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## **Targeted analysis of glycerophospholipids and mono-, di- or tri-acylglycerides in liver cancer**

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**Running head:** Targeted analysis of glycerophospholipids in liver cancer

## **Abstract**

The metabolic rearrangements of hepatic metabolism associated with liver cancer are still incompletely understood. There is an ongoing need to identify novel and more efficient diagnostic biomarkers and therapeutic targets based on the metabolic mechanisms of these diseases. In comparison to the traditional diagnostic biomarker, metabolomics is a comprehensive technique for discovering chemical signatures for liver cancer screening, prediction, and earlier diagnosis. Lipids are a large and diverse group of complex structured biomolecules that are at the heart of the liver physiology and play an important role in the development and progression of cancer. In this chapter, we described two detailed protocols for targeted lipids analysis: glycerophospholipids and Mono, Di, Tri-acylglycerides, both by Flow Injection Analysis (FIA) HPLC coupled to a SelexIon/QTRAP 6500+ system. These approaches provide a targeted lipidomic metabolomic signature of dissimilar metabolic disorders affecting the liver cancers.

## **Keywords**

Liver cancer, Lipids, targeted analysis, glycerophospholipids, mono-acylglycerides, bi-acylglycerides, tri-acylglycerides, SelexIon/QTRAP 6500+ system.

## **1. Introduction**

The liver plays a cardinal role in metabolism since it assimilates nutrients absorbed in the small intestine, removes toxins from the digestive tract and peripheral blood, maintains the balance of blood sugar levels, and synthesizes plasma protein including albumin and blood clotting factors *(1)*. Perturbation of liver metabolism plays a major pathogenic role in hereditary hemochromatosis, alpha-I antitrypsin deficiency, Wilson disease and local cancers. The incidence and mortality rates of liver cancer are continually rising, making it the third most common cancer-related cause of death worldwide *(2)*. Hepatocellular carcinoma (HCC), which accounts for up to 90% of cases, and cholangiocarcinoma (CCA), which accounts for 10% to 15%, are the two most common kinds of primary liver cancer *(3)*. Common risk factors for the development of HCC include chronic viral infections *(4)*, alcohol abuse *(5)*, and metabolic diseases *(6)*, such as non-alcoholic fatty liver disease (NAFLD), obesity or diabetes *(7)*. All these risk factors are associated to hepatic damage, including inflammation and steatosis, which may progress to fibrosis/cirrhosis and HCC *(8)*.

Early diagnosis and efficient treatment of HCC and CCA are rendered difficult by the complex heterogeneity of these malignancies. The lack of effective screening methods for early diagnosis has been a major obstacle to improving the survival rate of patients with HCC *(9)* and CCA *(10)*. Biomarkers have been identified for the clinical management of HCC and CCA, such as alpha-fetoprotein (AFP) and carbohydrate antigen 19.9 (CA19-9) respectively. Moreover, HCC can be detected in patients belonging to high-risk groups by abdominal ultrasound (US), although this technique is still relatively insensitive *(11-14)*. Metabolomic profiling has been proposed to identify circulating metabolites as potential biomarkers *(15, 16)*. However, this requires the identification of cancer-associated alterations in the metabolome as potential diagnostic or prognostic ‘signatures’, followed by their validation in independent cohorts. Targeted metabolomics reportedly detects specific metabolites to be enriched in HCC

patients. Such HCC-associated metabolites include aminoacids and their derivatives, such as methionine, proline and ornithine, as well as some acylcarnitines, such as pimelylcarnitine and octanoylcarnitine (17). Advances in targeted lipid-specific metabolomics (lipidomics) have highlighted alterations of lipids in HCC and in CCA, pinpointing potential mechanisms for carcinogenesis and disease progression (18-20). Specifically, dynamic changes in fatty acids, glycerophospholipids and mono-, di- and triglycerides were found in the development and progression of primary liver cancer (21, 22).

Taken together, metabolomics may constitute a comprehensive approach for discovering molecular signatures that will turn out useful for the identification of high-risk patients, early diagnosis of liver cancer, as well as for its clinical management (8). At this point, liver biopsy, which bears the significant risk with respect to morbidity and mortality, is the standard method for diagnosing HCC (23) and CCA (24) calling for alternative methods.

To explore the role of lipid metabolism during hepatic carcinogenesis, we developed two novel strategies for targeted lipidomic detection of glycerophospholipids and mono-, di- and triacylglycerides by flow injection analysis (FIA) high-performance liquid chromatography (HPLC) coupled to a SelexIon/QTRAP 6500+ system.

## **2. Materials**

### **2.1. Disposables**

1. 1.5 mL polypropylene Safe-lock centrifugation microtubes
2. Precellys tubes 1.5 mL CK28-R
3. 96 wells u-shape polypropylene plates
4. Standard plastic tips (10  $\mu$ L, 200  $\mu$ L 1000  $\mu$ L)
5. Filter, 0.22 $\mu$ m, 250ml

### **2.2. Equipment**

1. Tissue homogenizer Precellys 24
2. MilliQ water generator Reference A+
3. Precision balance LA 214i
4. Mass spectrometer QTRAP 6500+ (Sciex)
5. SelexIon+ module (Sciex)
6. Vortex
7. Centrifuge
8. Evaporator (under a chemical hood)
9. SpeedVac Concentrator

### **2.3. Software**

1. Analyst (v.1.7.1) (Sciex)
2. LipidView (v.1.2) (Sciex)
3. R software (freely available at <https://www.r-project.org>) for image analysis and statistical evaluation.

### **2.4. Reagents**

1. Methanol (MeOH)
2. Methyl tert-butyl ether (MTBE)
3. Isopropanol (IPA)

4. Formic acid

5. Ammonium acetate

### **3. Methods**

#### **3.1. Sample preparations**

Sample preparation of the liver is similar to Durand et al. 2021 (*the intracellular metabolome of starving cells*) (25) (Figure 1).

1. Weigh about 30 mg of liver and place them in 1.5 ml polypropylene microtubes containing ceramic beads.
2. On ice, add 150  $\mu$ l of water, 600  $\mu$ l of MTBE and 180  $\mu$ L Methanol in a ceramic bead tube for protein precipitation and liquid-liquid extraction (*see Note 1*).
3. Homogenize tubes in a tissue homogenizer 3 times 20s at 5500 rpm.
4. After homogenization, transfer the upper phase (500  $\mu$ l of MTBE) to microtubes and evaporate them with an evaporator under a chemical hood.
5. Spike the dry extracts with 200  $\mu$ l of injection buffer Methanol/MTBE (1/1) and 10 mM Ammonium acetate (*see Note 2*).
6. Vortex 5 minutes at 2500 rpm, and centrifuge 10 minutes at 15000g (+4°C).
7. Transfer 20  $\mu$ l of each sample to a microtube to create a pooled sample used as quality control monitoring and correction (see part Quality control pool) (*see Note 3*).
8. Transfer 150  $\mu$ l of each sample to a polypropylene 96well plate until the mass spectrometry (MS) analysis (*see Note 4*).
9. From this plate, transfer 100  $\mu$ l to another polypropylene 96-well plate to create a second aliquot (*see Notes 5 and 6*).
10. Store at -80°C until the analysis or direct HPLC analysis (*see Note 7*).

#### **3.2. Targeted analysis of glycerophospholipids by Flow Injection Analysis (FIA) high-performance liquid chromatography (HPLC) coupled to a SelexIon/QTRAP 6500+ system**

The targeted analysis is performed on a RRLC 1260 system (Agilent Technologies) coupled to a 6500+ QTRAP (Sciex) equipped with a modified electrospray ion source (*see Note 8*) and a SelexIon module.

