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Validation of metabolomics analysis of human perilymph fluid using liquid chromatography-mass spectroscopy

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ABSTRACT:

Although there is some data from animal studies, the metabolome of inner ear fluid in humans remains unknown. Characterization of the metabolome of the perilymph would allow for better understanding of its role in auditory function and for identification of biomarkers that might allow prediction of response to therapeutics. There is a major technical challenge due to the small sample of perilymph fluid available for analysis (sub-microliter). The objectives of this study were to develop and validate a methodology for analysis of perilymph metabolome using liquid chromatography–high resolution mass spectrometry (LC-HRMS). Due to the low availability of perilymph fluid; a methodological study was first performed using low volumes (0.8 μ L) of cerebrospinal fluid (CSF) and optimized the LC-HRMS parameters using targeted and non-targeted metabolomics approaches. We obtained excellent parameters of reproducibility for about 100 metabolites. This methodology was then used to analyze perilymph fluid using two complementary chromatographic supports: reverse phase (RP-C18) and hydrophilic interaction liquid chromatography (HILIC). Both methods were highly robust and showed their complementarity, thus reinforcing the interest to combine these chromatographic supports. A fingerprinting was obtained from 98 robust metabolites (analytical variability <30%), where amino acids (e.g., asparagine, valine, glutamine, alanine, etc.), carboxylic acids and derivatives (e.g., lactate, carnitine, trigonelline, creatinine, etc.) were observed as first-order signals. This work lays the foundations of a robust analytical workflow for the exploration of the perilymph metabolome dedicated to the research of biomarkers for the diagnosis/prognosis of auditory pathologies.

Keywords : fingerprinting; LC-MS; analytical optimization; CSF; inner ear fluid; metabolic profiling.

Abbreviations:

CSF, cerebrospinal fluid ; CV, coefficient of variation; ESI, electrospray ionization modes; GC-MS, gas chromatography–mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HPLC, high pressure liquid chromatography; LC-HRMS, liquid chromatography–high resolution mass spectrometry; MeOH, methanol; QC, quality control; RP, reverse phase; TIC, total ion current (TIC) chromatogram

1. Introduction

Characterizing the metabolic composition of perilymph fluid is important to understand basic mechanisms of auditory function, to increase knowledge regarding the physiopathology of deafness, and to identify biomarkers for diagnosis or prognosis of pathologies of the inner ear. However, the composition of perilymph fluid, as well as exchanges and interactions with the different compartments of the inner ear are largely unknown (Rask-Andersen et al., 2006).

The omics approaches go from detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a specific biological sample since a complex system is a whole. Perilymph has been study at a proteome level (Alawieh et al., 2015; Schmitt et al., 2018; Schmitt et al., 2017), but few have been done by metabolomics. The aim of metabolomics is to characterize the metabolome (a set of low-molecular compounds present in biological fluids, cells, or tissues) in order to study metabolism (Dunn et al. (2011); Madji Hounoum et al., 2016; Nicholson et al., 2002). Metabolomics studies typically use several types of analytical instruments, mainly nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled with gas chromatography (GC-MS) or liquid chromatography (LC-MS). One of the challenges of perilymph metabolome analysis is the micro-quantity of sample available; in humans, the volume of a perilymph fluid sample is $<1 \mu\text{L}$. Due to the higher sensitivity of mass spectrometry compared to NMR, LC-MS platform may be preferable for perilymph metabolome analysis because of its broad applicability to metabolites of all classes present in complex biological samples (metabolites are separated before analysis by chromatography), combined with its relative ease of use in generating global metabolite profiles (Theodoridis et al., 2008).

Because of the low availability of perilymph fluid samples, a methodology for metabolome analysis was developed using another biological matrix - cerebrospinal fluid

(CSF), which communicates with perilymph (Salt et al., 2015; Zou et al., 2003), and is similar to perilymph fluid in terms of composition (Scheibe et al., 1985). After validating this methodology using CSF, liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS) was used to analyze a small medical collection (0.8 μ L) of perilymph to determine its metabolic profile.

2. Methods

2.1. Perilymph sample

Human perilymph samples were obtained from 23 patients undergoing cochlear implantation surgery for bilateral sensorineural hearing loss. The perilymph was sampled via the round window using a 22g needle, before the insertion of the cochlear implant electrodes in the scala tympani. The perilymph samples were stored in polypropylene test tubes and frozen (-80°C) until analysis.

