Technical basics and requirements for on-line capillary LC/MS

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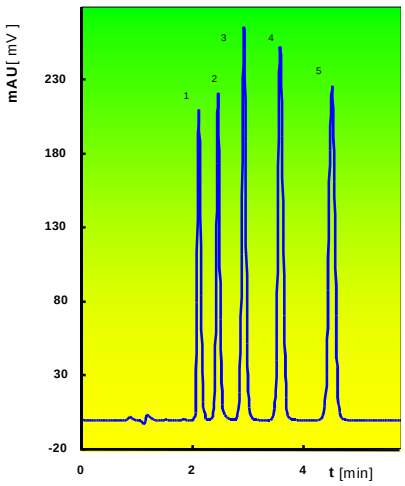
Analyses performed with capillary LC and nano LC methods, using columns with inner diameters (ID) of 50 to 800 µm, can be every bit as safe and trustworthy as HPLC separations employing conventional 4 or 4.6 mm ID columns. An unalterable prerequisite for this approach is the development and application of appropriate test procedures—for example, the measurment of a column's efficiency and selectivity (Figs. 1, 2).

Column efficiency test

- benzoates -

Fig. 1

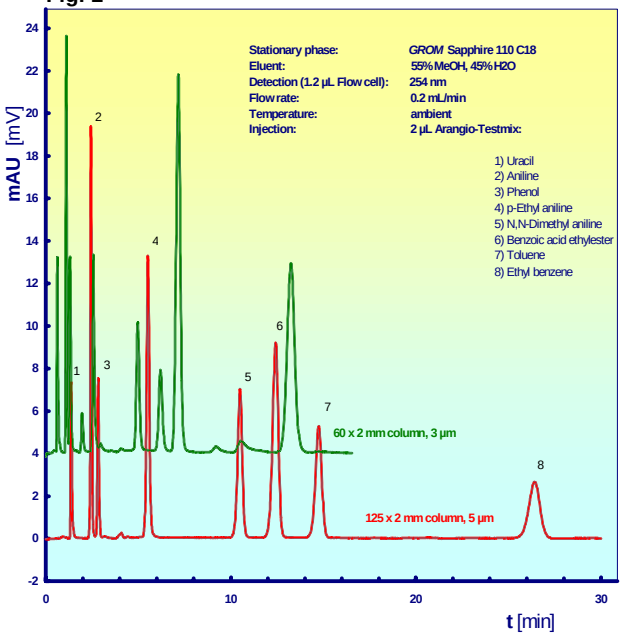


Stationary phase: *GROM* Sapphire C18 - 5 µm, **Column size:** 125 x 2 mm, **Eluent:** H_2O : ACN = 20 : 80, **Flow rate:** 200 µL / min, **Pessure:** 2.6 MPa, **Temperature:** RT, **Detection:** 254 nm, **Flow cell:** 1.2 µL/ 3 mm, **Sample:** 1) methyl benzoate, 2) ethyl benzoate, 3) propyl benzoate, 4) butyl benzoate, 5) pentyl benzoate, **Injection:** 2 µL (~ 18.5 - 50 µg/mL of each)

Column selectivity test

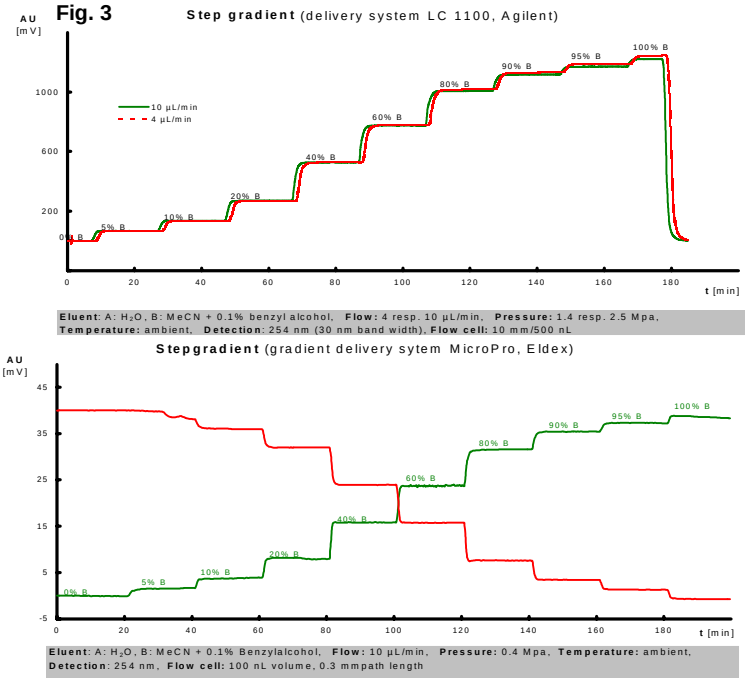
modified Arangio and Engelhardt procedure

