MDxL

BioAnalytical Laboratory, Inc.

1171 E. Rancho Vistoso Blvd.
Suite 155
Tucson, AZ 85755
V: 520.638.6476
F: 520.638.6476
www.MDX-Bio.com

Final Report

Identification and Quantification of Phospholipids in Beaudoin Oil

No. Lab: 09-1651

Sponsored by

Neptune Technologies and Bioressources

December 22, 2009

By:

Earl L. White, Ph.D.
President & CEO

Kenyon Hood, B.S.
Chemist

Introduction

A sample labeled Beaudoin Oil No. Lab: 09-1651 was submitted in a brown opaque 250 mL bottle to MDx BioAnalytical Laboratory for LC/MS and LC-MS/MS analysis to determine the identify of three classes of phospholipids (PL) present and to obtain relative concentrations of each lipid with the hope of obtaining quantitative information about phospholipids containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the sn-1 and sn-2 positions of the glycerol backbone. The PLs of interest included phosphatidylcholine (PC), phosphophatidylethanolamine (PE) and phosphatidylinositol (PI). These PL classes were believed to be present at 85%, 7% and 8% for PC, PE and PL, respectively.

Method

The Beaudoin Oil sample No. Lab: 09-1651 was analyzed by LC/MS. Initially the sample was dissolved in ethyl acetate (~1mg/mL). A white precipitate was observed in the bottom of the flask. The supernatant was removed and the precipitate dried under a stream of nitrogen and found to be water soluble. The ethyl acetate solution was evaporated to dryness and reconstituted to a concentration of 100 ng/µL in 20% ACN with 0.1% formic acid (FA) and subjected to LC/MS. Subsequently, chloroform was used to dissolve the oil sample. A 5 µL aliquot of this sample was injected onto a C18 column and separated on a Waters MicroMass CapLC and detected with a Waters MicroMass Q-TOF Micro quadrupole time-of-flight mass spectrometer with nano electrospray ionization (ESI).   The instrument was operated in the full scan positive ion mode for these samples. Continuum mass spectra were acquired over the mass range of 400-2000 amu with a scan time of 1.80 s and an inter-scan delay of 0.1 s. The capillary, sample cone and extraction cone voltage were 3654, 30 and 2.5 V, respectively. The source temperature was held at 120°C, the cone N_2 gas flow was 50 L/h and the collision energy was held constant at 10 V. The HPLC column was a CVC Micro-Tech C18WS-150EU nano column. HPLC/MS procedure; mobile phase A (0.1% FA H_2O:ACN, 95:5%) was held at 20% B (ACN: 0.1% FA in H_2O, 95:5%) for 4 min and then programmed to 70% B at 5 min, 80% at 15 min then to 100% B at 30 min and held for 36 min. The flow rate was 7 µL/min with a split ratio of about 6:1 yielding a flow through the column of approximately 600 nL/min. For MS/MS analysis, the Q-TOF was operated in the survey scan mode where data-dependent MS/MS analysis was perform on all ions detected above a pre-determined intensity threshold. Optimum collision energies for compound dissociation were determined.

Results:

There were approximately 45 PLs detected in the oil sample and approximately 16 with a relative percentage ≥1%. A list of phospholipids detected in the Beaudoin oil sample labeled No. Lab: 09-1651 is given in the table below with relative percentages based on area under the curve with the caveat that all PLs do not necessarily respond the same. Therefore, these values should be considered an approximate representation of the various PLs and classes of PLs only. Absolute quantification could be obtained by comparing these areas with those of an

authentic standard for each PL. Also, the sample contained major polyethylene glycol (PEG) contaminants which inhibited confirmation by MS/MS for several of the PLs. Other PLs are contained within these chromatograms at lower concentrations. This list is by no means exhaustive, but does contain most of the major PLs.

Figure 1 shows a representative total ion chromatogram for a 5 µL injection of a 0.5 µg/µL oil sample dissolved in CHCl$_3$. Peaks appearing in the first 12 minutes of the chromatogram were primarily low molecular weight PLs which contained essentially OH in the sn-1 or -2 position. A full scan summed mass spectrum for the time 9.5-12.5 min of the chromatogram is shown in Figure 2. Figure 3 shows the portion of the spectrum that contains the major low molecular weight PLs ranging from about 416 to 570 Da. The zoomed portion of the spectrum shown in Figure 4 depicts PEGs in the range of 600-900 Da. The zoomed region shown in Figure 5 represents dimers of the low MW PLs. Dimers and trimers (Figure 6) are characteristic for PLs and are often used as diagnostic ions for confirmation. Representative full scan ESI specta for major peaks in the chromatogram are shown in Figures 7 through 18. Figures 8,10 and 13 are zooms of portions of spectra that contain PLs. Figures 7, 9, 11 and 12 all contain PEGs in the MW range of 600-1000 Da. Figure 11 has two different ranges of PEGs. Those in the 800 Da region caused some inteference with sample and data analysis. Two major PLs were detected in each of the peaks at 19.4 and 20.1 min as shown in Figures 14 and 15.

