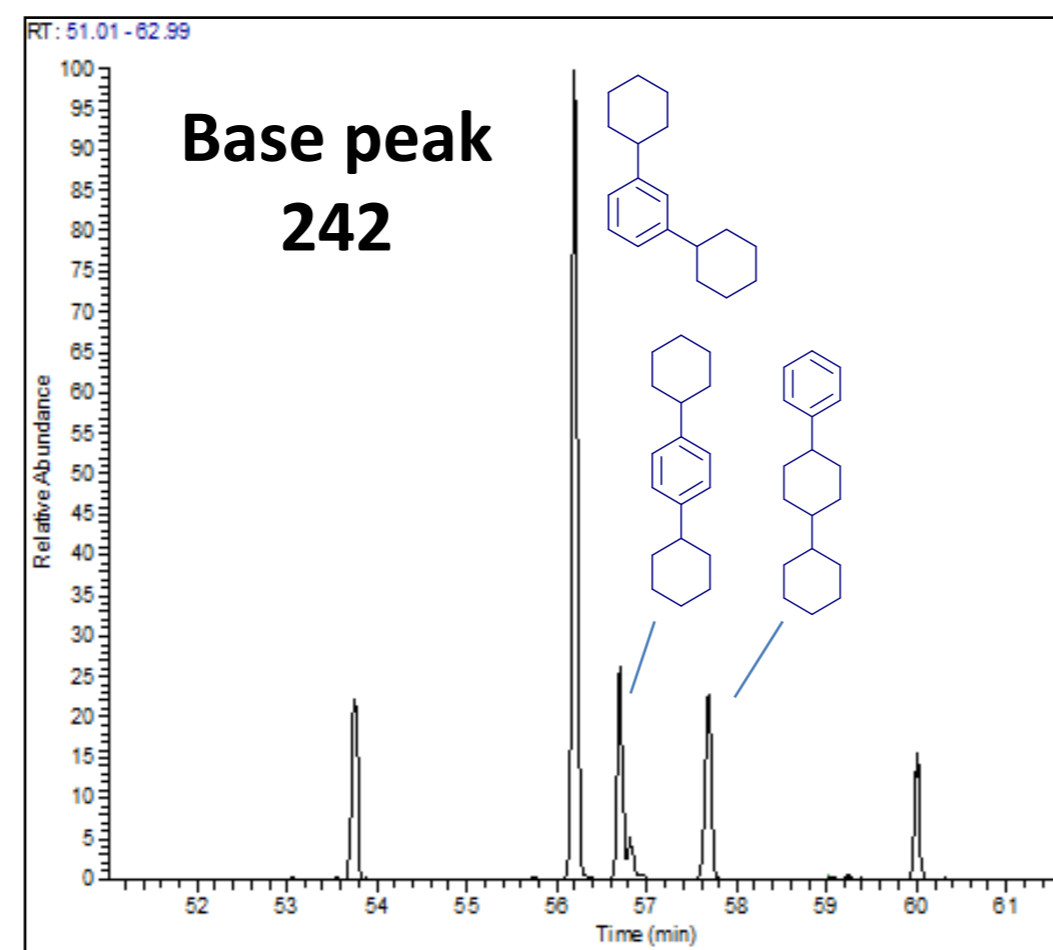
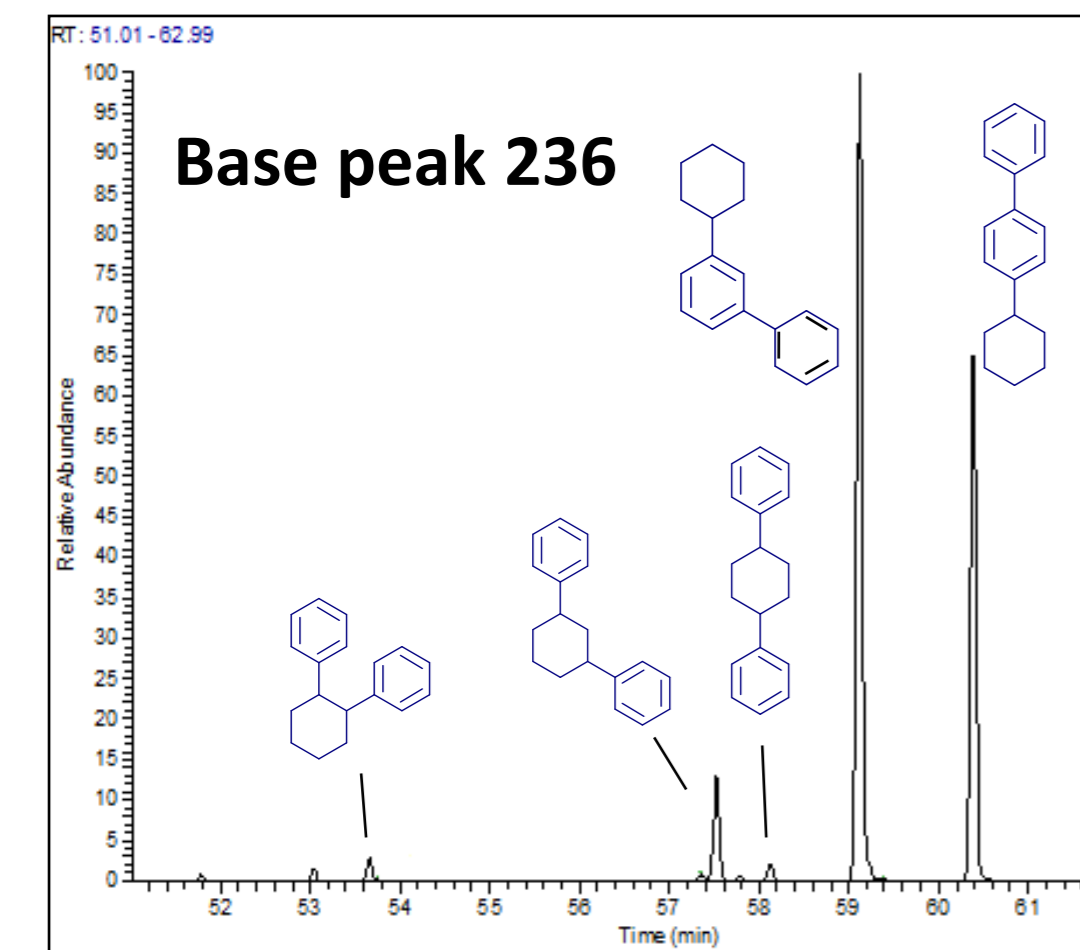
Results : Extraction of terphenyls and hydrogenated terphenyls using HLLME