Fig. 2



Microgradients: Sophisticated chromatographic separation of complex mixtures, such as pharmaceuticals or metabolites, as well as reproducible analytical results depend, to a large extent, on the quality of the gradient itself. This is especially true for capillary and nano HPLC procedures, which often have flow rates ≤100 µl/min. As seen in Fig. 3, elution gradients can be evaluated rapidly by employing two simple expedients: replacement of the HPLC column with a fused silica capillary restrictor and addition of benzyl alcohol to eluent B to permit its detection at 254 nm.

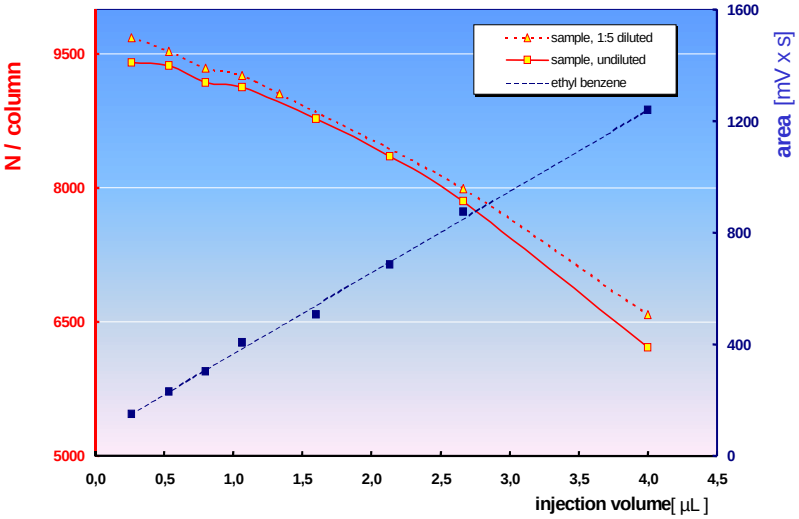
Fig. 3



Effect of injection volume: The amount and volume of analyte applied to a capillary HPLC column is an especially critical parameter to be considered in the development of an optimal assay. Figure 4 shows that even for a diluted sample not more than 500 nl may be applied to an 800 µm ID column. This means that the injection volume for a 300 µm ID column, for example, must be ≤ 100 nl for optimal separations. Such incredibly small sample volumes can be reproducibly applied only via "hair cutting", that is, by time-controlled injection or control of the injection valve. In this method, the loaded sample loop is in series with the column for only a brief time interval.

Fig. 4

Influence of the injection volume on the quality of the separation (plates/column)



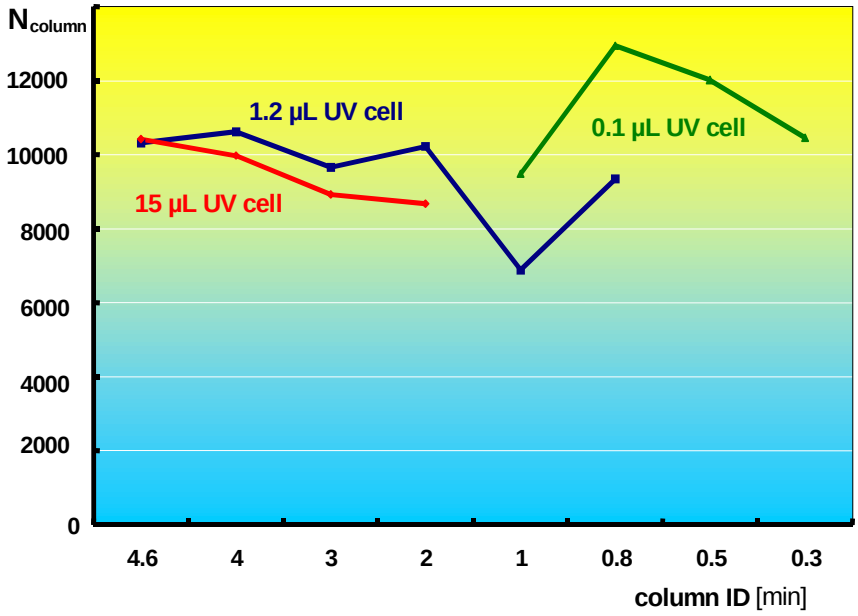
Stationary phase: *GROM* Sapphire C18 5 µm, **Column size:** 125 x 0.8 mm, **Flow rate:** 16 µL / min, **Eluent :** H_2O : ACN = 20 : 80, **Temperature:** RT, **Detection:** 254 nm, **Flow cell:** 0.1 µL, **Sample:** 1) benzoic acid, 2) anisol, 3) benzene, 4) toluene, 5) ethyl benzene; undiluted ~ 1-3 mg/mL, resp. diluted 0.2-0.6 mg/mL