2.2. CSF sample

Lumbar punctures were performed according to the standardized procedure for routine diagnosis. The CSF samples were collected into polypropylene test tubes and centrifuged (4000g, 10 min, 4°C). The supernatants were all pooled and aliquoted into polypropylene test tubes and frozen (-80°C) within 2 hours of puncture.

2.3. Metabolite extraction

Metabolism of perilymph fluid or CSF was quenched and metabolites were extracted with (i) 100 μ L or (ii) 200 μ L of cold methanol added to 0.8 μ L of biological fluid. After vortexing for 1 min, samples were stirred for 10 min at 4°C on a planar stirrer and then left in a cold bath at -20°C for 30 min. The samples were then centrifuged at 15,000 g for 15 min at

4°C, and the supernatant (95 µL) was (iii) harvested for analysis or (iv) evaporated with a SpeedVac concentrator at 40°C. In this last case (iv), the dry residue [obtained from 100 µL of methanol (MeOH) as extraction solvent] was re-suspended in 100 µL of methanol/water (50:50 v/v), subjected to vortex for 1 min and centrifuged at 20 000 g at 4°C for 10 min. Quality control (QC) samples were prepared from a mixture of equal volumes (20 µL) of all diluted extracted samples.

2.4. LC-HRMS analysis

LC-HRMS analysis was performed as previously described (Dieme et al., 2015; Madji Hounoum et al., 2015) using a UPLC Ultimate WPS-3000 system (Dionex, Germany) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and operated in positive (ESI+) and negative (ESI-) electrospray ionization modes (analysis for each ionization mode). Liquid chromatography was performed using a Phenomenex Kinetex 1.7 µm XB – C18 column (100 mm x 2.10 mm) maintained at 40°C. Two mobile phases gradients (preceded by an equilibrium time of 3 min) were used (see supplementary data, Table S1) (n=3 and confirmed with n=10 for gradient A and n=3 for gradient B). The gradient (Table S1) operated at a flow rate of 0.4 mL/min over a runtime of 26.5 min. Analyses were also performed on a hydrophilic interaction liquid chromatography (HILIC) column (100 mm x 2.10 mm, 100 Å). During the full-scan acquisition, which ranged from 58 to 870 m/z , the instrument operated at 70,000 resolution ($m/z = 200$). The impact of the injection volume on the quality of the chromatographic profile was studied and 2 volumes were taken into account (i) 10 µL and (ii) 20 µL, with gradient A (n=5). For both studies (the impact of the gradient and the impact of the injection volume), the samples were obtained after an extraction with 100 µL of MeOH [case (i)] without evaporation of the extraction phase [case (iii)]. Instrumental stability was obtained by 17 successive injections of a QC

sample. After each of the 6 injections, and at the end of the run, a QC sample was injected to evaluate instrument stability from the repeatability of these 5 injections.

2.5. Data processing

Two types of data processing were used: non-targeted and targeted. The non-targeted approach was used first, as it allows for global study of the spectral fingerprint to evaluate the gradient impact, the volume of extraction solvent, and the volume of injection impact. For the non-targeted analysis, XCMS software implemented in the Galaxy platform (<http://workflow4metabolomics.org/the-galaxy-environment/>) was used to pre-treat raw data (including chromatogram alignments and identification of peaks with a detection intensity threshold of 1,000,000).

Once the robustness of the non-targeted approach was evaluated, the targeted approach was used to describe and compare CSF and perilymph metabolomes sourced from an in-house chemical library. For this targeted analysis, a library of standard compounds (Mass Spectroscopy Metabolite Library (MSML[®]) of standards, IROA Technologies[™]) was analyzed with gradient A of mobile phases. The identity of each metabolite was characterized according to several criteria: (1) the retention time of the detected metabolite must be within ± 20 s of the standard reference, (2) the exact measured molecular mass of the metabolite must be within a range of 10 ppm around the known molecular mass of the reference compound, and (3) the isotopic ratios of the metabolite must correspond to the standard reference. The signal value was calculated using Xcalibur[®] software (Thermo Fisher Scientific, San Jose, CA) by integrating the chromatographic peak area corresponding to the selected metabolite.

The coefficient of variation for the area of each metabolite was calculated in control quality injections (QCs) [$CV\% = (\text{the standard deviation}/\text{the mean}) \times 100$]. Metabolites

having a CV in QCs higher than that in samples and/or with CV >30% were excluded. Venn diagrams were generated using software designed by GenoToul Bioinfo (Bardou et al., 2014).

The pathway analysis was done by using the free web software Metaboanalyst (www.Metaboanalyst.ca), based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Xia et al., 2016).

The workflow of the methodology employed for this paper is illustrated in Fig. 1.

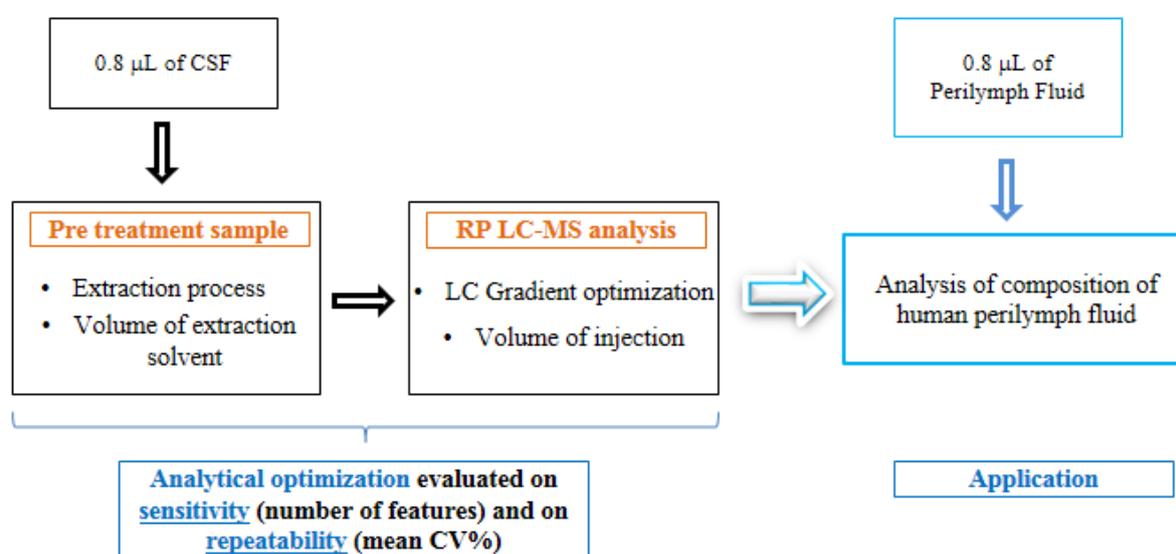


Fig. 1. Workflow protocol for pre-treatment and LC-MS optimization from sub-microliter CSF sample. From these, application to perilymph fluid analysis was performed.

3. Results and discussion

The conventional chromatographic support for LC-MS metabolomics is a reverse phase (RP), leading to the separation of metabolites of medium and low polarity. For polar/ionic molecules that are not well retained on RP phase (e.g., amino acids, sugars), separation using HILIC is an option (Theodoridis et al., 2008). An RP column was used for

optimization of the methodology, and RP gradient and HILIC were used to obtain complementary information.

3.1. CSF samples useful to optimize metabolomics analysis of human perilymph fluid

As perilymph is a valuable sample, not readily accessible and with low available volume (<1 μL), the metabolome of perilymph was first compared to that of CSF to validate that CSF could be used to develop the analytical method for human perilymph fluid. For this comparison, an in-house spectral library constructed from standard metabolites was analyzed under the same conditions; 147 compounds were cumulatively detected in both fluids (analyzed in the quality control (QC) samples). The comparative analysis showed that 94 and 74 metabolites were robustly detected in CSF and perilymph fluid, respectively (CV <30%), for a total of 88% metabolites detected from a very low sample volume (0.8 μL) (Fig. 2). Approximately a third of these metabolites (38) were detected in both biological fluids with similar mean signal intensities (CSF: $4.33\text{E} + 08$, perilymph: $3.68\text{E} + 08$) and satisfactory reproducibility (CV CSF: $9.4\% \pm 7.3\%$; CV perilymph: $3.9\% \pm 3.3\%$). Among the metabolites common to both fluids, a large majority of amino acids previously described in perilymph and CSF of guinea pigs (Medina et al., 1981; Thalmann et al., 1982) was found. These results show that perilymph fluid and CSF possess metabolomes of similar richness even from a submicroliter sample and share a substantial number of common metabolites, due to the exchange between their biological compartments. These results also validated using CSF as biofluid model to optimize and validate the methodology used to analyze the perilymph metabolome.

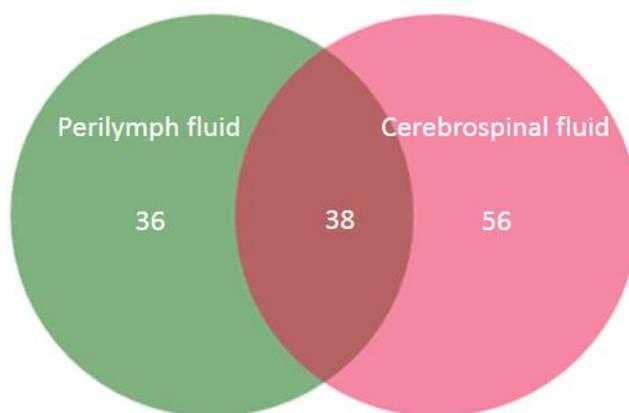


Fig 2. Venn diagram analysis. Comparison of number of metabolites detected by LC-MS from 0.8 μL of perilymph fluid and CSF with a $\text{CV} < 30\%$: 38 were common (29%), 36 and 56 were specific, respectively. These metabolites were observed in the 5 repeatedly injecting quality control (QC) samples.

3.2. Analytical optimization from sub-microliter sample volume (0.8 μL) of CSF

Because high-resolution full-scan acquisition allows for analysis of the spectral data contained in chromatograms (non-targeted) and a selection of previously identified metabolites (targeted), the effects of optimization parameters were evaluated according to these two approaches. Four parameters were studied: extraction without evaporation, extraction with evaporation then suspension in MeOH/water, optimization of the chromatographic gradient, and injection volume (Tables 1 and 2). Evaluation was performed with the non-targeted and targeted approaches for features with a $\text{CV} < 30\%$ and on the mean reproducibility of these features (see the typical total ion current (TIC) chromatograms in the supplementary data, Figure S1).

First, extraction with methanol followed by evaporation and re-suspension in the mobile phase allowed for a higher number of features to be detected, compared to no evaporation. This procedure was therefore used to evaluate the other optimization parameters. Increasing the extraction volume from 100 μL to 200 μL resulted a slight decrease in the number of features (Table 1) and metabolites (Table 2) detected, as well as slightly increased

variability. This may be due to a longer evaporation time needed for the 200 μL volume, resulting in a slight poorer detection. Extraction with 100 μL of MeOH was therefore used to evaluate the other optimization parameters. The solvent gradient of the mobile phase was also evaluated. The use of gradient B (with a lower percentage of methanol; see supplementary data, Table S1) led to a lower detection of features; thus, gradient A was used to evaluate the other optimization parameters. Finally, the volume of injection in the HPLC system (10 or 20 μL) had a very little impact on the number of features detected; thus, a volume of 10 μL was therefore satisfactory.

Table 1. Non-targeted analysis from 0.8 μL of CSF. Optimization of gradient, volume of extraction solvent, extraction process, and injection volume for non-targeted LC-HRMS analysis with a C18 column, in positive and negative ionization mode (HESI). Only one parameter was modified at each condition.

		Number of features with CV < 30%		Mean CV (%)	
		HESI +	HESI -	HESI +	HESI -
Extraction process ¹	Injection without evaporation (n=5)	861	249	11.2	13.3
	Evaporation, then suspension in MeOH/water (n=10)	1052	277	10.3	11.0
Gradient A ²	MeOH 100 μL (n=10)	1084	360	10.7	11.4
	MeOH 200 μL (n=10)	1036	273	13.9	15.0
Gradient B ²	MeOH 100 μL (n=3)	432	215	13.6	13.2
	MeOH 200 μL (n=3)	367	131	11.5	15.1
Injection volume ³	20 μL (n=5)	961	299	13.3	13.5
	10 μL (n=5)	987	298	12.2	14.1

¹ Gradient A, extraction with 100 μL of MeOH, without concentration

² Injection done after extraction, with evaporation

³. Gradient A, extraction with 100 μL of MeOH, injection done after extraction, with evaporation

The above parameters were also evaluated using a targeted analysis. As shown in Table 2, the 100 μL MeOH extraction volume was preferable with evaporation and then re-suspension in the mobile phase. The injection volume of 10 μL was preferred because it allowed the sample to be preserved for analysis with a subsequent re-assay.

Table 2. Targeted analysis from 0.8 μL of CSF. Optimization of gradient, volume of extraction solvent, extraction process, and injection volume for targeted LC-HRMS analysis with a C18 column, in positive and negative ionization mode (HESI). Only one parameter was modified at each condition.

		Number of metabolites with CV <30%		Mean CV (%)	
		HESI +	HESI -	HESI +	HESI -
Extraction process ¹	Injection without evaporation (n=5)	56	25	12.6	16.2
	Evaporation, then suspension in MeOH/water (n=10)	70	39	13.0	14.1
Volume of extraction solvent ²	MeOH 100 μL (n=10)	63	36	15.8	17.2
	MeOH 200 μL (n=10)	41	30	17.8	15.9
Injection volume ³	20 μL (n=5)	66	37	14.3	15.2
	10 μL (n=5)	75	26	13.1	14.4

¹Gradient A, extraction with 100 μL of MeOH

²Gradient A, injection done after extraction, without concentration

³Gradient A, extraction with 100 μL of MeOH, injection done after extraction, with concentration

Thus, a sub-microliter sample of CSF was sufficient for non-targeted and targeted metabolomics study with good repeatability according to the low CV (10-14%) in RP C18

LC-MS analysis. The optimized protocol described above was used for analysis of human perilymph samples.

3.3. Application: analysis of human perilymph using LC-MS (with RP C18 and HILIC support)

The optimized protocol was used to analyze 23 human perilymph samples, using two complementary chromatographic supports (RP C18 and HILIC) and ESI + and ESI- ionization modes (see typical TIC chromatograms in supplementary data, Fig. S2). By combining these two types of columns, 98 metabolites were detected; 25 of these were found in both the C-18 and HILIC supports. This result clearly shows the advantage of using several types of chromatographic columns, which allow for a broader metabolite detection, as only 43 metabolites (63%) were detected by C18 and 30 metabolites (54%) by HILIC (Fig. 3).

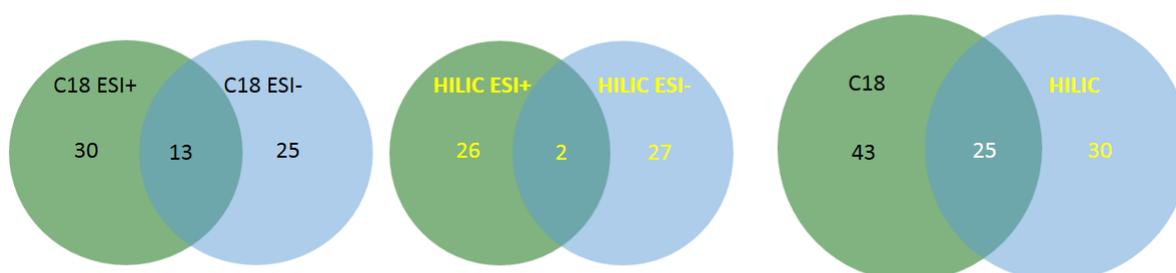


Fig. 3. Venn diagram analysis (Bardou et al., 2014). Comparison of number of metabolites detected by LC-MS in perilymph fluid with a C18 or a HILIC column, in positive and negative ionization mode (HESI)

The 20 metabolites with greatest intensity for each type of column are listed in Table 3. All metabolites detected and repeatability (CV) are listed in supplementary data (Table S2).

Table 3. The 20 metabolites with greatest intensity for each type of column detected in human perilymph by RP C18 and HILIC LC-MS analyses.

