# Immunoaffinity Capture Mass Spectrometry for Endogenous Lipid Biomarker Analysis

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## Introduction

Immunoaffinity (IA) capture has multiple benefits over traditional SPE for targeted trace analysis. The specificity of the antibody provides minimal risk of column overloading, which allows for the use of large sample volumes necessary for trace analyte enrichment. Samples are cleaner, which minimizes the problems associated with matrix interferences. The promiscuous nature of antibodies, which is the critical concern being raised about their use in assays, is overcome by the improved specificity and resolving power of the LC-MS/MS instrumentation.[1,2] The combination of these techniques offers an optimal solution for trace biomarker analysis.

Leukotrienes (LTs) are acute inflammatory mediators and downstream products of arachidonic acid oxidation by the enzyme 5-lipoxygenase (5-LO).[3] Due to their putative role in the pathogenesis of inflammatory diseases such as asthma, neurological diseases, and diabetes, LTs are under investigation to further understand their function in disease progression and to determine their potential value as biomarkers.[4-6] However, their low abundance and rapid clearance make LTs difficult to measure in biological matrices. GC-MS has been successfully used to measure LTs but requires multiple derivatization steps. Additionally, suspected antibody cross reactivity from other isobaric oxylipids raises concern about the use and accuracy of ELISAs.[7] We have developed a robust and sensitive IA capture LC-MS/MS method to isolate and quantify $LTB_4$, $LTC_4$, $LTD_4$, and $LTE_4$ in biological samples.

## Immunoaffinity Capture

The polymer-based IA resins were manufactured in-house using proprietary methods.

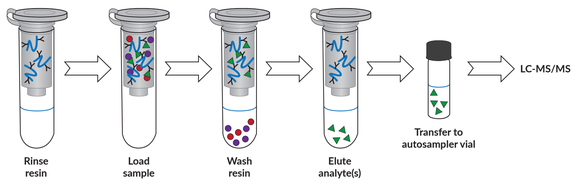
In order to measure the cysteinyl LTs (CysLTs) and $LTB_4$, we prepared two separate IA resins using Cayman's CysLT Monoclonal Antibody and $LTB_4$ Monoclonal Antibody.

Prior to use, the analytical performance of each resin was verified by applying plasma spiked with $LTB_4$ or $LTC_4$, running the standard enrichment protocol, and analyzing the eluents by ELISA (Leukotriene $B_4$ Express ELISA Kit (Cayman Item No. 10009292) and Cysteinyl Leukotriene ELISA Kit (Cayman Item No. 500390)). Precision, recovery over linear range, carryover, loading capacity, and specificity of each resin was measured and shown to meet predetermined quality requirements (data available upon request).

Sample extraction was performed using the following procedure:

1. $LTB_4$ and CysLT resins were mixed in a 1:1 ratio prior to applying the sample: 250 µl (as slurries in $K_2PO_4$ buffer) of $LTB_4$ IA resin and CysLT IA resin were aliquoted into a 2 ml spin column, rinsed with 700 µl of $K_2PO_4$ buffer, and the rinse was removed by centrifugation at 5,000 x g.
2. Brain tissues (flash frozen and stored at -80°C until use) were homogenized at 100 mg/ml in buffer (0.1 M $K_2PO_4$, pH 7.0, containing 1 mM EDTA and 10 µM indomethacin) and centrifuged to produce the supernatants. 1 ml of supernatant was diluted to 2 ml total volume with $K_2PO_4$ buffer, and homogenate was loaded in 4 x 500 µl portions onto the resin for extraction.
3. The resin was washed with 700 µl of homogenization buffer, and the wash was removed by centrifugation at 5,000 x g. The resin was washed a second time using an additional 700 µl of water followed by centrifugation at 8,000 x g.
4. The target analytes were eluted form the resin with 700 µl methanol (at 5,000 x g) into 10 µl of a proprietary trapping solution that minimizes loss of $LTC_4$.
5. Samples were dried under nitrogen and reconstituted in 100 µl of 50:50 acetonitrile/water for analysis.

### Figure 1. Immunoaffinity Capture Workflow




## LC-MS/MS Method

**HPLC**
Waters Acquity UPLC I-Class
Mobile Phase: (A) Water + 0.1% Formic Acid
(B) Acetonitrile + 0.1% Formic Acid
Column: Waters BEH C18 (2.1 x 100 mm, 1.7 µm) at 30°C
Gradient: 15%B 0 min, 44%B 15 min, 95%B 16 min

**MS**
Waters Xevo TQ-Sµ
Gases/Temps: 600°C Desolvation Temp; 1,000 L/hr Desolvation Gas; 100 L/hr Cone Gas
Voltages: (ESI- $LTB_4$) 2.4 kV Capillary; 40 V Cone (ESI+ CysLTs) 1.1 kV Capillary; 50 V Cone

