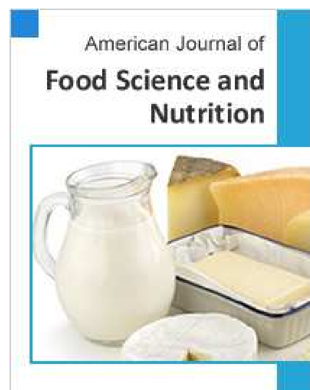
American Journal of Food Science and Nutrition
2015; 2(6): 95-100
Published online October 30, 2015 (http://www.aascit.org/journal/ajfsn)
ISSN: 2375-3935



Keywords
Evaporative Light Scattering Detector (ELSD),
Glucosylceramides,
Normal-Phase Chromatography,
Wheat Extracts

Received: October 7, 2015
Revised: October 20, 2015
Accepted: October 22, 2015

Quantification of Glucosylceramides in Wheat Extracts Using High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection

Xun Yan*, Douglas J. Vredeveld, Stephen R. Missler

Analytical Sciences, Amway Corporation, Ada, MI, USA

Email address
xun.yan@amway.com (Xun Yan)

Citation
Xun Yan, Douglas J. Vredeveld, Stephen R. Missler. Quantification of Glucosylceramides in Wheat Extracts Using High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection. *American Journal of Food Science and Nutrition*. Vol. 2, No. 6, 2015, pp. 95-100.

Abstract
In this report, we describe a simple and rapid HPLC method to analyze glucosylceramide content in wheat extracts and product formulations containing wheat extracts. The HPLC separation of glucosylceramides from other glycolipids was performed on a normal-phase (diol) column followed by evaporative light scattering detection to quantify the glucosylceramide content. The method was validated for selectivity, precision, and accuracy. The recovery of glucosylceramides from spiked matrix samples was approximately 100%, and the limits of detection and quantification were 0.03 mg/mL and 0.1 mg/mL, respectively. The electron light scattering detector response fits well into a power equation for calibration. The assay precision (relative standard deviation values) was less than 3% for repeatability and less than 4% for reproducibility.

1. Introduction

Glucosylceramides are a major class of sphingolipids widely present in plants, animals, and microbes.[1-3] They consist of hydrophilic glucose residues linked to a hydrophobic ceramide backbone via glycosidic bonds.[4, 5] In plants, the ceramide backbone contains a long chain sphingoid base, and a hydroxy fatty acid linked via an amide bond (Fig. 1).[6, 7] In wheat extracts, glucosylceramides are primarily comprised of a sphingadiene or sphingenine base with 2-hydroxypalmitic acid, hydroxystearic acid, or hydroxyeicosanoic acid linkages.[8-11]

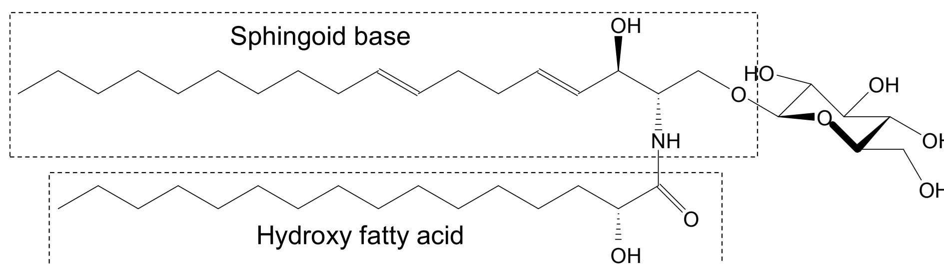


Fig. 1. *Chemical structure of glucosylceramides in plants.*

Glucosylceramides are present mostly in the outer layers of cell membranes and play an important role in many physiological functions.[12] A number of chromatographic techniques have been reported to analyze glucosylceramides including thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC).[13-17] Liquid chromatography mass spectrometry (LC-MS) has become a useful tool for the structural elucidation of complex sphingolipids, providing specific structural data for the identification of glycosphingolipid species.[18] Both reversed-phase and normal-phase chromatography can be used for lipid analysis. Reversed-phase chromatography separates lipids to molecular species based on their different hydrophobicity[9, 19-21] using mixtures of water and organic solvents (e.g., acetonitrile, isopropanol, and methanol) for the mobile phases and carbon-18-, carbon-8-, or phenyl-bonded silica as the stationary phase. Normal-phase chromatography separates lipids based on their hydrophilicity, and is capable of separating glycolipids by class, such as, monoglycodiacylglycerol, diglycodiacylglycerol, or phospholipids.[15, 22-24] The stationary phase is commonly made of silica with cyano, amino, or diol surface modifications and the typical mobile phases used were hexane, ethers, chloroform, isopropanol, methanol, and water. Supercritical or liquid carbon dioxide can also be used for normal-phase lipid separation.[25, 26]

