Gas chromatography mass spectrometry: key technology in metabolomics

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Metabolic profiling of ultra small sample volumes with GC-MS: from microlitres to nanolitres samples

ABSTRACT

Profiling of metabolites is increasingly used to study the functioning of biological systems. For some studies the volumes of available samples is limited to only a few µL or even less, for fluids, such as cerebrospinal fluid (CSF) of small animals like mice or the analysis of individual oocytes. Here we present an analytical method using in-liner silylation coupled to gas chromatography-mass spectrometry (GC-MS), that is suitable for metabolic profiling in ultra small sample volumes of two 2 µL down to 10 nL. Method performance was assessed in various biosamples. Derivatization efficiencies for sugars, organic acids and amino acids were satisfactory (105 – 120%) and repeatabilities were generally better than 15%, except for amino acids that had repeatabilities up to about 35 – 40%. For endogenous sugars and organic acids in fetal-bovine serum, the response was linear for aliquots from 10 nL up to at least 1 µL.

The developed GC-MS method was applied for the analysis of different sample matrices, i.e. fetal bovine serum, mouse CSF and aliquots of the intracellular content of *Xenopus laevis* oocytes. To the best of our knowledge, we present here the first comprehensive GC-MS metabolite profiles from mouse CSF and from the intracellular content of a single *Xenopus laevis* oocyte.

INTRODUCTION

The aim in metabolomics research is to measure as many metabolites as possible in body fluids, tissue samples or even cells, in order to gain insights in the functioning of biological systems. Gas chromatography coupled to mass spectrometry (GC-MS) is a very suitable technique for this purpose and several GC-MS based metabolomics methods have been reported.[1-7] Most of these methods rely on derivatization with a silylation reagent prior to analysis to convert the polar functional groups that are problematic in GC-MS analysis to less-polar groups.

Previously, the development and validation of a one-dimensional GC-MS method[8] and a comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GC×GC-ToF-MS) method[9] were described using oximation and subsequently silylation prior to analysis. Both methods allowed the measurement of a broad range of small, medium-polar to polar metabolites. These methods are very suitable for the analysis of cell extracts, body fluids and tissues when reasonable amounts of sample are available. Usually approximately 100 µL of cell extract or body fluids and 5-10 mg of tissue are needed for sample workup, in order to obtain good and repeatable profiles of the metabolites. In some cases, this amount of sample is not available. Cerebrospinal fluid (CSF) is an optimal body fluid to study pathophysiology of neurological diseases and mouse models have become available to study human diseases such as Alzheimer[10], Parkinson[11] or migraine[12] but the amount of CSF is limited to a few µL. The acquisition of metabolite profiles in mouse CSF sample is potentially very promising for translational studies and the application of proven methods such as LC-MS and GC-MS is very desirable. An even more demanding drive behind miniaturization of the sample workup is the growing interest to measure differences in metabolite concentrations at the level of individual cells or even in cellular compartments. To acquire metabolite profiles from sample volumes of nL up to a few µL, or ultimately from a single cell, is an enormous challenge for analytical chemists. For example, a human oocyte has a diameter of about hundred micrometers with a volume of only approximately one µL.

Most reports on the analysis and separation of biomolecules from single cells involve the use of capillary electrophoresis or microfluidic devices.[13-16] Thus far, only a limited number of target compounds have been measured and no comprehensive metabolite profiles have been obtained from single cells. Notable, Edwards et al.[17] described a two-dimensional liquid chromatography mass spectrometry method for the analysis of metabolites in complex samples, allowing for ultra low injection volumes of 2.5 – 9 nL. A preconcentration of the samples prior to injection was used to obtain sufficient sensitivity. The present study is the first to GC-based metabolite profiling of single cells and other ultra low volumes of sample.

With our existing GC-MS metabolomics methods[8,9], bodyfluid or microbial samples are usually derivatized in 300- or 600-µL autosampler vials. For an efficient and repeatable derivatization of the sample and to be able to automatically inject samples from these vials about 100 – 150 µL of total volume (derivatization reagents and solvent) is necessary. Finally, usually only 1-µL aliquots of the derivatized sample are injected splitless in the analytical system. The use of smaller autosampler vials (100 – 150 µL inserts) might be possible, but using an autosampler vial for derivatization and subsequent injection of a 1-µL aliquot into the GC-MS would not provide the required sensitivity to study metabolite concentrations in samples of only a few µL, or even smaller sample volumes from single cells.