TIC profile from HLLME of a non smoker plasma

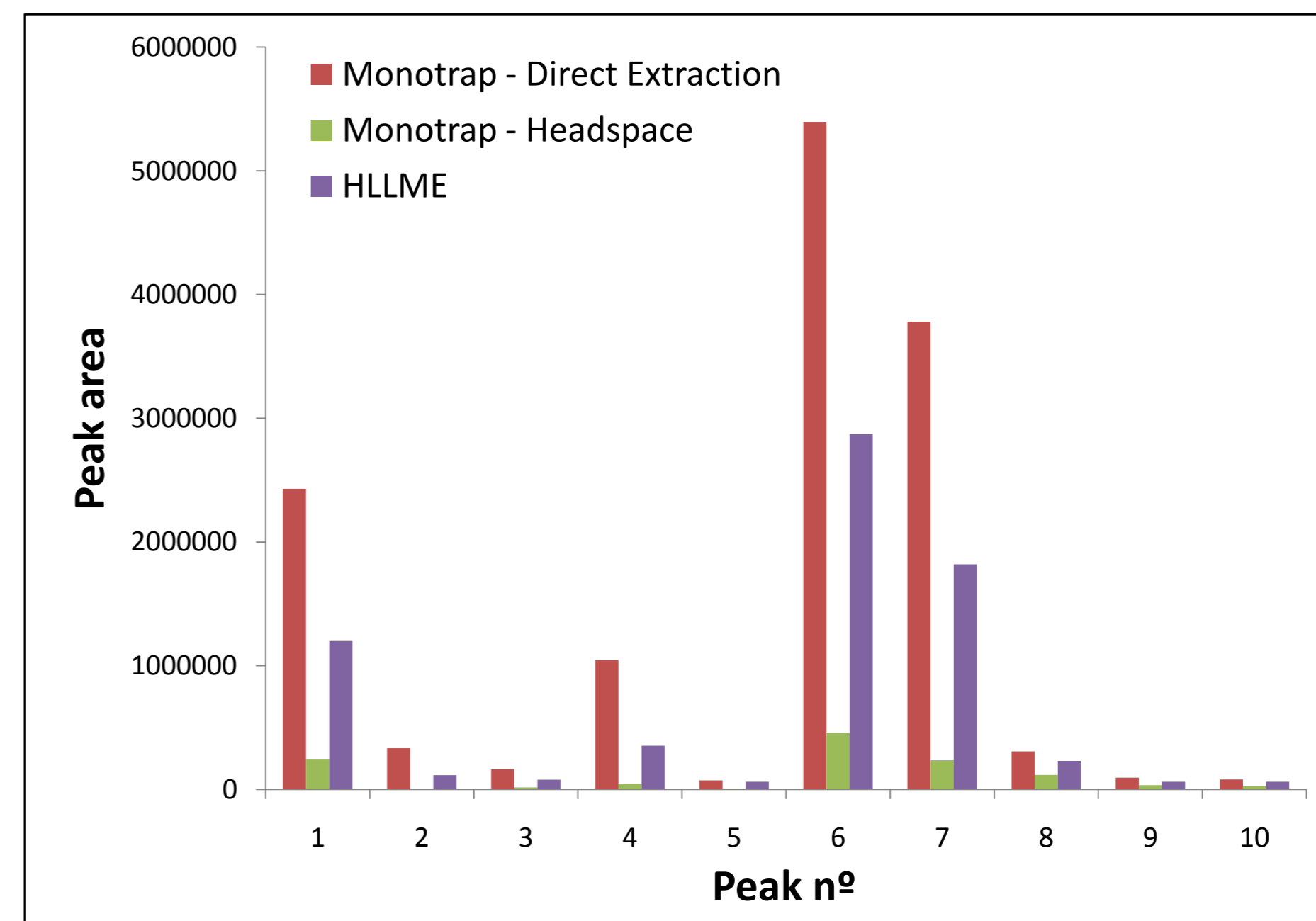


Peak nº	Name	Base peak area (m/z= 230)		
		MT-DE	MT-HE	HLLME
1	o-terphenyl	2429532	241665	1200775
2	m-terphenyl	333761	-	115186

Peak nº	Name	Base peak area (m/z= 236)		
		DE	HE	HLLME
3	1,2-diphenylcyclohexane	164368	17267	79147
4	1,3-diphenylcyclohexane	1045938	45041	353403
5	1,4-diphenylcyclohexane	72594	5981	61418
6	3-cyclohexylbiphenyl	5395533	457725	2872927
7	4-cyclohexylbiphenyl	3780512	237143	1819588