1. Operate the instrument by using Multiple Reaction Monitoring (MRM) in negative ion mode with a unit resolution for Q1 and Q3 (*see Note 9*).
2. Set the intervals between mass ranges to 20 ms.
3. Set the ion spray source temperature to 200°C.
4. Set the curtain (CUR) gas pressure to 35 psi.
5. Set the gas 1 (GS1) pressure to 15 psi and gas 2 (GS2) pressure to 0 psi.
6. Set the ion-spray voltage (IS) to +4500 V.
7. Set the collision-activated dissociation (CAD) to medium.
8. Set the entrance potential (EP) to -10 V.
9. Set the declustering potential (DP) to -120 V.
10. Set the DMS temperature to medium.
11. Use isopropanol as a modifier.
12. Set the modifier composition (MDC) parameter at low.
13. Set the separation voltage to 3800 V and the DMS Offset to 3.0 V.
14. Set the DMS Resolution Enhancement to low (*see Note 10*).
15. Inject 40  $\mu\text{L}$  of the sample (*see Note 11*).

The isocratic mobile phase (*see Note 12*) consists of Methanol/MTBE (1/1), and 10 mM ammonium acetate (*see Note 13*).

16. Set the flow rate to 0.02 mL/min.
17. Set the duty cycle to 6 minutes.
18. Use 863 MRM transitions in positive mode (*see Notes 14 and 15*).

Molecules begin to reach the MS in half a minute and are totally eluted from the LC system in 5 minutes.

### **3.3. The targeted analysis of mono-, di- and tri-acylglycerides by flow injection analysis (FIA) high-performance liquid chromatography (HPLC) coupled to a SelexIon+/QTRAP 6500+ system**

Targeted analysis is performed on a RRLC 1260 system (Agilent Technologies) coupled to a 6500+ QTRAP (Sciex) equipped with a modified electrospray ion source and a SelexIon+ module.

1. Operate the instrument by using multiple reaction monitoring (MRM) in positive ion mode with a unit resolution for both Q1 and Q3.
2. Set intervals between mass ranges to 50 ms.
3. Set the ion spray source temperature to 0°C.
4. Set the curtain (CUR) gas pressure to 20 psi.
5. Set the gas 1 (GS1) pressure to 15 psi and gas 2 (GS2) pressure to 0 psi.
6. Set the ion-spray voltage (IS) to +4500 V.
7. Set the collision-activated dissociation (CAD) to medium.
8. Set the entrance potential (EP) to 10 V.
9. Set the declustering potential (DP) to 60 V.
10. Set the differential mobility spectrometry (DMS) to off (*see Note 16*).
11. Inject 40 µL of the sample.

The isocratic mobile phase consists of Methanol/MTBE (1/1) and 10 mM ammonium acetate.

12. Set the flow rate to 0.02 mL/min.
13. Set the duty cycle to 6 minutes.
14. Use 1132 MRM transitions in negative mode (*see Note 15*).

Molecules begin to reach the MS in half a minute and are totally eluted from the HPLC system in 5 minutes.

### **3.4. Quality control pool**

During the sample preparation, a small aliquot of each biological sample has to be mixed in a pool of quality control (QC) samples. The pool must be injected before and through the batch to monitor and eventually correct sensitivity drifts (see **Note 17**).

### **3.5. LC/MS system rinsing**

At the end of the batch analysis:

1. Rinse the LC/MS system with 0.15 mL/min of MilliQ water/ acetonitrile (CAN) (1/1) for 30 minutes (see **notes 18 and 19**).
2. Shut down the system.

### **3.6. Data processing with LipidView 1.2**

LipidView is a branded software supplied by Sciex. This software allows the handling of data acquired with the QTRAP 6500+ equipped with a Selexion+.

1. Open a new LipidView session.
2. Load the data files of the samples to process.
3. Gather the samples by condition.
4. Choose the MRM mode and follow the data acquired, either negative/DMS or positive/noDMS mode.
5. Process data and analyze the results (see **Note 20**).