Capillary columns well-packed with quality phases have, for all practical purposes, the same stability as analytical columns. Their effective useful lifetime depends primarily on the purity of the eluents employed and the samples analyzed.

Effect of the column diameter: The chromatographic efficiency or resolution should be independent of the inner diameter of a series of HPLC columns, provided each is filled with one and the same batch of a particular stationary phase. In contrast, "fused silica" capillary columns of 500 and 800 µm packed with ***GROM* Saphir** C18 particles demonstrate a plate count (N) 10 to 20% higher than analytical columns packed with the same material (Figs. 5-7). The relatively poor performance of the microbore column (1 mm ID)(Fig. 7). is due to un-necessary dispersion within the column head

Fig. 5

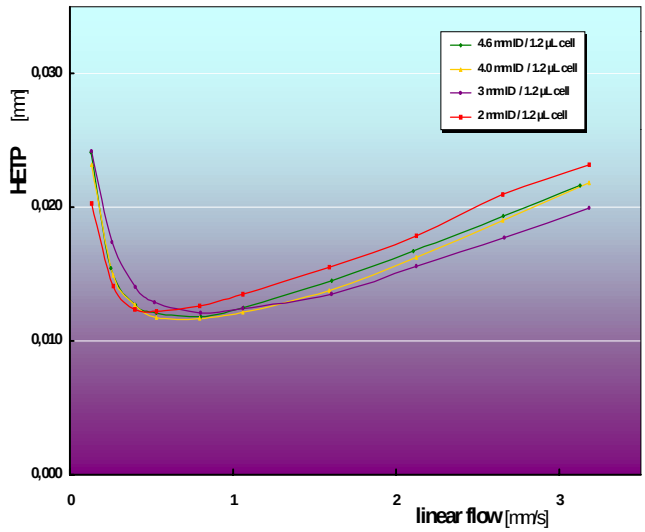
Influence of the column diameter on the plate count in combination with different UV detector cells



Stationary phase: *GROM* Sapphire C18 - 5 µm, **Column size:** 125 x 0.3 – 4.6 mm, **Flow** (lin. Vel.)**:** 0.5 mm/s, **Eluent:** H_2O : ACN = 20 : 80, **Temperature:** RT, **Detection:** 254 nm, **Flow cell:** 15 µL, 1.2 µL , 0.1 µL, **Sample:** 1) methyl benzoate, 2) ethyl benzoate, 3) propyl benzoate, 4) butyl benzoate, 5) pentyl benzoate, **Injection :** 2 - 10.6 µL (~ 18.5 - 43.8 µg/mL of each),