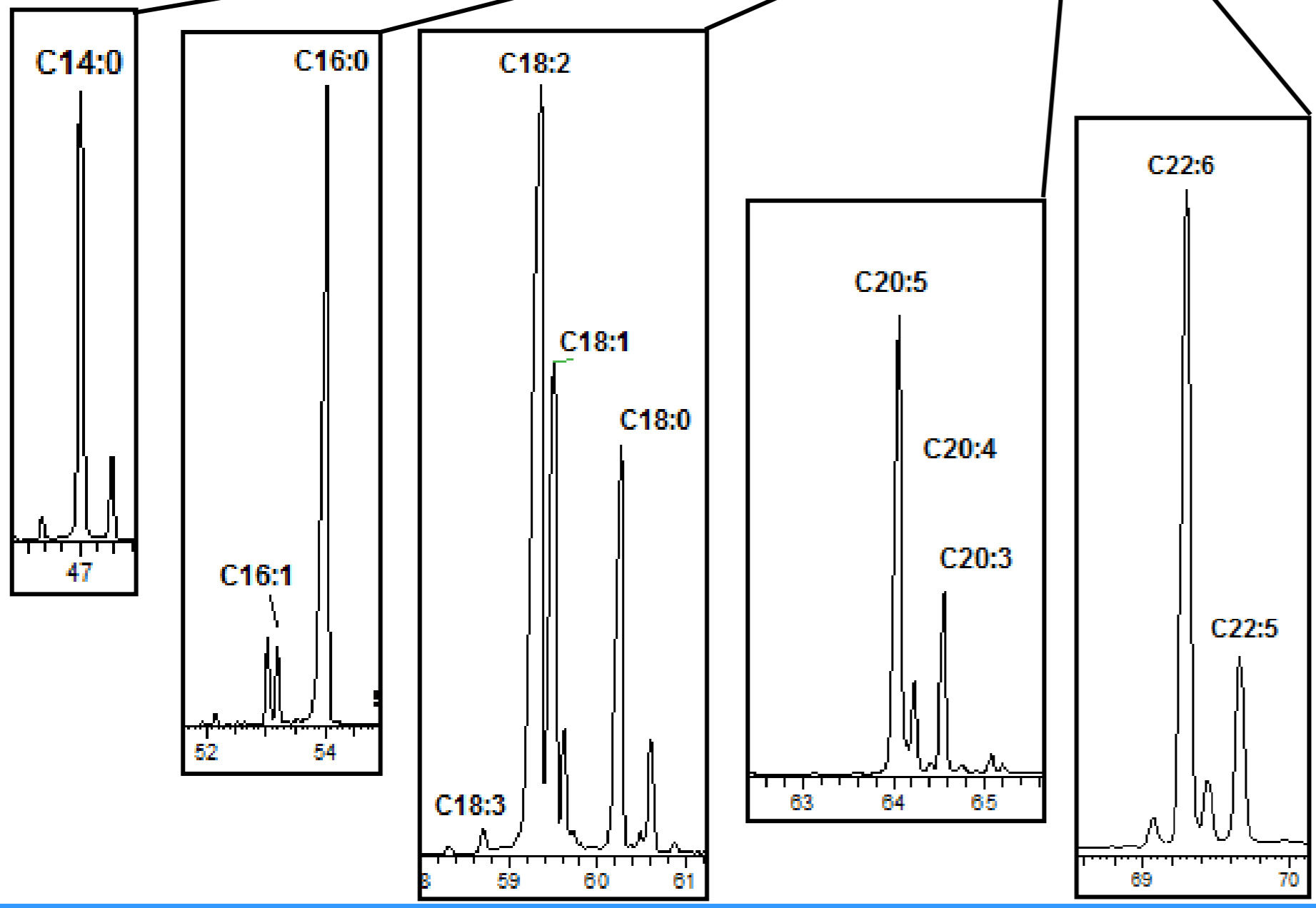