Detection of sphingolipids can be difficult since many unsaturated lipids exhibit ultraviolet (UV) absorption in the 200 to 220 nm range, overlapping with the absorbance of the mobile phase. Derivatization of the lipids to add a chromophore is often required for UV or fluorescence spectrophotometry.[11, 27] The use of MS is available for the detection of glucosylceramide and its moieties but due to its complexity, an MS detection method is limited to structure determination rather than routine quantitative analysis. Evaporative light scattering detectors (ELSD) detect the light scattering of analyte droplets or particles generated after rapid evaporation of the mobile phase, and can serve as a universal mass detector, provided the analytes are less volatile than the mobile phase. ELSD coupled with HPLC has become widely adapted for lipid analysis.[15, 28-32]

In this report, we present a simple and rapid normal-phase HPLC/ELSD method to separate and quantify glucosylceramides in wheat extracts and in products containing wheat extracts. The method is validated and can be applied to the quantitative analysis of glucosylceramide content in both raw materials and dietary supplements.

2. Experimental

2.1. Chemicals and Reagents

Glucosylceramides from wheat (>99% by TLC) were purchased from Nacalai USA, Inc. (San Diego, CA). Wheat lipophilic extracts (Ceramosides®) were obtained from Seppic, Inc. (Fairfield, NJ). Chloroform (Chromasolv grade) with ethanol as stabilizer, methanol (LC-MS grade), and acetic acid (LC-MS grade) were purchased from Sigma-Aldrich (MO). Water (LC/MS grade) was obtained from Fisher Scientific (NJ).

2.2. Sample Preparation

Sample extractions were performed by weighing a well-mixed sample of wheat extract (200mg) or a prepared formula containing 200 mg wheat extract (approximately 3 g of formula) into a polytetrafluoroethylene (PTFE)-lined glass vial, adding 10 mL chloroform/methanol (3:1, v/v) solution, and sonicating at 25°C for two minutes. Once extracted, the sample was cooled to room temperature, and then filtered through a 0.45μm polyvinylidene fluoride (PVDF) syringe filter into a glass HPLC autosampler vial for analysis.

2.3. HPLC Conditions

The HPLC system was the Agilent 1100 system equipped with a pump, degasser, 6 port switch valve, autosampler, Alltech 3300 ELSD detector (installed after the switch valve), and a LiChrospher® 100 Diol (5 μm), 125 mm x 4 mm, normal-phase column (EMD Millipore, Billerica, MA). The mobile phase solutions used for gradient separation were: A, chloroform/acetic acid (1000/20, v/v); B, chloroform/methanol/water/acetic acid (600/350/50/20, v/v/v/v); and C, methanol/acetic acid (1000/20, v/v). The mobile phase gradient (A/B/C), at a flow rate of 1 mL/min, was initially 92/8/0 and linearly changed to 70/30/0 from 0 to 15 min; 20/50/30 from 15 to 17 min and held for 5 min; then set back to initial condition at 23 min and held till 26 min. Column temperature was maintained at 40°C, and the ELSD evaporator temperature was 50°C with a nitrogen gas feed of 2 L/min. A switch valve was used to direct non-glucosylceramide components away from the ELSD to extend ELSD run-time between cleanings. The injection volume was 10 μl and the chromatograms were recorded and analyzed using Empower 3 chromatography data software (Waters Corporation, Milford, MA).

2.4. LC-MS Conditions

LC-MS was performed using a Synapt G2 Quadrupole Time-of-flight MS (Waters, Milford, MA) equipped with an Acquity ultra-performance liquid chromatography (UPLC)-H chromatograph with photodiode array (PDA) UV-visible detector. Liquid chromatography conditions were as previously described for HPLC. The column effluent was directed to the PDA and then, to the ion source of the mass spectrometer. The mass spectrometer was equipped with an atmospheric pressure photoionization (APPI) source operated in positive ion mode, with a 10,000 resolving power, and the spectra acquired at 0.5 sec intervals from m/z 100-1500. Cone voltage was 60 eV, probe desorption temperature was 550°C, source temperature 140°C, desorption gas 800 L/h, and cone gas 10 L/h. Data were processed using Waters MassLynx mass

spectrometry software, version 4.1.