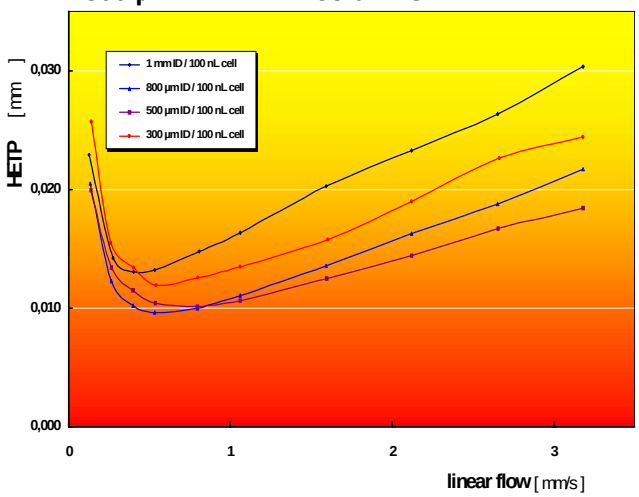
Optimal flowrates – v. Deemter diagrams

Fig. 6
2 – 4.6 mm ID columns



Stationary phase: *GROM* Sapphire C18 - 5 µm, **Column size:** 125 x 2 – 4.6 mm, **Flow rate:** variabel, **Eluent:** H_2O : ACN = 20 : 80, **Temperature:** RT, **Detection:** UV- (254 nm), **Flow cell:** 1.2 µL / 3 mm, **Sample:** 1) methyl benzoate, 2) ethyl benzoate, 3) propyl benzoate, 4) butyl benzoate, 5) pentyl benzoate, **Injection:** 2 - 10.6 µL (~ 18.5 - 43.8 µg/mL of each)

Fig. 7
300 µm – 2 mm ID columns



Stationary phase: *GROM* Sapphire C18 - 5 µm, **Column size:** 125 x 0.3 - 1 mm, **Flow:** variabel, **Eluent:** H_2O : ACN = 20 : 80, **Temperature:** RT, **Detection:** UV- (254 nm), **Flow cell:** 0.1 µL / 0.3 mm, **Sample:** 1) methy lbenzoate, 2) ethyl benzoate, 3) propyl benzoate, 4) butyl benzoate, 5) pentyl benzoate, **Injection:** 0.04 – 0.5 µL (~ 18.5 - 50 µg/mL of each),

Effect of flow cell volume: It is well-known that the dead volume of an HPLC system (for example, of the capillary connectors, column fittings, flow cell, etc.) must be minimized to guarantee the highest possible resolution in any analysis. This is independent of the type of detector employed (Figs. 3, 4). Nonetheless, for UV detectors, small cell volumes are often synonomous with short path lengths and thus a reduction in sensitivity due to the Beer-Lambert law (Fig. 8). The same holds true for radioactivity detectors, since counts per time interval (cps) are measured and eluents analyzed in small cells have a short residence time (Fig. 9).

Fig. 8 ***UV detection*** **(v. Deemter diagram)**

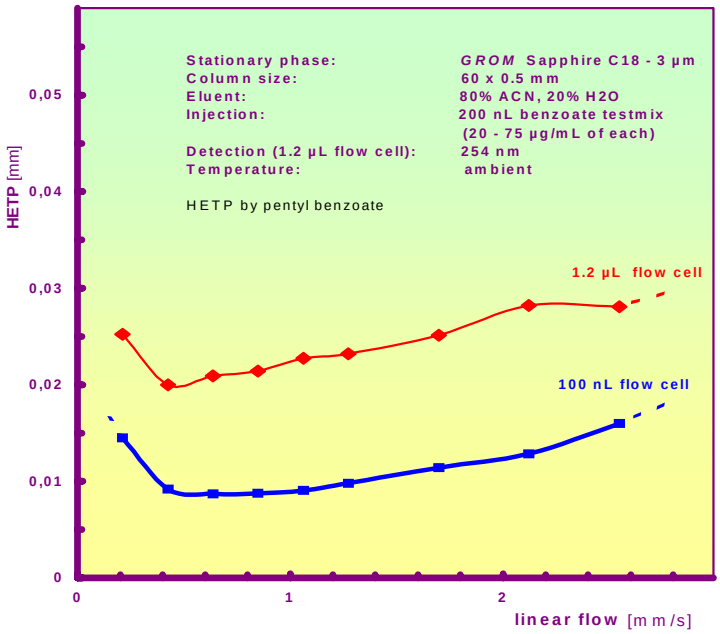
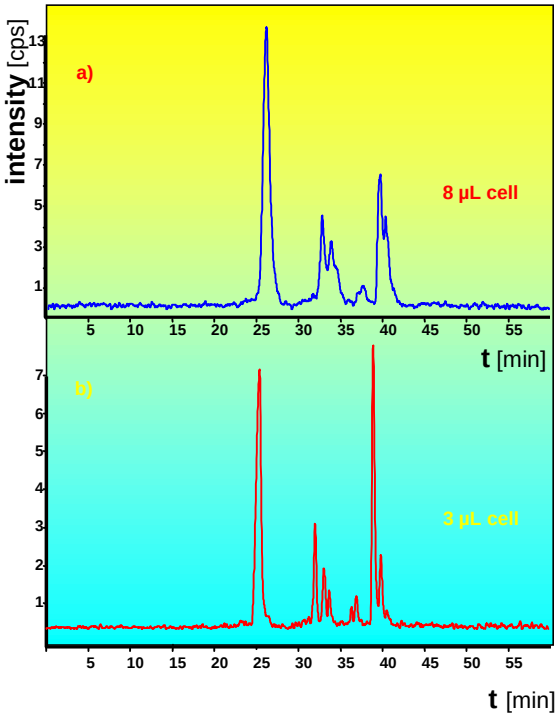