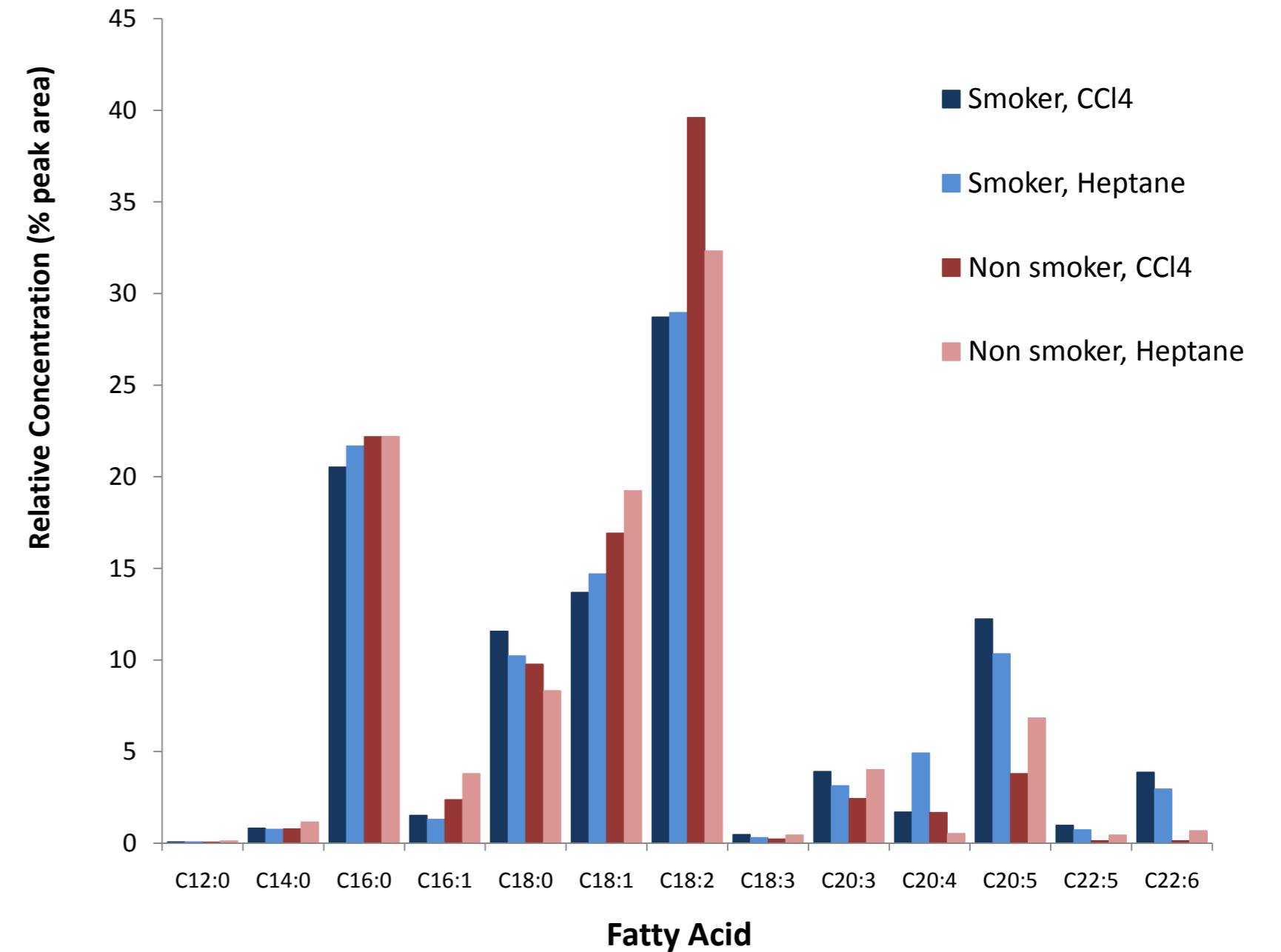