### Table 1. Mass Transition List

| Parent | Transition | IS | Transition |
|---|---|---|---|
| $LTB_4$ | 335.4 > 195.1 | $LTB_4$-$d_4$ | 339.4 > 197.1 |
| $LTC_4$ | 626.3 > 308.1 | $LTC_4$-$d_5$ | 631.3 > 194.2 |
| $LTD_4$ | 497.3 > 189.2 | $LTD_4$-$d_5$ | 502.2 > 194.2 |
| $LTE_4$ | 440.2 > 189.2 | $LTE_4$-$d_5$ | 445.1 > 194.2 |

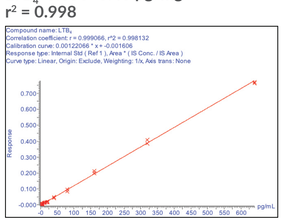
### Table 2. Precision

| | %CV (n=6) | | |
|---|---|---|---|
| | 20 pg/ml | 100 pg/ml | 500 pg/ml |
| $LTB_4$ | 7.1 | 3.19 | 2.6 |
| $LTC_4$ | 10.5 | 3.08 | 3.6 |
| $LTD_4$ | 13.1 | 4.6 | 2.2 |
| $LTE_4$ | 10.9 | 6.9 | 3.5 |

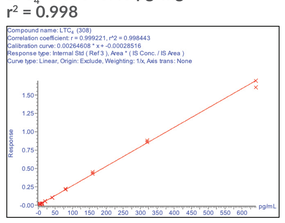
Due to the specificity of the IA enrichment method matrix interferences were not observed. This allowed for the preparation of calibrators directly in buffer over the range of 5-600 pg/ml. Linearity, precision, and recovery were within acceptable ranges (Figure 2, Tables 2 and 3).

### Figure 2. Linear Range

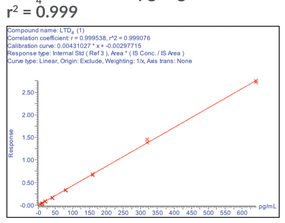
$LTB_4$ 0.050–6.40 pg/mg
$r^2$ = 0.998



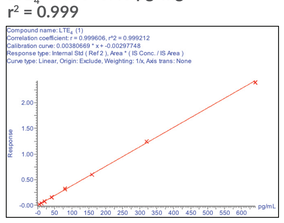
$LTC_4$ 0.050–6.40 pg/mg
$r^2$ = 0.998



$LTD_4$ 0.050–6.40 pg/mg
$r^2$ = 0.999



$LTE_4$ 0.050–6.40 pg/mg
$r^2$ = 0.999



### Table 3. Recovery

100 pg/ml extracted *versus* unextracted

| $LTB_4$ | $LTC_4$ | $LTD_4$ | $LTE_4$ |
|---|---|---|---|
| 94.4 | 90.7 | 99.1 | 97.2 |

### LLOQ

The lower limit of quantitation (LLOQ) of the method as defined by a %CV of <15% was determined to be 0.020 pg/mg for all analytes.

### Carryover and Breakthrough

No method carryover was detected for any of the analytes in blank samples injected after analysis of the high calibrators. A small amount of analyte loss was observed as breakthrough in the washes of the resin (possibly due to resin loading capacity); however, method recovery was determined to be >90% for all analytes.

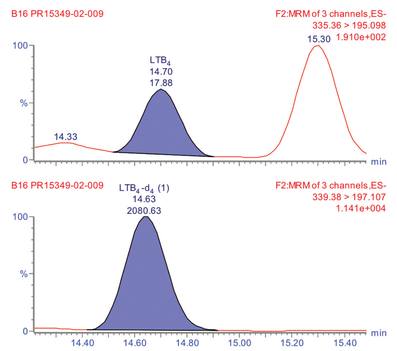
## Results

LTs have previously been found to be present at very low levels in normal mouse brain tissue.[8-10] To test the performance of the method in this matrix, whole brains from healthy C57BL/6 mice were obtained using standard practices and stored at -80°C until use. Brains from three adult (10-week-old) mice and four 2-week-old (p14) mice were analyzed in the initial study.