Fig. 9 ***Radioactivity detection***

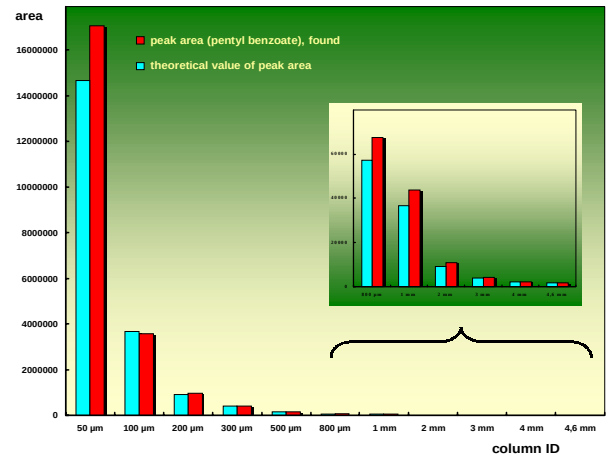


The driving force and really most important argument for employing capillary LC and nano HPLC is not the oft-cited reduction in solvent consumption, with attendent reductions in costs and pollution, nor is it the small amounts of sample required, but rather is the dramatic increase in sensitivity (~ 200-fold, Fig. 10) and the simple, straight-forward procedure for on-line LC/MS coupling, which renders splitting of the eluate stream unnecessary. Figure 11 shows that a properly constructed splitter makes possible simultaneous UV or radioactivity dectection and on-line MS coupling with negligble losses in resolution.

Fig. 10

Sensitivity

signal height vs. Column ID

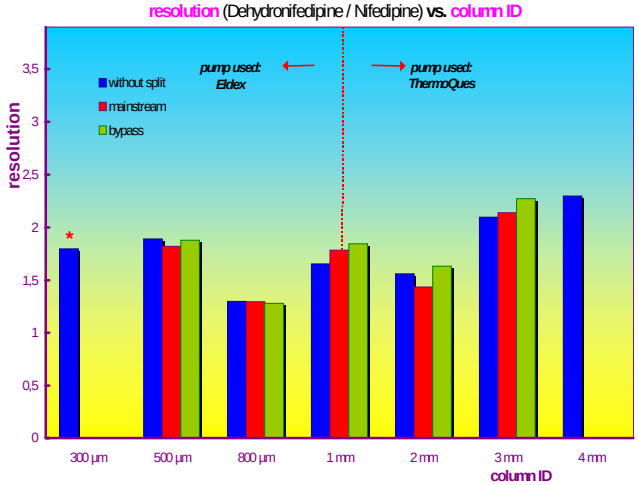


Stationary phase: *GROM* Sapphire 110 C18 - 5 µm, **Column size:** 125 mm x id, **Eluent:** 20% H_2O, 80% MeCN (v/v), **Flow** (lin. vel.)**:** 0.80 mm/s, **Temperature:** ambient, **Detection:** 254 nm, **Flow cell:** 0.3 mm, 100 nL, **Injection:** 200 nL, **Sample:** alkyl benzoates (20-75 mg/mL)

Fig. 11

Splitting

resolution (Dehydronifedipine / Nifedipine) **vs.** **column ID**



Stationary phase: *GROM* Sapphire 110 C18 - 5 µm, **Column size:** 125 mm x .. ID, **Eluent:** A H2O + 0,1% HCOOH, B MeCN + 0,1% HCOOH, **Gradien:** 5 - 70% B (0-20 min), **Flow** (lin. vel.)::0.80 mm/s, **Temperature:** ambient, **Detection:** UV-(225 nm), **Flow cell:** 100 nL / 0.3 mm, **Injection:** 200 nL drugs (50 - 250 µg of each)

<u>Summary:</u> ***Results obtained with capillary LC and nano HPLC are every bit as valid and reliable as those obtained with conventional HPLC. Figures 11 and 13 additionally demonstrate the ≥ 8000-fold enhanced sensitivity and the superior resolution of a modern HPLC system, particularly one employing on-line capillary LC/MS.***

Analysis of pharmaceuticals and metabolites.