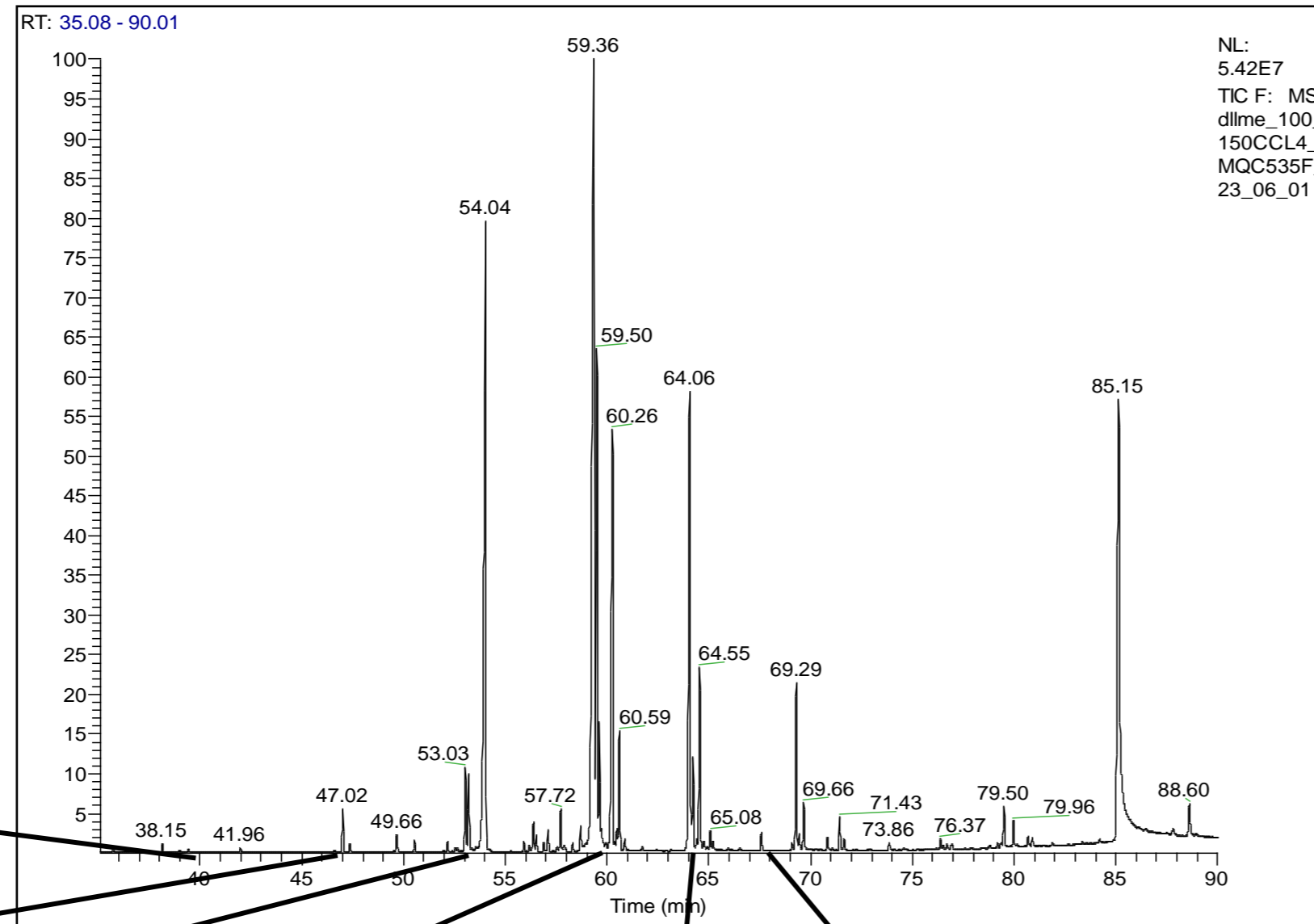