Metabolite	Column	Chemical class (from HMDB)
12-Hydroxydodecanoic acid	C18+	Fatty acid
2,4-Hexadienoic acid	C18+	Medium-chain fatty acid
2-Hydroxybutyric acid	C18-	Hydroxy acid
3-Hydroxybutyric acid	Hilic-	Hydroxy acid
3-Hydroxymethylglutaric acid	Hilic-	Hydroxy fatty acid
5-Hydroxylysine	Hilic +	Alpha-amino acid
Acetylcarnitine	Hilic +	Carboxylic acid
Adenosine	Hilic +	Purine nucleoside
Adipic acid	Hilic-	Dicarboxylic acid
Alanine	C18+	Amino acid
Allantoin	C18-	Aliphatic acid compound
Allothreonine	C18-	Alpha-amino acid
α -D-Glucose*	Hilic- C18-	Carbohydrate
α -Hydroxyisobutyric acid	Hilic-	Alpha hydroxy acid
α -Lactose	C18-	Polyol
Arabitol	C18-	Polyol
Arginine	Hilic- Hilic +	Amino acid
Asparagine*	C18- Hilic- Hilic +	Carboxylic acid
Butyric acid	Hilic-	Carboxylic acid
Carnitine*	Hilic + C18+	Carboxylic acid
Citrulline	Hilic +	Amino acid
Creatine*	Hilic + C18+	Carboxylic acid
Creatinine	Hilic + C18+	Aliphatic heteromonocyclic compound
Galactitol	C18-	Polyol
Gamma-Aminobutyric acid	C18+	Amino acid
Gluconic acid	C18-	Polyol
Glutamine*	C18+ Hilic +	Amino acid
Glutaric acid	Hilic-	Dicarboxylic acid
Glycerol 1-myristate	C18+	Alcohol/polyol
Glycerophosphocholine	Hilic +	Glycerophospholipid
Glycine	C18+	Amino acid
Histidine	Hilic +	Amino acid
Homoserine	C18- C18+	Amino acid
Hydroxyphenyllactic acid	C18-	Phenylpropanoic acid
Hypoxanthine	Hilic +	Bicyclic aromatic compound
Isobutyric acid	Hilic-	Carboxylic acid

Ketoleucine	C18-	Keto acid
Lactic acid*	Hilic-	Hydroxy acid
Lysine	Hilic + C18+	Amino acid
Maltose	C18-	Polyol
Mannitol	Hilic-	Polyol
Methylglutaric acid	Hilic-	Methyl-branched fatty acid
Methylmalonic acid	C18-	Carboxylic acid
Monoethyl malonic acid	Hilic-	Dicarboxylic acid
N-Acetylleucine	Hilic +	Carboxylic acid
N-Acetylputrescine	Hilic +	Carboximidic acid
Niacinamide	Hilic +	Pyridinecarboxylic acid
Pantolactone	C18-	Lactone
Phenylalanine	C18+	Aromatic acid
Pimelic acid	Hilic-	Fatty acyl
Proline	Hilic + C18+	Amino acid
Pyroglutamic acid	Hilic +	Alpha amino acid
Ribose	C18-	Carbohydrate
Serine	Hilic +	Amino acid
Suberic acid	Hilic-	Dicarboxylic acid
Succinic acid	C18-	Carboxylic acid
Taurine	Hilic- C18+ C18-	Aliphatic acyclic compound
Threonine	Hilic- C18+	Amino acid
Trigonelline	Hilic +	Heteroaromatic compound
Valeric acid	Hilic-	Fatty acyl
Valine	C18+	Amino acid
Xanthine	Hilic-	Heteroaromatic compound

* metabolites with the higher signal intensity

Fig. 4 shows the relative intensity for the 22 metabolites most strongly detected. Asparagine and lactate were the most intense signals. Lactate and hypoxanthine exhibited presented high cumulative biological and analytical CV (black error bars) but much smaller CV in QC, corresponding to the analytical variability (red error bars; <7% and <14%, respectively, as described in supporting data Table S3). In a quick and simple pathway analysis, these 22 most intense metabolites are present in the nitrogen metabolism, namely histidine, phenylalanine, glutamine and asparagine. These last two metabolites, as well as alanine, are implicated in the “alanine, aspartate and glutamate” metabolism. Glutamine,

asparagine are also implicated in the “arginine and proline” metabolism as well as creatinine and proline. We could also mention that lactate and glucose are implicated in the glycolysis or gluconeogenesis.

