

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Fotini Sampalis Confirmation No.: 3094  
Application No.: 10/485,094 Art Unit: 1614  
Filed: July 13, 2004 Examiner: Gregg Polansky  
Title: **NATURAL MARINE SOURCE PHOSPHOLIPIDS COMPRISING  
FLAVONOIDS, POLYUNSATURATED FATTY ACIDS AND  
THEIR APPLICATIONS**

**Mail Stop Declaration**  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION OF EARL L. WHITE, PH.D. UNDER 37 C.F.R. § 1.132**

I, Earl L. White, declare as follows:

1. I am a U.S. citizen.
2. I am presently President and CEO of MDx BioAnalytical Laboratory, Inc. in College Station TX, which offers, among other things, expert analytical services for identification of biosubstances.
3. I have a Ph.D. in analytical chemistry and mass spectrometry from University of North Carolina at Chapel Hill. Since 1985 I have worked professionally in the area of analytical chemistry and mass spectrometry (26 years). I have served as Adjunct Professor of Chemistry, Wake Forest University, Dept. of Chemistry and have published 27 peer reviewed journal articles. I presently serve as a Peer Reviewer for *J. Chromatographic. Science* and *J. Chromatography*. I am a member of the American Chemical Society (CNCS-Section Chairman '93, Chair-Elect/Program Chair '92, Secretary '91 and Treasurer '90), Mississippi Academy of Science, Society of Analytical Chemists of Pittsburgh, Applied Spectroscopy Society, Phi Lambda Upsilon Honorary Chemical Society, America Society for Mass Spectrometry, National Technical Association and Alpha Phi Alpha Fraternity, Inc., Triangle Area Mass Spectrometry.

Discussion Group, National Organization of Black Chemists and Chemical Engineers (NOBChCE), Bay Area Mass Spectrometry Discussion group (BAMS) and the SF Bay Area Proteome Society. My CV is attached and labeled as Attachment A.

4. I am considered an expert in the area of identification of biomolecules using analytical techniques in general, and mass spectrometry, specifically.

5. MDx BioAnalytical Laboratory, Inc. was contracted by Neptune Technologies and Bioressources of Québec, Canada to analyze certain phospholipid samples (the “Beaudoin Oil Fractions”) and report which fatty acid phospholipids are present in the Beaudoin Oil Fractions. Furthermore, I was asked to opine on whether certain fatty acid phospholipids were present or absent in the Beaudoin Oil Fractions. The result of this analysis follows.

6. I received three Sample Sets from Neptune Technologies and Bioressources, each of the first two Sample Sets containing three separate Fractions labeled Beaudoin Oil Fractions I, II and III, and the third Sample Set containing two separate Fractions labeled Beaudoin Oil Fractions I and II<sup>1</sup>, for liquid chromatography/mass spectrometry (“LC/MS”) and liquid chromatography-mass spectrometry/ mass spectrometry (“LC-MS/MS”) analysis. LC/MS is an widely accepted separation and identification technique used in the pharmaceutical industry to characterize organic molecules. I was to analyze the eight Fractions in the three Sample Sets and identify the fatty acids attached to the phospholipids (PL) present in each of the Fractions in each Sample Set. The PLs of interest include phosphatidylcholine (PC), phosphophatidyl-ethanolamine (PE) and phosphatidylinositol (PI).

## METHODS

7. All Fractions received were analyzed by LC/MS. Each Fraction was dissolved in chloroform and diluted to a concentration of 500 ng/μL in 20% MeOH in 0.1% formic acid (FA) and subjected to LC/MS. A 1 or 2 μL aliquot of each sample was injected onto a C18 column, 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 or MS/MS positive ion mode for these Fractions.

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<sup>1</sup> When the third run was performed for the third Sample Set there was not enough material remaining after the ethyl

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 3672, 30 and 2.5 V, respectively. The source temperature was held at 120°C, the cone N<sub>2</sub> 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<sub>2</sub>O:ACN, 95:5%) was held at 20% B (ACN: 0.1% FA in H<sub>2</sub>O, 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 performed on all ions detected above a pre-determined intensity threshold. Optimum collision energies for compound dissociation were determined. Each Fraction was analyzed in duplicate for MS scan and at least once in the MS/MS mode.

8. My full report is attached to this Declaration as Attachment B.

## RESULTS

9. A list of fatty acid phospholipids detected in the Beaudoin Oil Fractions is given in the Tables 1 and 2 (see Attachment B) with relative percentages based on area under the curve with the caveat that all PLs do not necessarily respond the same. These values should therefore be considered a semi-quantitative representation of the various PLs.

10. Table 1 presents the results of Fractions I, II and III for each Sample set. Table 2 presents the same results grouped by Sample Sets 1, 2, and 3 for each Fraction therein.

11. PLs detected in the Fractions were primarily phosphatidylcholines as confirmed by the characteristic *m/z* 184 ion in the MS/MS spectra. With the exception of PL with molecular weight (MW) of 732.5 Da, PLs in an amount of  $\geq 5\%$  were low MW PLs. Six of the PLs detected in the samples (*m/z* 542, 568, 666, 808, 826 and 852) have molecular weights, which, without further analysis, could be considered evidence of a PC containing DHA and EPA, or a combination of DHA and EPA, however MS/MS spectra shown in the figures below confirm that these PLs do not have attached to the sn-1 and sn-2 positions of the glycerol backbone DHA and DHA. EPA and EPA. DHA and EPA. or EPA and DHA.

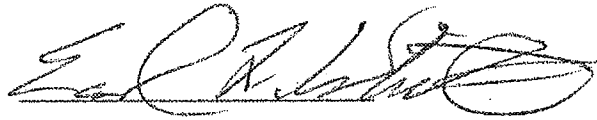
12. Figures 1 – 3 show representative total ion chromatograms for 500 ng/ $\mu$ L of each oil sample dissolved in  $\text{CHCl}_3$ . Peak areas were calculated from the protonated molecular ions for each of the major peaks in the spectra. MS/MS spectra for a representative Beaudoin Oil Fraction sample are shown in Figures 4-9, and 11. Figure 10 is a representative MS/MS spectrum for a PC 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 is indicative of the loss of a C20:5 (EPA) and C18:0 fatty acids from the protonated molecular ions. However, there was no ion observed at  $m/z$  of 499 which would have been indicative of a loss of 327 which is DHA from the protonated molecular ion at  $m/z$  of 826, which is indicative that there is no DHA attached to the phospholipid.

13. It is my opinion that the Beaudoin Oil Fractions received and tested by me do not contain PLs which have attached to them DHA and DHA, EPA and EPA, DHA and EPA, or EPA and DHA, at the detection levels described above in paragraph 7 or represented in the spectra of Figures 1-11.

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14. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By:



Earl L. White, Ph.D.

Dated: May 31, 2011

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