An efficient way to improve the sensitivity of the overall method is to ensure that the complete sample or a larger aliquot of the sample is transferred to the analytical column. One could use large volume injection (LVI), however, the relatively polar solvents and reagents used in the derivatization are not very suitable for LVI. Another option is to use a programmed temperature vaporiser (PTV) injector (so-called Direct Thermal Desorption (DTD) interface) in combination with disposable microvials that fit inside an injection liner. The liner with the microvial containing the sample is placed inside the PTV injector and after analysis the liner is exchanged with a new liner containing the next sample. This setup is especially suitable for analysis of solid samples or samples containing high boiling and/or non-volatile components in which sample constituents remain in the liner after transfer of the volatiles. However, the DTD interface in combination with the microvials can also be used to derivatize compounds inside the injector and transfer the analytes on-line to the analytical column. For example, Blokker et al. used a DTD method to perform methylation of fatty acids in algae in the DTD liner.[18] This method was also applied and further optimized by Akoto et al.[19-21]

Some studies describe the use of on-column silylation.[22-25] In these methods the underivatized sample and subsequently the derivatization reagent were injected, to derivatize the compounds on-column. The setup in these studies is not suitable for the analysis of metabolites in aqueous samples of only a few µL or even smaller, as the sample handling will be problematic. The steps needed prior to derivatization, that is water removal, redissolution in an organic solvent, and injection of the small sample, increase the risk of losing metabolites. Furthermore, the described methods all involve the silylation of compounds with only hydroxylic functional groups. However, the aim in metabolic profiling is to derivatize also other functional groups. In a previous study metabolites were classified into three groups based on their analytical performance and ease of silylation.[8] Class-1 metabolites contained hydroxylic and carboxylic functional groups, while Class-2 type metabolites contained amine or phosphoric functional groups. Class-3 type metabolites had amide, thiol or sulfonic functional groups. The performance of silylation decreased in the following order: Class1 (hydroxyl >

carboxyl) > Class 2 > Class 3. The silylation of, for example, carboxylic and amine functional groups is relatively slow compared to the silylation of hydroxylic groups.[26] To derivatize these functional groups, longer contact times between the sample and the derivatization reagent is needed than those encountered under on-column derivatization conditions.

In summary, for the derivatization of a small sample and the complete transfer of the derivatized products onto the GC column the in-liner derivatization using a DTD interface seems most promising for metabolite profiling. In this setup, the amounts of derivatization reagent and the initial temperature of the PTV injector can be varied to achieve optimal contact times between the sample and the silylation reagent, and no redissolution of the sample is required.

The feasibility of using the DTD interface to perform in-liner silylation coupled to GC-ToF-MS was investigated for the metabolic profiling of ultra small volumes of biological samples, ranging from a few µL of body fluid (i.e. serum, CSF) down to an aliquot of only ten nL of fetal bovine serum. Finally, the method was applied to obtain metabolite profiles from an aliquot of the intracellular content of a single *Xenopus leavis* oocyte.

EXPERIMENTAL

Chemicals and materials

Pyridine (Baker analyzed) was obtained from Mallinkrodt Baker, Deventer, The Netherlands and pyridine hydrochloride (analytical grade) came from Sigma-Aldrich (Zwijndrecht, The Netherlands). N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA; Alltech, Breda, The Netherlands) was used for silylation. The OV1701-deactivated microvials (DMI inserts) were obtained from BGB Analytik (Boeckten, Switzerland).

Standards

All reference compounds and metabolites used as internal standards and for method optimization were obtained from Sigma-Aldrich, unless mentioned otherwise. The 2H, 15N labelled amino acid mix standard (20 labelled amino acids) was purchased from Cambridge Isotope Laboratories (Andover, Massachusetts, USA). US defined fetal bovine serum was obtained from Hyclone (Logan, Utah, USA).

A standard of phenylalanine, malic acid and glucose (25 ng/uL) in water was used for method optimization and to determine the recovery and repeatability of the derivatization reaction. An internal standard mixture (IS mixture) was prepared containing 4,4-difluorobiphenyl (25 ng/µL), trifluoroantracene (25 ng/µL) and dicyclohexyl phthalate (25 ng/µL) in aceton/pyridine hydrochloride solution 9:1 (v/v) (pyridine hydrochloride solution is a saturated solution of pyridine hydrochloride in pyridine). The IS mixture was spiked to all samples prior to analysis to control and/or compensate for variations due to the transfer of components from the sample onto the analytical column and for variations of the detector response. Furthermore, the pyridine hydrochloride solution increased the solubility of the underivatized metabolites and pyridine catalyzed the silylation reaction.