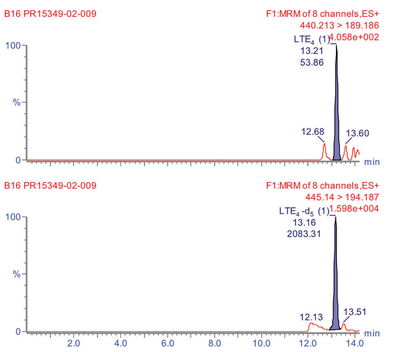
Whole brains were homogenized in a Precellys® Tissue Homogenizer (tissues and buffer were added to 7 ml Precellys® Tubes containing 2.8 mm ceramic beads; samples were homogenized for 2 cycles of 30 seconds at 5,000 rpm with a 30 second rest between cycles) and 100 mg of tissue homogenate was used in the extraction procedure. Sample chromatograms for each analyte are shown in Figure 3. All four LTs were detected in all samples. In one instance (p14-4 mouse), the $LTC_4$ level fell just below the lowest point on the calibration curve, and in this case, the $LTC_4$ level was estimated by extrapolation from the curve. $LTB_4$ ranged from 0.0226–0.1881 pg/mg, $LTC_4$ ranged from 0.0158–0.0892 pg/mg, $LTD_4$ ranged from 0.0454–0.1085 pg/mg, and $LTE_4$ ranged from 0.0295–0.0869 pg/mg. Additionally, LT levels were observed to be lower in the 14-day-old mice compared to the 10-week-old mice; however, the number of samples was too low in this initial method development work to draw any significant conclusions regarding age-related differences in the LT levels in mice.

### Figure 3. Sample Chromatograms for Adult WT-2 Mouse

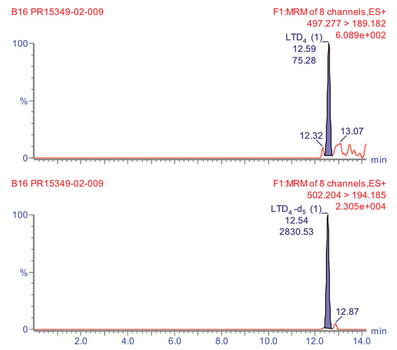
$LTB_4$ (top) and $LTB_4$-$d_4$ (bottom)



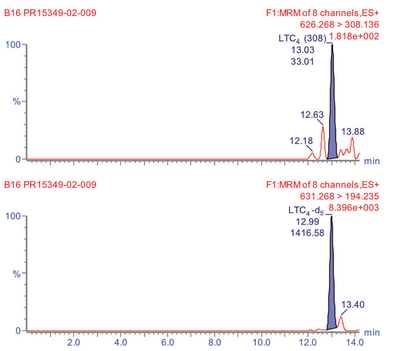

$LTE_4$ (top) and $LTE_4$-$d_5$ (bottom)




$LTD_4$ (top) and $LTD_4$-$d_5$ (bottom)



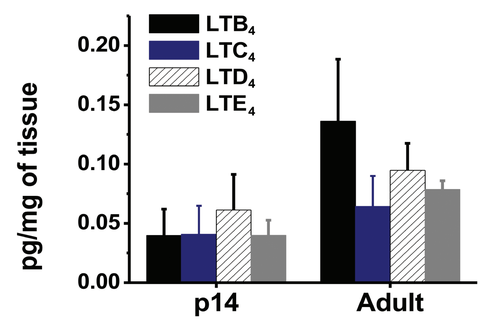

$LTC_4$ (top) and $LTC_4$-$d_5$ (bottom)




### Table 4. Summary of Brain LT Data

| Sample ID | $LTB_4$ | $LTC_4$ | $LTD_4$ | $LTE_4$ |
|---|---|---|---|---|
| p14_WT-1 | 0.0317 | 0.0678 | 0.1027 | 0.0583 |
| p14_WT-2 | 0.0724 | 0.0260 | 0.0626 | 0.0376 |
| p14_WT-3 | 0.0325 | 0.0534 | 0.0340 | 0.0343 |
| p14_WT-4 | 0.0226 | 0.0158 | 0.0454 | 0.0295 |
| *AVG* | 0.0398 | 0.0408 | 0.0612 | 0.0399 |
| *STD DEV* | 0.0222 | 0.0240 | 0.0301 | 0.0127 |
| adult_WT-1 | 0.1368 | 0.0659 | 0.1085 | 0.0734 |
| adult_WT-2 | 0.0836 | 0.0892 | 0.0686 | 0.0757 |
| adult_WT-3 | 0.1881 | 0.0380 | 0.1072 | 0.0869 |
| *AVG* | 0.1362 | 0.0644 | 0.0948 | 0.0787 |
| *STD DEV* | 0.0523 | 0.0256 | 0.0227 | 0.0072 |

### Figure 4. Average LT Levels in Young and Adult Mouse Brain Tissue




## Conclusions

An improved method for measuring trace LT levels in brain tissue was developed that involves the use of IA capture to both enrich the LTs and clean up the sample from other interfering matrix components. Combined with UPLC-MS/MS, this method allows for very sensitive, simultaneous measurement of $LTB_4$, $LTC_4$, $LTD_4$, and $LTE_4$.

The use of IA enrichment to assist LC-MS/MS analysis of targeted, trace biomarkers provides an opportunity to overcome the challenges of immunoassays and improve methods for research and clinical laboratories.

**References**

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