2.5. Method Validation

To validate the method, ELSD detector response to glucosylceramide standards were analyzed for precision, repeatability, and accuracy. To characterize ELSD detector response to analyte concentration, 10 mg of glucosylceramides standard was dissolved in chloroform/methanol extraction solution to 2 mg/mL and then, a serial dilution was prepared (1.5, 1, 0.5, 0.25 mg/mL) for analysis. The standards were used for detector calibration and for detector response measurement.

To analyze method repeatability, five replicates of wheat glucosylceramide extract and test formula were extracted and analyzed, and the results analyzed for repeatability. For method reproducibility, a second set of samples were prepared and analyzed by a second analyst. The results were compared to the first set of results for equivalency. A typical test formula containing plant extracts, stearate, and silica, common ingredients in dietary supplement products, was prepared for method performance analysis.

To evaluate method accuracy, a matrix blank (3 g) was spiked with 75 mg, 100 mg, and 150 mg of wheat extract in triplicates. The spiked samples were shaken with 10 mL chloroform/methanol extraction solution, filtered, and analyzed for the recovery yield. Data analysis was performed using Minitab, version 17, statistical software (State College, PA).

3. Results

3.1. Glucosylceramide Separation

Glucosylceramides in wheat extracts were separated from other major extract components using normal-phase HPLC followed by electron light scattering detection, as shown in Fig. 2. The glucosylceramides were eluted in a group of overlapping peaks at 10 min, and were well-separated from other glycolipids, such as, digalatosyldiacylglycerols, sterylglucosides, and monogalactosyldiacylglycerols.

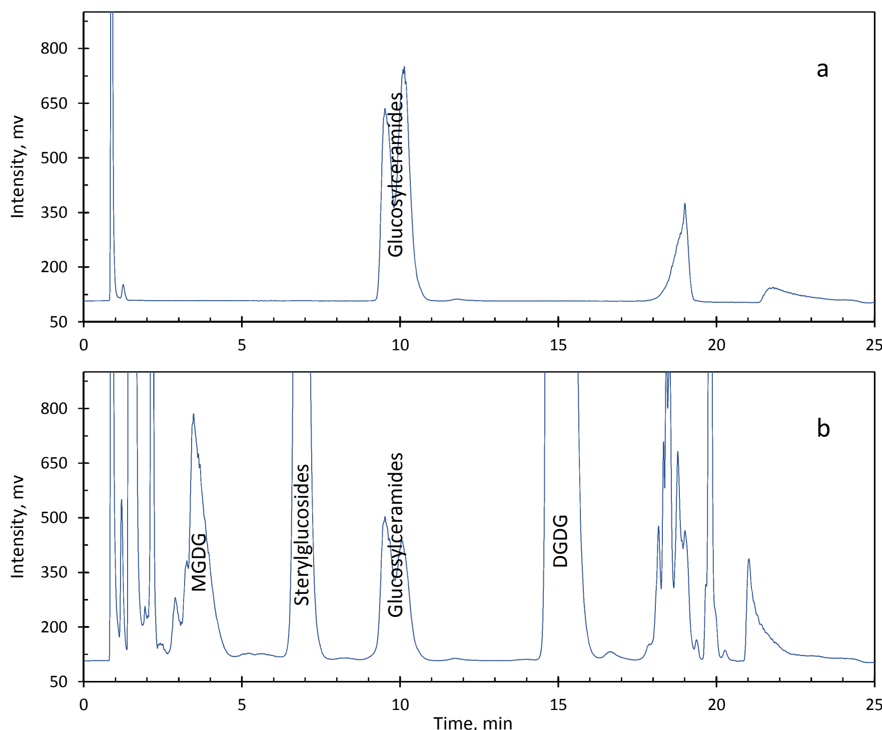


Fig. 2. Chromatograms of glucosylceramide standards (a) and wheat extract (b) using normal-phase HPLC. DGDG, digalatosyldiacylglycerols; MGDG, monogalactosyldiacylglycerols.

3.2. Mass Spectroscopy Analysis

Analysis by mass spectrometry found that the major components in the overlapping glucosylceramide peaks have molecular weights of 715, 743, 771, and 799 Da (spectrum not shown). The distribution of molecular weights is due to the variation of double bonds and aliphatic chain length in the ceramide back bone and the hydroxyl fatty acids, and the results are consistent with the range of 713 to 800 Da reported in previous mass spectrometry studies [8, 9].

3.3. Method Validation

3.3.1. Linearity

To verify the detector response linearity, the five glucosylceramide standards were analyzed and the results plotted as peak area counts against concentration, as shown in Fig. 3. The correlation between the two parameters fits well into a power equation, peak area = a·concentration^b (a and b are fitting parameters), with a correlation coefficient (R^2) of 0.9999.

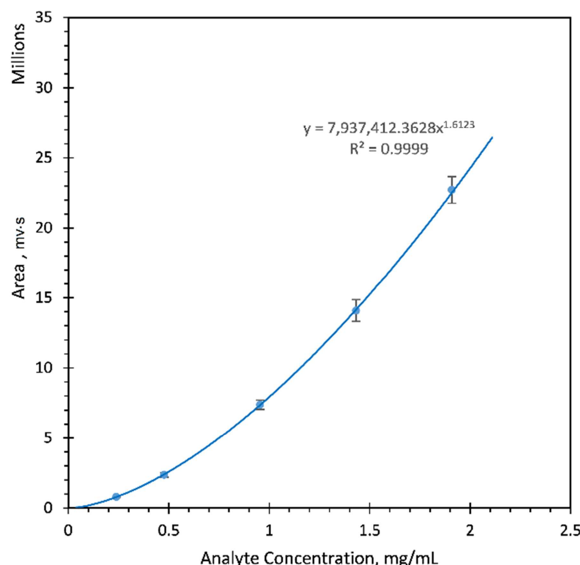


Fig. 3. *A plot of evaporative light scattering detector (ELSD) response to glucosylceramide concentration. Results plotted as peak area counts (mv·s) against analyte concentration (mg/mL)*

3.3.2. Precision

The analyses of method precision (repeatability and reproducibility) are presented in Table I. Individual analysts obtained relative standard deviations (RSD) less than 3%, for both the analysis of raw material and finished product. The assay reproducibility was evaluated for all test results from the two analysts. The RSD of the pooled results from both analysts was less than 4%. The results from the two different analysts were shown to be equivalent, within 5% of the overall average, and with a p-value of 0.009 at a confidence level of 95%.

Table I. *Method precision performance.*

	Analyst I		Analyst II		Overall
Sample	**Average (mg/g)**	**RSD (%)**	**Average (mg/g)**	**RSD (%)**	**RSD (%)**
Raw	52.15	2.5	54.63	2.3	3.4
Formula	2.35	0.75	2.4	1.03	1.4

RSD, relative standard deviations

3.3.3. Recovery and Matrix Effect

The percent recovery of glucosylceramides from the spiked matrix samples (75 mg, 100 mg, and 150 mg) was 107%, 107%, and 105%, respectively.

The method detection and quantification limits were estimated by comparing the noise level in a matrix blank with a spiked glucosylceramide sample. There is no visible peak in the analyte elution window and the detector noise is estimated by a forced integration over the elution window. At a signal to noise ratio (S/N) of 3, the limit of detection was extrapolated to be 0.03 mg/mL and limit of quantification (S/N= 10) at 0.1 mg/mL.

4. Discussion

Following a simple one-step chloroform/methanol extraction, we were able to use HPLC to separate the analytes into different lipid classes based on their polar functionality; thus, allowing for the efficient and accurate quantification of glucosylceramides in wheat extracts and formulations containing wheat extract. Using normal-phase liquid chromatography, several species of glucosylceramides were grouped and well-separated from other lipids, including sterylglucosides and digalactosyldiacylglycerols, which enable the glucosylceramides to be quantified as a group. The differences in alkyl substitution or molecular weight had little effect on the retention and separation of the glucosylceramides. Molecular unsaturation, such as the number of double bonds, can affect analyte retention, as illustrated by the two closely eluting peaks under the glucosylceramides envelope (Fig.2).

Evaporative light scattering detection is an effective detection method for non-volatile analytes without distinctive chromophores. The detector response fits well into a power equation against the analyte concentration. Multilevel standards can be used for calibration and a power equation is effective for glucosylceramide quantification. The method performance was shown to be selective, precise, and accurate for glucosylceramide determination; therefore, demonstrating assay efficacy.

5. Conclusion

In summary, we have developed an efficient and effective HPLC/ELSD method to characterize and quantitate glucosylceramide content in wheat extracts and formulations containing wheat extracts. A typical sample can be prepared with a simple one-step chloroform/methanol extraction and the glucosylceramides separated from other lipid classes using a normal-phase diol chromatography column. The method was validated for selectivity, precision, and accuracy. It was also shown to have reasonable detection limits, permitting routine quantitative analysis of glucosylceramide content of both raw materials and product formulations.

Acknowledgements

The authors wish to thank Mr. Guillaume Lamy, Seppic, Inc., for providing the wheat extract samples used in this study. We also thank Dr. Mark Proefke and Dr. Amit Chandra, Research and Development, Amway Corporation, for supporting this project.

References

[1] Heinz, E.; Plant Glycolipids: Structure, Isolation and Analysis. In: Christie, W.W. (ed). Advances in Lipid Methodology, Volume 3. The Oily Press, Dundee, (1996), pp. 211-332.

[2] Sperling, P., Warnecke, D. and Heinz, E.; Plant Sphingolipids. In: Daum, G. (ed). Lipid Metabolism and Membrane Biogenesis. Springer, Berlin Heidelberg, (2004), pp. 337-381.

[3] Kolter, T.; Glycosphingolipids. In: Nicolaou, A., Kokotos, G. (eds). Bioactive Lipids. The Oily Press Bridgewater, (2004).

[4] Fujino, Y. and Ohnishi, M.; Structure of Cerebroside in Aspergillus Oryzae. *Biochimica et Biophysica Acta* (1976); 486(1):161-71.

[5] Duartea, R.S., Polycarpo, C.R., Wait, R., Hartmann, R. and Bergter, E.B.; Structural Characterization of Neutral Glycosphingolipids from Fusarium Species. *Biochimica et Biophysica Acta (BBA)* (1998); 1390(2):11.

[6] Carter, H.E., Hendry, R.A., Nojima, S. and Stanacev, N.Z.; The Isolation and Structure of Cerebrosides from Wheat Flour. *Biochimica et Biophysica Acta* (1960); 45:3.

[7] Carter, H.E., Johnson, P. and Weber, E.J.; Glycolipids. *Annual Review of Biochemistry* (1964); 34:34.

[8] Sullards, M.C., Lynch, D.V., Merrill, A.H.J. and Adams, J.; Structure Determination of Soybean and Wheat Glucosylceramides by Tandem Mass Spectrometry. *Journal of Mass Spectrometry* (2000); 35(3):7.

[9] Zhu, Y., Soroka, D.N. and Sang, S.; Structure Elucidation and Chemical Profile of Sphingolipids in Wheat Bran and Their Cytotoxic Effects against Human Colon Cancer Cells. *Journal of Agricultural and Food Chemistry* (2013); 61:9.

[10] Laine, R.A. and Renkonen, O.; Ceramide Di- and Trihexosides of Wheat Flour. *Biochemistry* (1974); 13(14):7.

[11] Goto, H., Nishikawa, K., Shionoya, N., Taniguchi, M. and Igarashi, T.; Determination of Sphingoid Bases from Hydrolyzed Glucosylceramide in Rice and Wheat by Online Post-Column High Performance Liquid Chromatography with O-phthalaldehyde Derivatization. *Journal of Oleo Science* (2012); 61(12):8.

[12] Messner, M.C. and Cabot, M.C.; Glucosylceramide in Humans. In: Chalfant, C., Del Poeta, M. (eds). Glycosphingolipids in cell surface recognition. Springer Science, New York, (2010), pp. 9.

[13] Svennerholm, E. and Svennerholm, L.; The Separation of Neutral Blood-Serum Glycolipids by Thin-Layer Chromatography. *Biochimica et Biophysica Acta* (1963); 70(10):432.

[14] Horning, M.G., Murakami, S. and Horning, E.C.; Analyses of Phospholipids, Ceramides, and Cerebrosides by Gas Chromatography and Gas Chromatography-Mass Spectrometry. *The American Journal of Clinical Nutrition* (1971); 24:11.

[15] Christie, W.W.; Rapid Separation and Quantification of Lipid Classes by High Performance Liquid Chromatography and Mass (Light-Scattering) Detection. *Journal of Lipid Research* (1985); 26:6.

[16] Alvarez, J.G., Touchstone, J., Storey, B.T. and Grob, R.L.; Determination of Sphingolipid Sphingoid Bases by HPTLC Fluorescence Spectrodensitometry. *Journal of Liquid Chromatography* (1989); 12:6.

[17] Li, M., Yang, L., Bai, Y. and Liu, H.; Analytical Methods in Lipidomics and Their Applications. *Analytical Chemistry* (2014); 86.