Preparation of cell homogenate from *Xenopus laevis* oocyte

Twenty oocytes (stage IV-VI) were washed three times with 1 mL of water. Next, 0.5 mL water and 0.5 mL methanol were added to the cells, and the cells were homogenised immediately by disrupting the cell wall by a pipette. The homogenate was mixed thoroughly and then stored at -20°C prior to analysis. Before starting sample workup, the homogenate was centrifuged for 10 min at 10000 rpm.

Samples for the optimization of the method

1-µL aliquots of standard mixture (25 ng/µL phenylalanine, malic acid and glucose in water) were placed inside microvials and dried in an exicator. Five microvials containing 1-µL aliquots of standard mixture (25 ng/µL phenylalanine, malic acid and glucose) and 1-µL aliquots of oocyte homogenate were dried in an exicator. The recovery was assessed by comparison with an off-line derivatization and GC-MS

analysis. For this purpose a larger sample containing 100 µL of the standard mixture was derivatized offline with 70 µL MSTFA and 30 µL pyridine for 45 minutes at 40 °C, so that 1-µL aliquots of these samples contained the same concentrations of metabolites (and internal standards) compared to the miniaturized setup.

Samples for the evaluation of the method performance

Repeatability
Five microvials were filled with 2-µL aliquots of fetal bovine serum and subsequently dried in an exicator.

Dynamic range
1-µL aliquots of fetal bovine serum and 1-µL aliquots of fetal bovine serum diluted ten or hundred times with water were transferred to microvials and dried in an exicator.

Applications

Mouse CSF
Cerebrospinal fluid from mice was aspirated from the cisterna magna using a glass capillary, connected via polyethylene tubing to a mouth pipette according to the method of Fischer et al.[27] The procedure was performed in such a manner that possibility of contamination with blood was kept to a minimum. Samples of approximately 2 µL were transferred directly to a microvial and immediately frozen in liquid nitrogen and stored at -80°C until analyses, which was at maximum 1 month after sampling. Prior to analysis, the samples were dried in an exicator.

Single cell analysis
Approximately 100 nL of cell content from *Xenopus Laevis* oocytes (stage IV-VI) were withdrawn from single oocyte cells. A micromanipulator (Alessi, P-5069, Irvine, CA, USA) was used for control of depth of the needle (fused silica capillary, internal diameter 30 µL). The sampling needle was inserted 100 µm into the cell. The cell content was sampled using pressure pulses. The samples were directly transferred to microvials and stored at -80°C until analysis. Prior to analysis, samples were dried in an exicator.

Miniaturized at-line derivatization and GC-MS analysis

Analyses were carried out with an Agilent 6890 gas chromatograph (Agilent technologies, Santa Clara, CA, USA) with an Optic 3 injector (AtasGL, Eindhoven, The Netherlands), coupled to a time-of-flight mass spectrometer (Pegasus III; LECO, Monchengladbach, Germany). The microvials containing the dried sample were placed inside the injection liner and 1 µL of IS mixture and 3 µL MSTFA were added just prior to GC-MS analysis. The liner with the microvial containing the sample and MSTFA was placed inside the PTV injector. The PTV injector was used in splitless

mode and the PTV temperature was kept at 70°C for 10 min to allow the metabolites to be derivatized. Subsequently the PTV temperature was raised to 325°C at a rate of 2°C/s. The samples were injected into an OV1701-deactivated retention gap (2 m x 0.32 mm ID, BGB Analytik) coupled with an HP5-MS capillary column (30 m x 250 µm I.D., 0.25 µm film thickness; J&W, Folson, CA, USA). The initial GC oven temperature was 70°C; 20 min after injection the GC oven temperature was increased at 15°C/min to 325°C and held for 5 min at 325°C. Helium was used as the carrier gas and pressure programmed such that the helium flow was kept constant at a flow rate of 1.7 mL per minute. Detection was achieved using MS in electron ionization mode. The temperature of the MS transfer line was set at 325°C and the ion-source temperature was 280°C. The detector voltage was set at -1700 V. Data acquisition rates were 5 Hz.

Quantification of metabolites

The peak areas of the metabolites were determined using an appropriate reconstructed ion chromatogram per metabolite. For all (automated) quantifications the ChromaTOF software (V3.34; Leco Corporation, St. Joseph, MI, USA) was used.