Fig. 12 **UV detection**

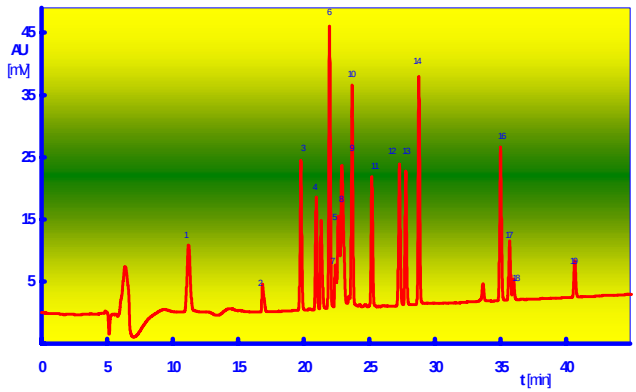
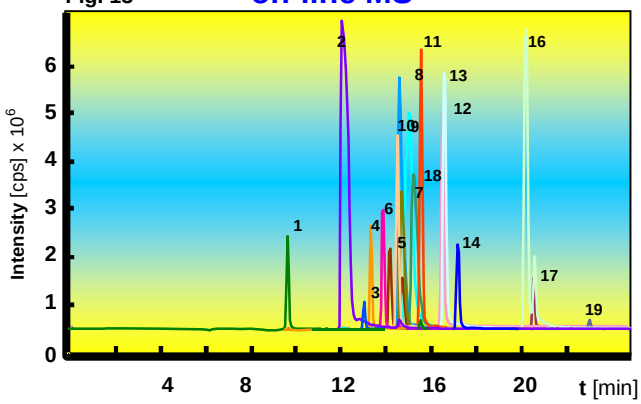


Fig. 13 **on-line MS**



1)	Paracetamol (70 µg/mL)	6)	7-OH-Coumarin (175 µg/mL)	11)	Phenacetin (90 µg/mL)	16)	Dehydronifedipin (90 µg/mL)
2)	Dextrorphan (140 µg/mL)	7)	1-OH-Midazolam (36 µg/mL)	12)	Coumarin (120 µg/mL)	17)	Nifedipin (70 µg/mL)
3)	6-OH-Chlorzoxazon (140 µg/mL)	8)	Methoxymorphinan (100 µg/mL	13)	Mephenytoin (210 µg/mL)	18)	Midazolam (100 µg/mL)
4)	4-OH-Mephenytoin (70 µg/mL)	9)	Dextromethorphan (100 µg/mL)	14)	Chlorzoxazon (140 µg/mL)	19)	Diclofenac (90 µg/mL)
5)	4-OH-Midazolam (100 µg/mL)	10)	Nirvanol (350 µg/mL)	15)	4-OH-Diclofenac		

Stationary phase: *GROM* Sil 120 ODS-4 HE, 3 µm **Column size:** 250 x 0.5 mm **Eluent:** A: H_2O + 0.1% HCOOH, B: MeCN + 0.1% HCOOH **Gradient:** 8-83% B, 0-50 min **Flow rate:** 10 µL/min **Pressure:** 28.2 MPa **Temperature:** ambient **Detection:** 225 nm **Flow cell:** 100 nL/ 0.3 mm **Injection:** 200 nL

Stationary phase: *GROM* Sapphire 110 C18, 5 µm, **Column size:** 125 x 0.5 mm, **Eluent:** A: H_2O + 0.1% HCOOH, B: MeCN + 0.1 % HCOOH; **Gradient:** 5-70% B (0-20 min), 70% B (20-25 min), **Detection:** MS/MS (atmospheric pressure ionisation), **Flow rate:** 10 µL/min (*main tream* 5 µL/min), **Temperature:** ambent, **Injection:** 0,2 µL

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the experiments presented have been a collaborative effort of: