Application Note

Simplified analysis of lipid or detergent content in biological samples using the IR-based Direct Detect® Spectrometer

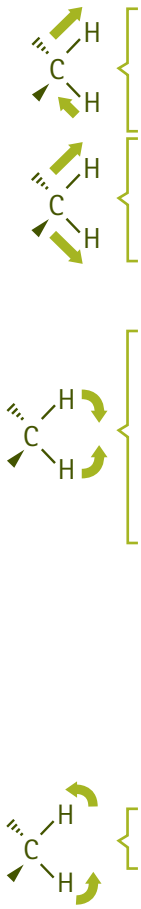
Abstract

Lipids are a fundamental class of biologically relevant compounds. The annual number of reports on lipid biochemistry, chemistry and physiology is increasing rapidly. Despite the growth in lipid research, analytical methods applied for their characterization typically involve multistep procedures, requiring extensive sample manipulation and separation from other biomolecules, like proteins, before the analysis. A new feature of the Direct Detect® spectrometer software (version 2.0) enables simultaneous protein quantitation and lipid analysis, in the same sample. The new application can also be used to monitor the efficiency of detergent removal during preparation of samples for downstream analysis and for quantitation of known lipid(s) in cases where a viable standard curve has been determined.

Introduction

Lipids are a diverse group of compounds that serve many key biological functions. Lipids, together with proteins and carbohydrates, constitute the principal structural components of all tissues. They account for the major (~50%) compositional and structural element of biological membranes[1]. A subgroup of lipids, triglycerides, is a major form of energy storage in animals and plants. Lipids also play a significant role as pathway intermediates in cell signaling cascades[2]. Interestingly, there is no precise and widely accepted definition of lipids. Most commonly, lipids are categorized as a group of naturally occurring organic compounds that are related by their solubility in nonpolar organic solvents (e.g. ether, chloroform, acetone and benzene) and general insolubility in water.

Analytical characterization of lipids typically requires that samples undergo laborious, multistep preparative processes prior to analysis. Lipids can be isolated by liquid–liquid extraction and separated into classes, often derivatized, and then analyzed. Conventional methods of analysis include measuring iodine value, elemental phosphorus, acid value (saponification equivalent), peroxide value and radiochemical techniques. More recently, classical methods have been replaced by thin-layer chromatography (TLC), gas chromatography (GC), and high performance liquid chromatography (HPLC) as well as mass spectrometry (MS).



Description	Approximate frequency (cm^{-1})
CH_3 asymmetric stretch	2956 (strong)
CH_2 asymmetric stretch	2920 (strong)
CH_3 symmetric stretch	**2870 (strong)**
CH_2 symmetric stretch	**2850 (strong)**
C=O stretch	1740 (strong)
CH_2 scissoring (triclinic)	1473 (medium)
CH_2 scissoring (hexagonal)	1468 (medium)
CH_2 scissoring (orthorhombic)	1472 and 1463 (medium)
CH_3 asymmetric bend	1460 (medium)
CH_3 symmetric bend	1378 (medium)
PO_2- asymmetric stretch	1228 (strong)
PO_2- symmetric stretch	1085 (medium)
CH_2 rocking	720-730 (medium)

Table 1. Selected infrared absorption bands of lipids[8]. Vibration types used by the Direct Detect® spectrometer to quantitate lipids and detergents are shown in **bold (highlighted in yellow).**

Previous reports have also identified infrared (IR) spectroscopy as a viable method for lipid analysis[3]. Due to their complex chemical composition, lipids absorb in many different regions of the IR spectrum. Approximate frequencies of selected vibrational modes are listed in Table 1. A more comprehensive list of lipid absorptions can be found in the literature[4-6]. Specifically, characteristic lipid bands, such as the aliphatic group stretching (2800 - 3000 cm^{-1}), ester C=O stretching (around 1740 cm^{-1}) or phosphate stretching (around 1235 cm^{-1}) permit qualitative and quantitative analysis of lipid content[7]. Moreover, given that each lipid possesses an IR signature uniquely defined by its chemical composition and structure, IR spectroscopy further offers a means of qualitative lipid discrimination.

Due to similarities in composition, detergents possess IR absorption spectra that closely resemble lipids present in the cell membrane. The Direct Detect® spectrometer utilizes the C-H symmetric stretching vibrational population between 2870 and 2840 cm^{-1} to determine lipid or detergent content (Figure 1). The Direct Detect® assay-free sample card enables analysis of aqueous-based biological samples, which are normally not compatible with infrared spectroscopy, due to their high water content. These assay-free cards are also compatible with many organic solvents.