Determination of the evaporation time

Microvials were filled with 3 µL MSTFA and 1 µL pyridine/aceton mixture (9:1) (v/v) and placed inside an injection liner in the injector. The samples were analyzed at different PTV injector temperatures to determine the evaporation times. The GC oven was set at 140°C to allow the solvent to flow through the column unretained. The column flow was set to 1.7 mL/min helium. The detector voltage was set at -1000 V. No solvent delay was programmed, in order to measure the complete solvent peak. The evaporation time was equal to the width of the solvent peak.

Comparing human and mouse CSF profiles

ChromaTOF software V3.34 was used for data processing. A human CSF sample was processed with the following settings: baseline tracking, default; baseline offset, 0.5; peak width, 2 s; segmented processing, peak find S/N 10; number of apexing masses, 2. All peaks found in the human CSF sample were added to the reference table. Known artefacts and peaks without the m/z 73 (specific for silylized compounds) were removed from the reference table leaving 359 different peaks. It should be noted that for some metabolites, e.g. monosaccharides, two or more isomers are formed after derivatization. These were all included in the reference table. Five mouse CSF samples (measured on the same day as the human CSF sample) were processed using the constructed reference table to search for the 359 peaks.

RESULTS AND DISCUSSION

Optimization of in-liner derivatization

The in-liner derivatization method was based on a derivatization method reported earlier consisting of an oximation and subsequent silylation of the sample carried out in autosampler vials prior to GC-MS analysis.[8] To reduce the total reaction time in the miniaturized in-liner setup and to minimize potential problems with injection of large amounts of relatively polar solvents and reagents, no oximation was used.

For the optimization of the in-liner derivatization a set of three test compounds from different compound classes were used, i.e. glucose, malic acid and phenylalanine. At first, derivatizations were carried out with the injector at 70°C in solvent venting mode. In this mode of operation the split exit of the injector was opened to vent off excess derivatization reagent. When only a small amount of derivatization reagent was left inside the microvial in the liner, the split exit was closed. However, loss of relatively volatile metabolites, such as silylated malic acid, was observed in this mode, even when the split exit was closed with large amounts of derivatization reagent present in the liner (e.g. 5 – 10 µL; data not shown). Evidently, the derivatized metabolites were not sufficiently trapped in the derivatization reagent to be able to perform the derivatizations in the solvent-venting mode.

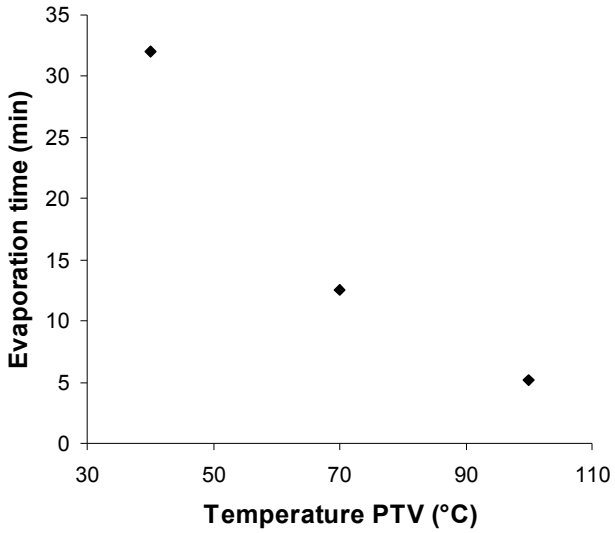

Figure 1 Evaporation time of 3 µL MSTFA at different temperatures.

Further experiments were therefore carried out in the splitless mode. Several parameters were optimized: (i) the amount of derivatization reagent, (ii) the reaction temperature, which is identical to the initial PTV temperature, (iii) the reaction time, which is equal to the evaporation time of the MSTFA, and (iv) gas flow. These

parameters could not be set independently. For example, an increase in derivatization reagent resulted in longer reaction times if the temperature and gas flow were kept constant, as the evaporation time of the MSTFA influences the reaction time. Additionally, a decrease in reaction temperature resulted in longer evaporation/reaction times (Figure 1).

The recoveries of malic acid and glucose compared to off-line derivatization (cf. Experimental) were determined at different reaction temperatures (Table 1). The recovery of glucose was already satisfactory at 40°C. However at this temperature malic acid and phenylalanine were not derivatized efficiently. At temperature of 70°C and above, both malic acid and glucose had satisfactory recoveries. The derivatization of phenylalanine was not repeatable and therefore no reliable derivatization efficiencies could be obtained. However, even when longer reaction times (equal or longer than 20 min) and/or more derivatization reagent was used (6 µL) at the highest temperature (140°C), no improvement in derivatization efficiency of phenylalanine was observed (data not shown). The amine functional group in phenylalanine is more difficult to derivatize[28,29] and, moreover, the bond between a trimethylsilyl group and a amine is relatively weak.[26] Therefore, derivatized amino acids are more prone to degrade and/or react in the liner or on the analytical column.