[18] Bielawski, J., Pierce, J.S., Snider, J., Rembiesa, B., Szulc, Z.M. and Bielawska, A.; Sphingolipid Analysis by High Performance Liquid Chromatography-Tandem Mass Spectrometry. In: Chalfant, C., Del Poeta, M. (eds). Sphingoliids as Signaling and REgulatory Molecules. Springer, New York, (2010), pp. 46-59.

[19] t'Kindt, R., Jorge, L., Dumont, E., Couturon, P., David, F., Sandra, P. and Sandra, K.; Profiling and Characterizing Skin Ceramides Using Reversed-Phase Liquid Chromatography−Quadrupole Time-of-Flight Mass Spectrometry. *Analytical Chemistry* (2011); 84:9.

[20] Fillet, M., Van Heugen, J.C., Servais, A.C., De Graeve, J. and Crommen, J.; Separation, Identification and Quantitation of Ceramides in Human Cancer Cells by Liquid Chromatography–Electrospray Ionization Tandem Mass Spectrometry. *Journal of Chromatography A* (2002); 949(1-2):9.

[21] Zhao, F., Xu, J., Chen, J., Yan, X., Zhou, C., Li, S., Xu, X. and Ye, F.; Structural Elucidation of Two Types of Novel Glycosphingolipids in Three Strains of Skeletonema by Liquid Chromatography Coupled with Mass Spectrometry. *Rapid Communications in Mass Spectrometry* (2013); 27:12.

[22] Watanabe, W. and Arao, Y.; A New Solvent System for the Separation of Neutral Glycosphingolipids. *Journal of Lipid Research* (1981); 22:5.

[23] Moreau, R.A., Doehlert, D.C., Welti, R., Isaac, G., Roth, M., Tamura, P. and Nunez, A.; The Identification of Mono-, Di-, Tri-, and Tetragalactosyldiacylglycerols and their Natural Estolides in Oat Kernels. *Lipids* (2008); 43:17.

[24] Wang, L., Wang, T. and Fehr, W.R.; HPLC Quantification of Sphingolipids in Soybeans with Modified Palmitate content. *Journal of Agricultural and Food Chemistry* (2006); 54:7422-7428.

[25] Deschamps, F.S., Lesellier, E., Bleton, J., Baillet, A., Tchapla, A. and Chaminade, P.; Glycolipid Class Profiling by Packed-Column Subcritical Fluid Chromatography. *Journal of Chromatography A* (2004); 1040(1):7.

[26] Lesellier, E.; Analsis of Non-saponifiable Lipids by Super-/Subcritical Fluid Chromatography. *Journal of Chromatography* (2001); 936(1-2):14.

[27] Groener, J.E.M., Poorthuis, B.J.H.M., Kuiper, S., Helmond, M.T.J., Hollak, C.E.M. and Aerts, J.M.F.G.; HPLC for Simultaneous Quantification of Total Ceramide, Glucosylceramide, and Ceramide Trihexoside Concentrations in Plasma. *Clinical Chemistry* (2007); 53(4):6.

[28] Lesellier, E., Gaudin, K., Chaminade, P., Tchapla, A. and Baillet, A.; Isolation of Ceramide Fractions from Skin Sample by Subcritical Chromatography with Packed Silica and Evaporative Light Scattering Detection. *Journal of Chromatography A* (2003); 1016:11.

[29] Kashima, M., Nakagawa, A., Sugawara, T., Miyazawa, T., Murakami, C., Miyashita, R., Ono, J., Deschamps, F.S. and Chaminade, P.; Method for Quantitative Determination of Cerebroside in "Plant Ceramide" Foodstuffs by HPLC with Evaporative Light Scattering Detection. *Journal of Oleo Science* (2002); 51.

[30] Homan, R. and Anderson, M.K.; Rapid Separation and Quantitation of Combined Neutral and Polar Lipid Classes by High Performance Liquid Chromatography and Evaporative Light Scattering Mass Detection. *Journal of Chromatography B* (1998); 708:6.

[31] Conforti, F.D., Harris, C.H. and Rinehart, J.T.; High Performance Liquid Chromatographic Analysis of Wheat Flour Lipids Using Evaporative Light Scattering Detector. *Journal of Chromatography* (1993); 645:6.

[32] Kobayashi, N., Noel, E.A., Barnes, A., Rosenberg, J., DiRusso, C., Black, P. and Oyler, G.A.; Rapid Detection and Quantification of Triacylglycerol by HPLC–ELSD in Chlamydomonas Reinhardtii and Chlorella Strains. *Lipids* (2013); 48(10):15.