Peak areas were calculated from the molecular ions for each of the major peaks shown in the spectra. Ion chromatograms for these *m/z*'s are shown in Figures 19 through 28. The bottom chromatogram for each figure represents the total-ion chromatogram for that region of the chromatogram. Areas from these chromatograms were entered into the table and used to calculate relative % for each peak representing the identified PL. Note that some chromatograms contain more than one integrated peak (Figures 22A, B & C, 25A, 26A & B, 27C, and 28B). These peaks represent different PLs or even classes of PL with the same nominal molecular weight. Figure 29 is a representative MS/MS spectrum for a PL with a molecular ion at *m/z* 826.5. Fragment ions detected at *m/z* 524.3 and 542.3 are indicative of 302 and 284 Da losses from the molecular ion which indicate loss of C20:5 and C18:0 fatty acids from the protonate molecular ions.

There were no phosphatidylinositols detected in the oil sample, however several phosphatidylserines were detected.

Table 1   Phospholipids Detected in Beaudoin Oil Sample

Phospholipid	PL Class	RT	m/z	Area	Relative%
C13:0/OH	PC	10.28	454	149	0.60
C18:2/OH	PE	11.27	478	127	0.51
C16:0/OH	PC	10.73	480	142	0.57
C14:0/OH	PC	10.1	468	642	2.58
C16:0/OH	PC	11.3	496	2535	10.20
C16:1/OH+Na*	PC	9.9	516	1318	5.31
C18:1/OH	PC	11.7	522	1013	4.08
C20:5/OH	PC w/EPA	10.4	542	4406	17.74
C22:6/OH	PC w/DHA	11.0	568	1650	6.64
C18:0/OH	PC	10.4	524	169	0.68
C18:1/OH	PE	12.7	524	122	0.49
C20:2/OH	PS	11.0	550	57	0.23
C20:1/OH	PC	13.1	550	77	0.31
C22:6/OH+Na*	PC w/DHA	11.0	590	15	0.06
C22:6/OH	PC w/DHA	14.7	578	208	0.84
Unidentified		16.6	606	28	0.11
C16:0/C22:2	PC	18.3	800	204	0.82
C20:5/C12:0	PC w/EPA	17.9	724	18	0.07
C20:5/C18:3	PC w/EPA	18.3	802	119	0.48
Unidentified		22.7	844	10	0.04
C20:4/C12:0	PC	18.9	726	216	0.87
C20:5/C20:5	PC w/EPA & EPA	18.9	826	543	2.19
C14:0/C20:5	PC	19.4	752	904	3.64
C22:6/C20:5**	PC w/DHA & EPA	19.5	852		
C21:0/C20:5**	PC w/EPA	19.5	852	452	1.82
C12:0/22:4*	PC	20.5	754	849	3.42
C22:6/C13:0**	PC w/DHA	20.5	764	61	0.25
C16:0/C20:5**	PC w/EPA	20.9	764	69	0.28
C22:6/C16:0**	PE w/DHA	21.3	764	19	0.08
C20:5/C18:1**	PE w/EPA	21.9	764	18	0.07
C20:5/C16:0	PC w/EPA	21.0	780	3861	15.54
C22:6/C16:1**	PS w/DHA	21.5	806	656	2.64
C22:6/C16:0**	PC w/DHA	22.0	806	1610	6.48
C18:0/C18:0**	PC	20.9	790	63	0.25
C22:6/C18:1**	PE w/DHA	22.1	790	41	0.17
C22:6/C22:6	PC w/DHA & DHA	20.1	878	162	0.65
C22:6/C14:0**	PC w/DHA	19.8	778	158	0.64
C20:5/C16:1**	PC w/EPA	20.2	778	329	1.32

C16:0/C22:0	PC	21.7	818	29	0.12
C22:0/C16:1	PS	24.5	818	49	0.20
C22:6/C18:1	PC w/DHA	22.5	832	601	2.42
C20:5/C18:0**	PC w/EPA	21.9	808	245	0.99
C22:6/C16:0**	PS w/DHA	23.4	808	150	0.60
C20:5/C21:0**	PE w/EPA	24.0	808	198	0.80
C20:4/C18:1	PC	24.4	808	134	0.54
C20:3/C18:2	PC	25.4	808	87	0.35
C20:5/C20:6**	PC w/EPA	24.8	834	328	1.32
C22:6/C18:0**	PC w/DHA	24.8	834		
			Total	**24841**	**100.0**

* - Unconfirmed
** - Could not differentiate
PC – Phosphatidylcholine
PE – Phosphatidylethanolamine
PS – Phosphatidylserine
EPA – Eicosapentaenoic acid
DHA – Docosahexanoic acid

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.

fastcase®
Smarter legal research.