Table 1 Recoveries at different reaction temperatures compared to the offline procedure [a)]

Compound	Recovery at various temperatures [b)]			
	40°C	70°C	120°C	140°C
Glucose	85	85	85	85
Malic acid	0	105	105	115

a) In-liner reaction conditions: 3 µL MSTFA, 10 minutes reaction time (equal to hold time initial PTV temperature), further parameters: see Experimental.

b) Initial PTV temperature.

For the final method a derivatization temperature of 70°C, 3 µL of derivatization reagent, and a gas flow of 1.7 mL/min helium were chosen, resulting in a reaction time of about 10 min. In this way, the derivatization temperature was kept to a minimum preventing breakdown of unstable metabolites and the total analysis time was kept as short as possible.

Table 2 Recovery compared to the off-line procedure and repeatability

Compound	Academic standards		*Xenopus laevis* samples [a)]	
	Recovery (%)	RSD (n=5)	Recovery (%)	RSD (n=5)
Glucose	92	4	n.d. [b)]	7
Malic acid	90	13	120	11
Phenylalanine	24	65	105	36

a) The metabolites were spiked to the sample.

b) n.d.: not determined. The recovery of glucose was not determined because of the presence of endogenous glucose.

Analytical characteristics

Recovery

The recovery of metabolites compared to off-line derivatization and the repeatability of derivatization of phenylalanine, malic acid and glucose in academic standards and spiked to *Xenopus laevis* lysates were determined with the optimized method (Table 2). The relative standard deviations (RSDs) of the response and the recoveries for glucose and malic acid were satisfactory for the standards and Xenopus laevis homogenates (i.e. with RSDs below 15% and recoveries between 80 – 120%). The recovery of glucose was not calculated in the Xenopus laevis homogenate because of the presence of endogenous glucose. However, the response of glucose and other sugars in the Xenopus laevis extract were comparable with a sample of the same extract that was derivatized with the off-line derivatization procedure (data not shown). In agreement with the results from the method optimization, the analytical performance for phenylalanine was not as good as that for malic acid and glucose. Still, the performance for phenylalanine in complex samples was better than in academic solutions. This was most likely caused by reduced adsorption and/or degradation of phenylalanine in the presence of matrix components (matrix enhancement effect[8]).

Table 3 Repeatability of endogenous metabolites in a 2-µL fetal-bovine-serum samples

Compound	m/z	RSD (%) (n=5)
Organic acids		
D-Glyceric acid	189	6
Fumaric acid	245	12
Malic acid	245	9
Fatty acids		
Nonanoic acid	215	17
trans-9-Octadecenoic acid	339	9
Octadecanoic acid	341	13
Amino acids		
Glycine	174	36
Glutamic acid	246	39
L-Phenylalanine	218	35
Sugars		
Fructose	204	7
Myo-Inositol	318	5
Other		
Glycerol-3-phosphate	299	19
Cholesterol	458	7

Repeatability in serum samples

Five fetal-bovine-serum samples were analyzed and the repeatability of the miniaturized in-liner derivatization and GC-MS analysis was calculated for thirteen metabolites (Table 3). The performance for organic acids, fatty acids and sugars was satisfactory with RSDs of 5 – 17%. The analysis of amino acids was less repeatable (RSDs of 35 – 39%).

Table 4 Intermediate precision of some endogenous metabolites in human 2- µL CSF samples

Metabolite	m/z [b]	Intermediate precision (n=8) [a]
Organic acids		
2,3,4-Trihydroxybutanoic acid	292	7
Adipate	111	8
Lactic acid	147	8
Sugars		
Fructose	437	11
Glucose	204	12
Mannitol	319	7
Myo-inositol	217	1
Amino acids		
Glycine	174	9
Isoleucine	158	14
Phenylalanine	192	20
Threonine	218	42
Other		
Glycolamide	188	6
Glycerol-3-phosphate	299	11
Creatinine	115	32
Nicotinamide	179	51

a) The inter-batch precision was calculated according to analysis of variance calculation (one-way ANOVA).[30]

b) m/z used for reconstructed ion chromatogram and determination of peak area.

Intermediate precision

The intermediate precision (i.e. the inter-batch precision across different days) was determined by eight GC-MS analysis of 2-µL aliquots of the same human CSF sample. These aliquots were analyzed as quality-control (QC) samples during the analysis series of a study of mouse CSF samples (data of the actual study not shown) measured over a period of four days. The QC samples were measured before and after every fifth mouse CSF sample. For organic acids and sugars the intermediate precision was generally very satisfactory (i.e. between 1 and 15%). The intermediate precision for the amino acids ranged from 9 – 56% (Table 4). As observed for the validation the type of the metabolite was the most important factor determining the performance. For worse-

performing metabolites, such as amino acids, the concentration was also an important factor. The RSD of glycine, for instance, which is present at a relatively high concentration in the samples, was much better than the RSD of threonine (which is present at a low concentration) (Table 4).

Linear range

The linear range of the method was determined by analyzing different aliquots of fetal bovine serum in duplicate (1 µL, 100 nL and 10 nL; Table 5), and by determining the ratio between the normalized response of a metabolite and the amount of serum in the microvial. For the sugars and succinic acid the ratio was constant down to the 10-nL aliquot. For the other compounds the relative response at the 10-nL level was somewhat lower than expected, i.e. smaller than 1, probably due to adsorption and/or degradation on active sites on the surface of the microvial, liner and/or analytical column. In conclusion, for organic acids and sugars the linear range based on constant response factors ranged from 10 nL up to at least 1 µL of sample, and for other compound classes from 100 nL up to at least 1 µL.

Table 5 Linear range determined by ratio of normalized response (n = 2) and amount in different volumes of fetal bovine serum

Compound	m/z	Response/amount for various aliquots of serum [a)] (µL)		
		1	0.1	0.01
Acids				
Succinic acid	247	0.99	1	0.97
Octadecanoic acid	341	1.02	1	0.72
Amino acids				
Glutamic acid	246	1.03	1	0.75
Sugars				
Glucose	204	0.96	1	0.97
Myo-inositol	318	1.02	1	0.91
Other				
Cholesterol	368	1.02	1	0.79

[a)] Ratio of response and amount of metabolite for different amounts of serum; for better comparability the ratio was set to 1 for the 0.1-µL aliquot by dividing all ratios by the ratio at the 0.1-µL aliquot.

In Figure 2 the metabolite profiles obtained from a 100 nL aliquot and a 10 nL aliquot of fetal bovine serum are shown. Most metabolites were present at low concentrations in the 10-nL aliquot, and peaks from the reagent, solvent and other artefact peaks were relatively high compared to the metabolite peaks. Still, approximately one hundred metabolites with a signal-to-noise ratio (S/N) > 10 in appropriate extracted ion chromatograms were detected. These metabolites were detected by comparing the 10-nL sample with the 100-nL sample, peaks that were increased in the 100-nL sample

were considered metabolites (Figure 2). Furthermore, the mass spectra of the remaining peaks were evaluated to remove possible artefacts and/or reagent peaks.

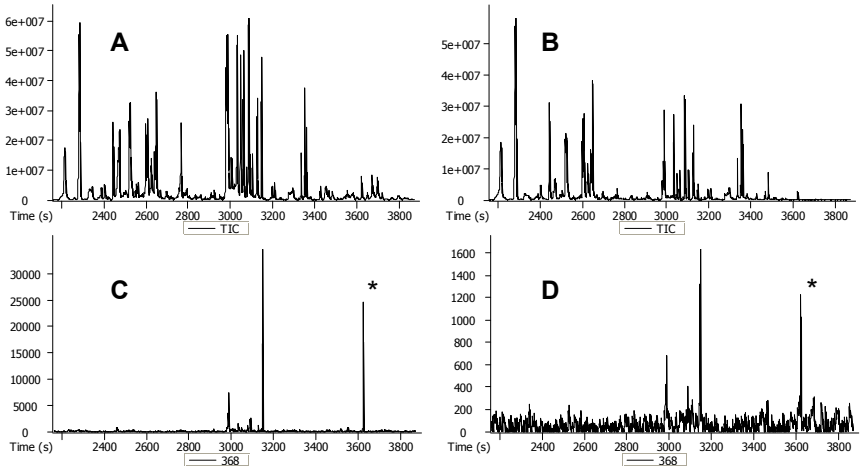

Figure 2 Full-scan chromatograms of 100 nL (A) and 10 nL (B) of fetal bovine serum and the corresponding reconstructed ion chromatograms of m/z 368 (C: 100 nL and D: 10 nL), for the targeted detection of metabolite cholesterol (S/N = 10) (*).