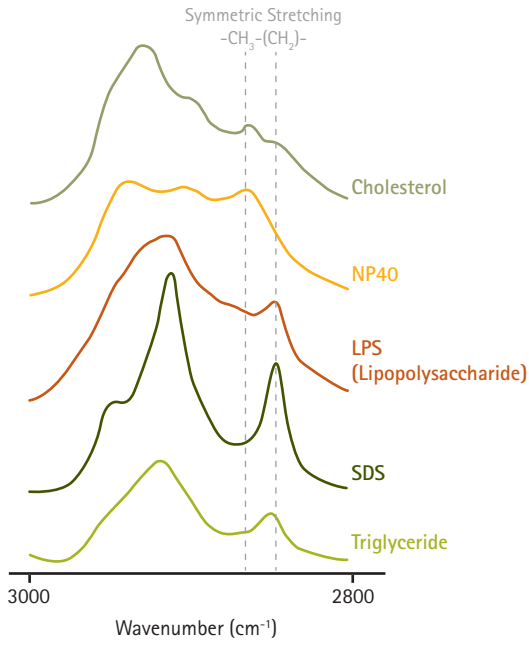


Figure 1. C-H symmetric stretching bands observed in the infrared spectra of lipids and detergents.

Materials and Methods

Measurements of sample concentration were acquired using Direct Detect® assay-free sample cards (Catalogue No. DDAC00010-8P) and the Direct Detect® spectrometer (Catalogue No. DDHW00010-WW). Each assay-free card contains four polytetrafluoroethylene (PTFE) membrane positions, sized for easy sample application and analysis. All measurements were performed using 2 µL of sample solution per membrane position. Unknown lipid mixtures were analyzed in the "Relative Absorbance" mode, where the system delivers information based solely on IR signal strength. Empirical sample concentration values were determined by interpolation from calibration curves developed for each specific lipid or detergent. For the experiments reported here, the system was calibrated using Tetracosanoic Acid (Supelco, Catalogue No. R420240) in chloroform and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) from Detergent Variety Pack (Catalogue No. 263458) in phosphate-buffered saline (PBS). A series of seven concentrations from 0.25 mg/mL to 1.75 mg/mL was used to generate an instrument calibration curve for Tetracosanoic Acid. For CHAPS, the calibration curve was formed using a series of seven concentration points spanning from 0.25% to 4%.

Robustness of the "Relative Absorbance" mode was further demonstrated through simultaneous analysis of protein and lipid content in samples containing mixture of both biomolecules as well as in breast cancer tissue lysate. Frozen tissue, derived from a breast ductal carcinoma, was obtained from Analytical Biological Services Inc. and divided into 2 equal samples. Tissue was covered with 2 mL RIPA buffer (Catalogue No. 20-188) or CytoBuster™ protein extraction reagent (Catalogue No. 71009-50mL), both supplemented with an inhibitor cocktail, and disrupted with a glass tissue homogenizer (Kimble Chase, Catalogue No. 885301-0002). Effective removal of the fatty fraction from the resulting tissue homogenate by a series of centrifugation steps was also monitored by the Direct Detect® spectrometer.

Results

Standard curve generation

As previously demonstrated, a universal calibration curve derived from a single protein standard can be used to quantitate a wide range of protein samples[9]. However, due to the great complexity and variability among lipids and detergents, their quantitation requires the generation of a standard curve for each of the compounds to be analyzed. In this study, the Direct Detect® spectrometer was initially calibrated using either Tetracosanoic Acid in chloroform or CHAPS in PBS. Two concentration ranges (performed in triplicate), spanning 0.25 mg/mL - 1.75 mg/mL (Tetracosanoic Acid) and 0.25% - 4% (CHAPS), were used to derive lipid and detergent calibration curves, respectively (Figure 2).

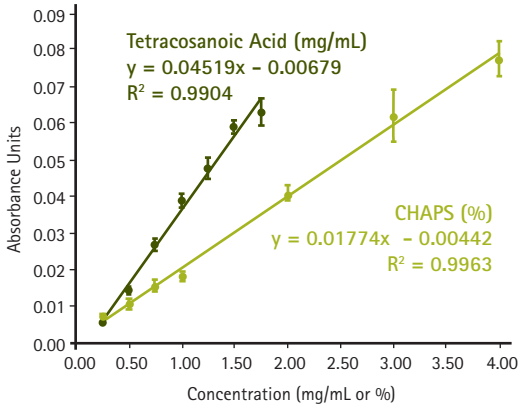


Figure 2. Calibration curves prepared by the Calibration Method using the Direct Detect® spectrometer's software (version 2.0). The calibration curve generated for Tetracosanoic Acid is shown in dark green while the curve obtained for CHAPS is lighter green.