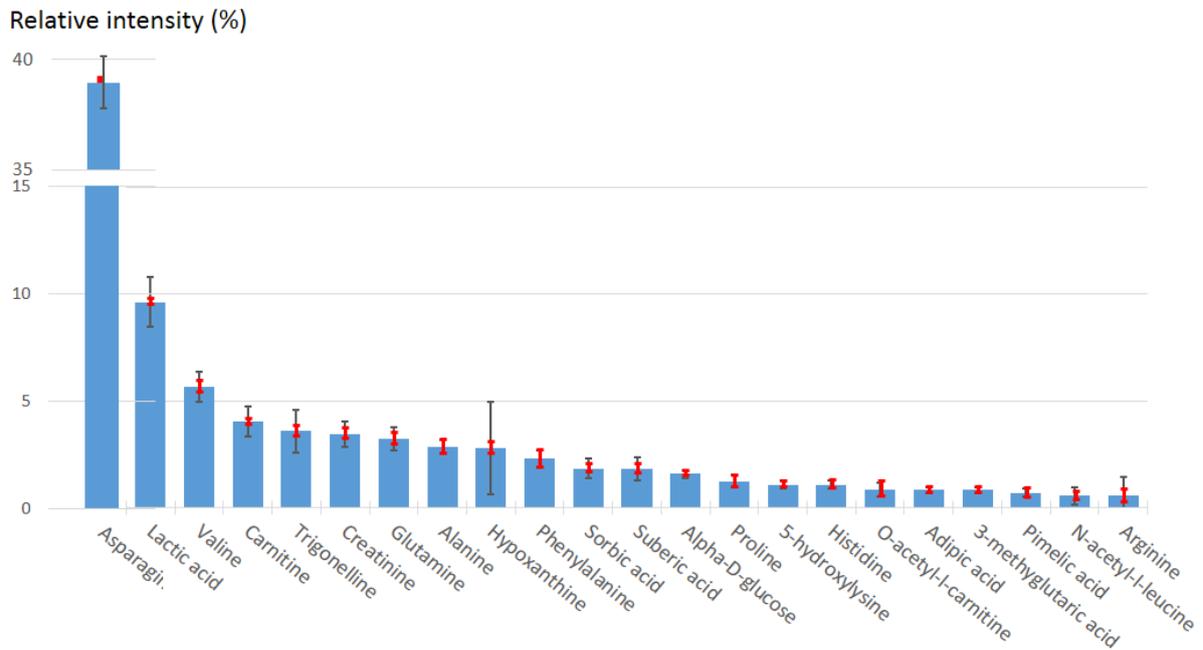


Fig. 4. Relative intensity for the 22 metabolites most strongly detected in 23 human perilymph fluids. The black error bars show CV obtained from the 23 biological samples. The red error bars show CV for QC samples (repeatability).

Fig. 5 shows signal intensities for 30 metabolites with low biological variability observed for 23 patients undergoing cochlear implantation surgery for bilateral sensorineural hearing loss; ribose exhibited the greatest intensity, reproducible irrespective of the patient.

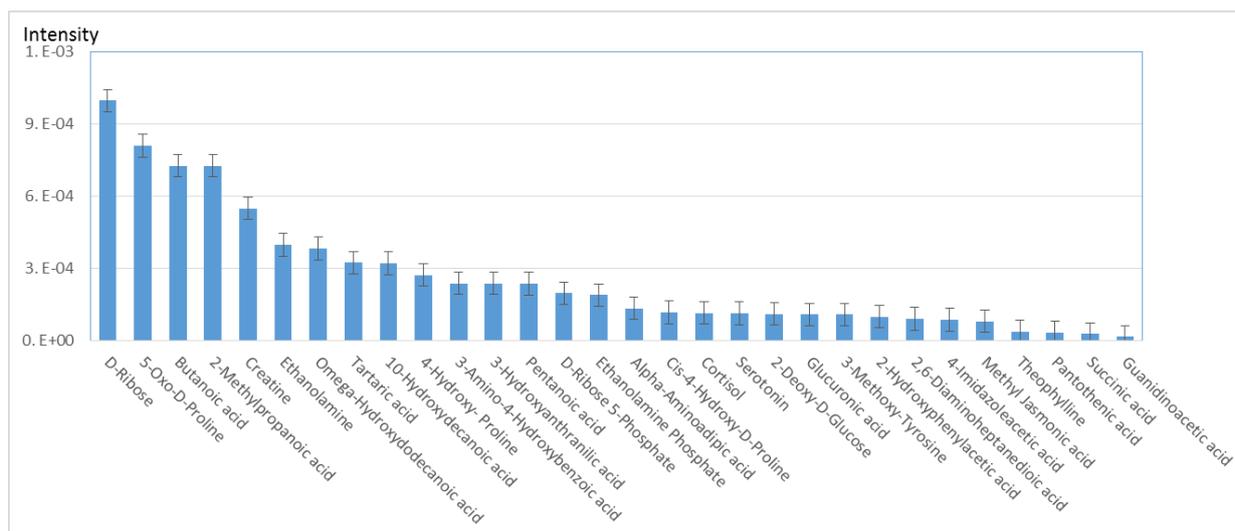


Fig. 5. Signal intensities of metabolites, from 23 perilymph fluids, with the lower biological (and analytical) variabilities irrespective of the patients.