Applications

Comparing metabolite profiles of cerebrospinal fluid (CSF) from mice and humans

To investigate the potential of the developed in-liner GC-MS method for future translational studies of mouse models of neurological disease, mouse CSF samples were analyzed. We obtained the first comprehensive GC-MS metabolite profiles from mouse CSF (Figure 3).

The metabolite profiles from the human subject (QC samples, cf. Experimental, subsection Analytical performance – Intermediate precision) were acquired in the same manner as the mouse CSF, and were qualitatively compared with the mouse CSF profiles in the following manner. After removal of artefacts, 358 metabolites were detected in human CSF and stored in a reference table. Based on this table, a total of 342 peaks, i.e. approximately 95% of all peaks measured in human CSF, were also found in the mouse CSF profiles. Therefore, we conclude that the majority of the metabolites in humans and mice overlap.

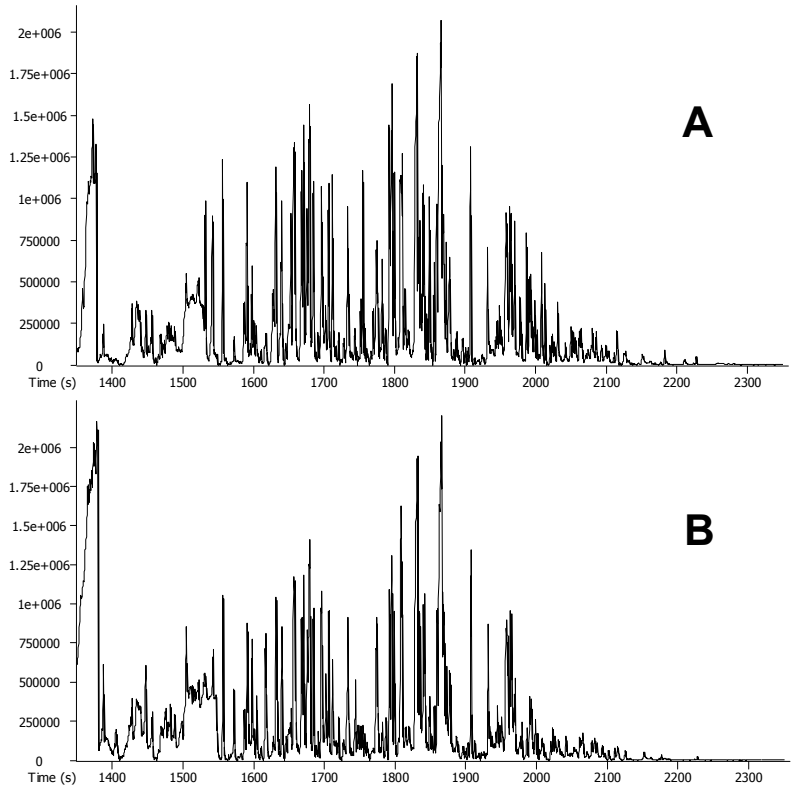

Figure 3 Full-scan GC-MS chromatogram of human (A) and mice (B) CSF; about 2 µL CSF was analyzed.

Single cell analysis

Approximately four hundred peaks with a signal-to-noise ratio over 100 in appropriate extracted ion chromatograms were detected with in-liner derivatization GC-MS of about 100 nL sampled from a *Xenopus laevis* oocyte (Figure 4). Compounds from different classes were detected, including organic acids (e.g. propane-, pentane-, and hexanedioc acid, succinic acid, fumaric acid, malic acid, gluconic acid), fatty acids (saturated, unsaturated and hydroxy fatty acids ranging from C8 to C28), amino acids (e.g. serine, phenylalanine, glutamic acid), alcohols and sugars.

Although the *Xenopus laevis* oocyte is a relatively large cell (with a volume of approximately 1 µL), the obtained profile is, to our knowledge, the most comprehensive metabolite profile obtained from a single cell, and in this case even of one tenth of the internal cell content. Moreover, the signal-to-noise obtained for many metabolites should allow the analysis of even smaller sample aliquots. In addition, the use of comprehensive two-dimensional GCxGC-MS should improve detection limits and detectability of metabolites further.[9]