The strength of IR signal for each concentration was fitted to a regression line represented by linear equations y = 0.04519x - 0.00679 (lipid) and y = 0.01774x + 0.00442 (detergent). These equations were used by the Direct Detect® software to determine the concentration of Tetracosanoic Acid and CHAPS in subsequent samples. Figure 2 clearly shows the difference in strength of IR signal displayed by each of the compounds that were analyzed. Comprehensive analysis of various lipids, including fatty acids, phospholipids, triglycerides, liposaccharides and many detergents, demonstrated a high degree of variability in the slope of the calibration curves (data not shown) strongly suggesting the requirement for individualized calibrations.

Accuracy of lipid and detergent quantitation

The accuracy of concentration estimation within the dynamic ranges established for Tetracosanoic Acid and CHAPS was assessed using 0.8 mg/mL Tetracosanoic Acid in chloroform and 1.8% CHAPS in PBS. The results showed that, within a well-defined calibration method, the instrument was capable of estimating lipid and detergent concentration with minimal error. The Direct Detect® spectrometer estimated the concentration of the Tetracosanoic Acid samples to be 0.853 ± 0.14 mg/mL (2.4%CV). The CHAPS samples were estimated to be 1.8 ± 0.004% (2.3%CV).

Analysis of complex mixtures

Virtually every organic compound absorbs infrared radiation at frequencies that correspond in energy signature to stretching and bending of its functional groups. The resulting spectral profiles of many biochemical compounds are distinct, thereby allowing detection and identification of a broad range of biomolecules. The spectral range of the Direct Detect® spectrometer is suitable for analysis of proteins, lipids/detergents, carbohydrates and many other molecules. Version 2.0 software for the Direct Detect® spectrometer enables the simultaneous quantitation of proteins and analysis of lipids in the same sample.

Protein quantitation and lipid content analysis performed in the same sample

Protein content can be quantified using one of the pre-loaded standard curves (all prepared in PBS pH 7.4[9]). Lipid analysis can be performed, on the same sample, using the "Relative Absorbance" mode. The software determines concentration values based on the signal strength of the IR profile for the C-H symmetric stretching region (Figure 3). Quantitation of lipids and detergents is further possible through creation of user-defined lipid calibration curves.

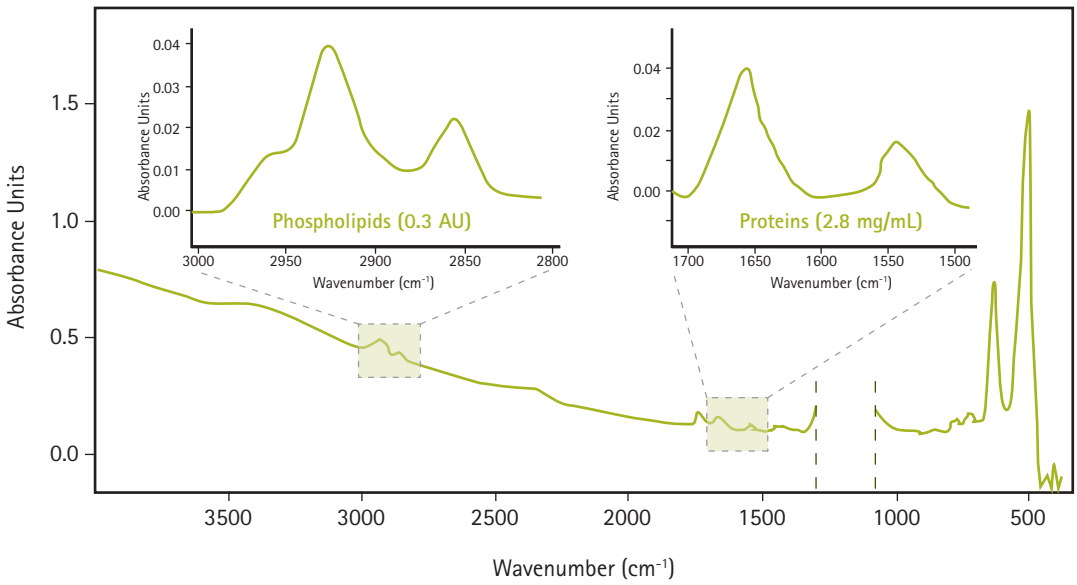


Figure 3. Analysis of protein and lipid content from a single sample measurement. The protein in the sample was quantified (using NIST BSA AM1 calibration method) at 2.8 mg/mL. Because the sample composition and ratio of individual components were unknown, the analysis of the phospholipids was performed using the "Relative Absorbance" mode.