#### 4. Notes

1. Water, MTBE and Methanol must be cooled at 4°C beforehand.
2. Buffer should be filtered with a 2 µm pore size vacuum filter during the last step of its preparation. This step is necessary to remove potential solid elements.
3. These steps of transfer of MeOH/MTBE should be done under the chemical hood because of the toxicity of these organic solvents and taking into account that MTBE is highly volatile: first spiked samples could be evaporated during the operation transfer if the operator does not proceed fast enough.
4. It is mandatory to use polypropylene at each step of the experience since polystyrene does not stand MTBE solvent.
5. As solvents in which the final aliquots are diluted (MeOH and MTBE) and are highly volatile, the sample evaporates by the hole opened, by the needle during the injection, in the folder. For this reason, it is necessary to have two aliquots for two injections, in negative polarity, and in positive polarity.
6. After injection of 40 µL from the second plate, the 60 µL are evaporated (see **Note 4**). 60 µL of buffer MeOH/MTBE (1/1) 10mM ammonium acetate are spiked and carefully sealed before being stored at -80°C for backup.
7. Because of evaporation at -80°C, it is advised to limit the duration of storage before injection.
8. The ESI (Electrospray Ionization) source was previously upgraded with elements adapted to the new Drift Mass Spectrometry (DMS) cell nest within the source.
9. Fast switch polarity is not used to avoid an unwarranted extension of the acquisition duty cycle.
10. DMS is here useful to disambiguate molecules with the same MRM transition but part of different class families (e.g. phosphatidylcholine/PC and phosphatidylethanolamine/PE).

11. This volume is a minimum when injected without any column (FIA).
12. To avoid ripple and air in our binary LC system, split the flow of the mobile phase 1 to 1, between lines A1 and B1.
13. As for the injection buffer, the mobile phase must be filtered with a 2  $\mu\text{m}$  pore size filter at the last step of its preparation.
14. It is important to not reach more than  $10^7$  counts of the intensity, to avoid damaging the MS detector.
15. These settings correspond to 15 lipid families (see Table 1).
16. We found no profit with DMS parameters, and there is no need to disambiguate class families in this MRM method.
17. QC samples are not used to remove non-specific interaction (this system does not have a LC column).
18. This rinse allows thorough cleaning of the system from the needle to the ESI probe to avoid ammonium acetate sedimentation.
19. To avoid electric arks between its two electrodes, the DMS cell is daily cleaned following each batch of analysis. It is recommended to follow the heavy cleaning procedure set by Sciex.
20. It is also possible to export data in a table file toward R to analyze them in a GRMeta workflow (Kroemerlab/GRMeta on github.com) (25).

## **Conflicts of interest**

GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Sotio, Tollys, Vascage and Vasculox/Tioma. GK has been consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders.

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## Figure legend

**Figure 1:** Graphical abstract of sample preparation for lipid analysis. (MeOH, methanol; MTBE, methyl tert-butyl ether).

**Table 1: Lipid coverage**

<b>Lipid class</b>	<b>Number of metabolites</b>	<b>Polarity</b>	<b>DMS</b>
Monoacylglyceride	19	POS	No
Diacylglyceride	190	POS	No
Triacylglyceride	445	POS	No
Phosphatidylethanolamine	114	POS	No
Lactosylceramide	19	POS	No
Glucosylceramide	19	POS	No
Ceramide	24	POS	No
CholesterylEster	21	POS	No
Sphingomyelin	12	POS	No
Phosphatidylcholine	188	NEG	Yes
Phosphatidylethanolamine	185	NEG	Yes
Phosphatidylglycine	189	NEG	Yes
Phosphatidylinosine	190	NEG	Yes
Phosphatidylserine	190	NEG	Yes
Phosphatic acid	190	NEG	Yes

Abbreviations: DMS, differential mobility spectrometry; NEG, negative; POS, positive