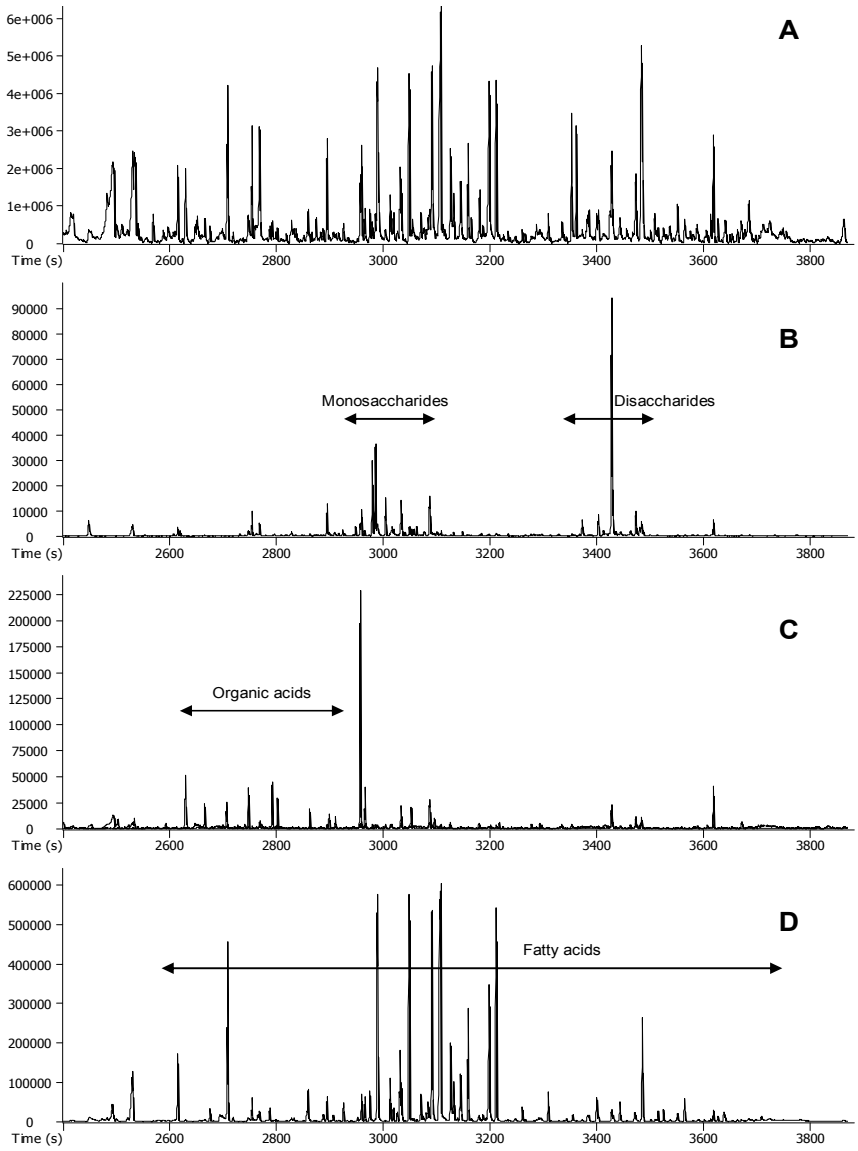

Figure 4 Single-cell analysis of an aliquot of intracellular content of an individual *Xenopus laevis* oocyte; total-ion-current trace (A) and metabolite-class specific reconstructed ion chromatograms, i.e. m/z 217 characteristic for sugars (B), sum of m/z 233 (x10) and m/z 292 characteristic for organic acids (C) and m/z 117 characteristic for fatty acids (D).

CONCLUSION

An in-liner silylation GC-MS method was developed that is suitable for metabolic profiling in ultra-small sample volumes. The analytical performance for class-1 metabolites (i.e. sugars, alcohols, organic acids and fatty acids) was comparable with the conventional off-line derivatization procedure; the performance of class-2 metabolites (amino acids) was somewhat worse than in the off-line procedure. The procedure can be automated by automatic exchange of the liner.

With the optimized method the first comprehensive GC-MS metabolite profiles could be obtained from mouse CSF. It could be demonstrated that the metabolites in human and mice CSF are to a large extent the same, supporting the potential of metabolomics for translational studies (i.e., comparing for instance CSF of patients with CSF of mice that are models for the same disease). The first comprehensive metabolite profile with GC-MS was obtained from an aliquot of intracellular content of a single *Xenopus laevis* cell. In addition, metabolite profiles could be measured in an aliquot of only 10 nL of fetal bovine serum. Many metabolites were detected in the samples and sensitivity can be even further improved using GC×GC-MS. The developed method can provide more insight into local biochemical processes, whether in an individual cell or in animals, by taking fluid samples locally.

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