Monitoring the lipid profile during the preparation of a breast cancer tissue lysate

In biological research, downstream detection and analysis methods are often strongly influenced by the mode of lysate preparation. Traditionally, the quantitation of proteins and lipids has been tedious, requiring large sample volumes and specialized methodologies. In many cases, the results for either component may be obscured by cross-interference. The Direct Detect® spectrometer has enabled rapid analysis of total protein with simultaneous monitoring of lipid content, thereby simplifying and improving the analytical process. The utility of this new feature in the Direct Detect® spectrometer software was demonstrated during analysis of breast cancer biomarkers[10]. Briefly, tissue lysates were prepared using RIPA buffer and CytoBuster™ protein extraction reagent. Following tissue homogenization, the Direct Detect® spectrometer was used to monitor the efficiency of fat removal and total protein concentration during centrifugal extraction. IR spectra collected using the Direct Detect® spectrometer showed gradual removal of a fatty fraction from the samples (Figure 4). The same spectra were also used to determine the total protein recovery across the various fractions (Table 2). From this limited study, it is clear that the Direct Detect® system offers a means for in-line process optimization for maximal yield and/or purity.

Conclusions

Version 2.0 software for the Direct Detect® spectrometer enables rapid analysis of lipids and detergents in addition to accurate and reproducible protein quantitation. The additional features allow changes in the lipid profile to be monitored over time and detergent removal to be tracked during sample processing. This functionality has been added to the previously proven capability for protein quantitation. The ability to simultaneously monitor protein concentration and fat removal during sample preparation provides a tool for assay optimization as well as greater confidence in final sample purity.

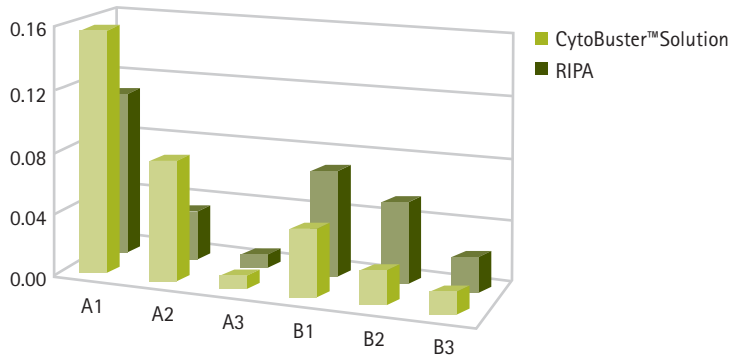


Figure 4. Lipid content as determined using the Direct Detect® spectrometer in the "Relative Absorbance" mode in 3 different fractions of breast cancer tissue lysates prepared with CytoBuster™ Protein Extraction Reagent or RIPA buffer.

		Total protein content (mg/mL)	
Sample	Spin condition and fraction collected	CytoBuster™ Protein Extraction Reagent	RIPA
A1	spin @10,000 xg (top fatty fraction)	5.0	14.0
A2	spin @15,000 xg (top fatty fraction)	2.7	20.0
A3	spin @15,000 xg (bottom layer)	5.0	17.0
B1	spin @10,000 xg (top fatty fraction)	3.3	3.6
B2	spin @15,000 xg (top fatty fraction)	2.1	5.0
B3	spin @15,000 xg (bottom layer)	2.8	5.0

Table 2. Total protein recovery from breast cancer tissue lysed with RIPA buffer or CytoBuster™ protein extraction reagent measured using the Direct Detect® spectrometer.

References

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Description	Qty/Pk	Catalogue No.
Direct Detect® Spectrometer and Starter Kit	1	DDHW00010-WW
Includes:		
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Universal Power Adapter	1	
Dell Latitude® 2120 Netbook and Power Adapter	1	
Direct Detect® Software	1	
Netbook Stand	1	
Spotting Tray	1	
Ethernet Cable	1	
Direct Detect® Assay-free Cards (50/pk)	1	
Direct Detect® Assay-free Cards (50/pk)	1	DDAC00010-GR
Direct Detect® Assay-free Cards (50/pk)	4	DDAC00010-4P
Direct Detect® Assay-free Cards (50/pk)	8	DDAC00010-8P

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