Furthermore, this approach could also be done to improve knowledge about circadian regulation in the auditory system. It was demonstrated that the auditory system is involved in circadian entrainment. However, little is known (Basinou et al., 2017) regarding the molecular components of the clock machinery that drive vulnerability or resilience to noise.

3.4. Comparison of human perilymph and inner ear fluid in guinea pig cochlea

The composition of inner ear fluid (mainly perilymph fluid) of guinea pigs cochlea was previously analyzed using GC-MS (Fujita et al., 2015). The present human perilymph samples were compared to inner ear fluid from guinea pig. From the 72 metabolites detected by GC-MS from guinea pig samples and the 74 metabolites detected by LC-MS from human perilymph, only 15 were found to be in common. The relative intensities of MS signals for these common metabolites were similar across samples, except for creatinine, which was very intense in human perilymph but only a minority compound in guinea pig (Fig. 6). Lactate was the one of the most intense signals for both samples.

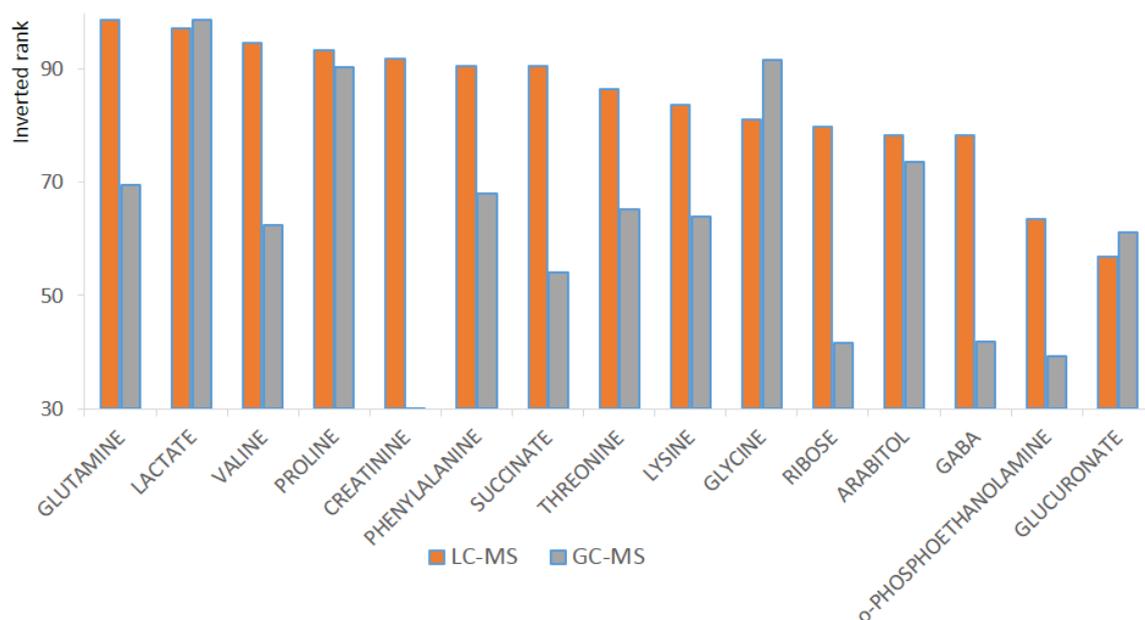


Fig. 6. Inverted rank $[100 - (\text{rank} \times 100 / \text{total number of metabolites})]$ of feature intensity, where the most intense metabolite = 100.

4. Conclusion

An optimization of RP LC-MS protocol was performed using 0.8 μL of CSF. This protocol was then applied to human perilymph fluid, leading to the robust detection of nearly 100 metabolites from two complementary chromatographic stationary phases (C18 and HILIC). The feasibility of metabolomics on very small volume of biological fluid such as perilymph was demonstrated, giving new opportunities to improve diagnosis and knowledge of pathogenesis.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the (doi:xxxxxxxxxxxxxxxx) with elution gradients for C18 LC-MS; typical TIC chromatograms obtained by LC-MS from CSF and from perilymph fluid ; and reproducibility assessment of all detected metabolites in perilymph fluid.

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