Peak nº	Name	Base peak area (m/z= 242)		
		DE	HE	HLLME
8	1,3-dicyclohexylbenzene	308491	117248	231020
9	phenyldicyclohexane	95245	35084	62574
10	1,4-dicyclohexylbenzene	80384	26736	62484



Results : direct transesterification + HLLME CCl₄ or heptane

Tic profile, FFA methyl esters from smoker plasma



- Fatty acid profile can be obtained with direct transesterification and HLLME either with heptane or with CCl₄.
- Comparison of 13 reference fatty acid showed no significant differences between smokers and non smokers.
- Although heptane extracts are less concentrated than CCl₄ extracts we could identify 26 fatty acid methyl esters.

26 Fatty Acid Methyl Esters Identified		
(C12:0) Lauric acid	(C18:3), Linolenic acid	(C20:2) Eicosadienoic acid
C14:0) Myristic acid	(C18:2), Linoleic acid	(C20:1) Eicosenoic acid
(C14:0) 12-Methyltetradecanoic acid	(C18:1), Oleic acid	(C20:0) Eicosanoic acid
(C16:1) Palmitoleic acid	(C18:1) Elaidic acid	(C22:6) Docosahexaenoic (2 isomers)
(C16:0) Palmitic acid	(C18:0) Stearic acid	(C22:5) Docosapentaenoic
(C16:0) 15-methylhexadecanoic acid	(C20:5) Eicosapentaenoic acid	(C22:4) Docosatetraenoic
(C16:0) 14-methylhexadecanoic acid	C20:4) Eicosatetraenoic acid (3 isomers)	
(C17:0), Heptadecanoic acid	(C20:3) Eicosatrienoic acid (2 isomers)	

Conclusions :

- Terphenyls and partially hydrogenated terphenyls were extracted from human plasma.
- Extracts of the materials in contact with the plasma samples and blanks of the analytical process revealed no contamination with these compounds.
- SPE of deproteinized and diluted plasma with a new monolithic adsorbant (MonoTrap™), both in direct contact or from the headspace, allows the selective extraction of these plasma contaminants without significant co-extraction of plasma metabolites. The method with direct contact provides better extraction yield than the headspace extraction.
- To the best of our knowledge this is the first report on the use of non halogenated solvents for any kind of liquid-liquid microextraction. Although showing smaller enrichment factors than halogenated solvents, the non halogenated solvents are generally less toxic and therefore can be an environmentally safer alternative.
- When performing DLLME with plasma samples diluted in water the addition of the organic phase (heptane or CCl₄), causes the formation of a gel like structure that prevents a good phase separation.
- A new method for the direct derivatization (transesterification) of plasma lipids and simultaneous extraction and concentration of the corresponding methyl esters was developed. The method uses organic solvents in the microliter range, is performed in a single vial and has a total derivatization + extraction time of 15 minutes or less.
- The homogeneous liquid-liquid microextraction coupled with direct transesterification allows the fast and sensitive determination of up to 40 fatty acid methyl esters derivatives of plasma lipids.
- Terphenyls and partially hydrogenated terphenyls were found, at variable concentrations in 39 out of 40 human plasma samples analysed.
- No significant differences were found between the smokers and the non smokers group in what concerns fatty acid profile or aromatic contaminants level.

Aknowledgements: The authors thank Dr. Etsudo Ikeda (GL Sciences, Japan) for the kind offer of MonoTrap™ samples. Thanks are also due to Dr. Carlos Marques (Nova Era Clinical Analysis Laboratory, Lisbon) for supplying the plasma samples.