Qualitative and Quantitative Analysis of Sphingomyelin in Whey Protein

A Senior Honors Thesis

by

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INTRODUCTION

Sphingolipids are a group of phospholipids found in eukaryotic cells and are especially prevalent in the plasma membrane and related cell membranes (golgi membranes and lysosomes) (Merrill et al., 1997). They contain a long-chain sphingoid base backbone, which is an amide-linked, long-chain fatty acid, and a polar head group. (Kolesnick, R., 2002).

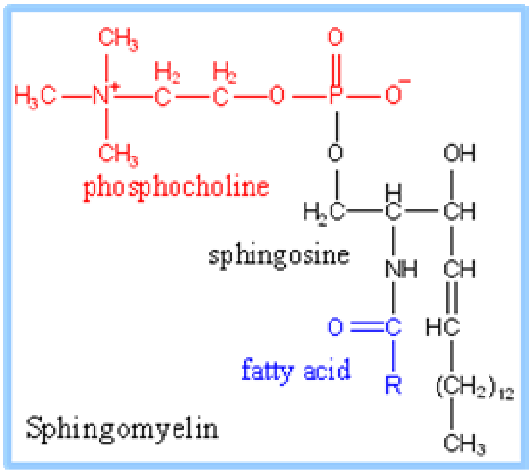

Figure 1: Structure of SM

Sphingomyelin (SM) is the predominant membrane sphingolipid and can be hydrolyzed to form ceramides and sphingosines which are involved in numerous cellular processes such as apoptosis, cell cycle progression, and cell differentiation (Vesper et al., 1999). Although only small amounts of free sphingoid bases are present in blood, they are associated with albumin and circulating cells (both erythrocytes and leukocytes). Sphingomyelin is the major sphingolipid of low-density lipoprotein and high-density lipoprotein, while very low-density lipoprotein contains mainly ceramide. The determination of the effect sphingolipids have on cell membranes can help solve question

in toxicology, such as possibly decreasing or stopping the proliferation of cancer cells (Merrill et al., 1997).

SM can be hydrolyzed to form ceramide, which is biologically beneficial. Ceramide has been proposed as a messenger for events as diverse as differentiation, senescence, proliferation, and cell cycle arrest, however, a larger body of research has focused on its role in apoptosis (Kolesnick, R., 2002). Ceramide can be formed through sphingomyelinase (SMase)-dependent catabolism of SM. SMases, specialized enzymes with phospholipase C activity, hydrolyze the phosphodiester bond of SM (Kolesnick, R., 2002).

Sphingolipids are important components of food, but unfortunately very little information is available regarding their content in foods. It is, however, found that dairy products in particular contain large amounts of SM and are the major sources of sphingolipids consumed per year in the United States (Vesper et al., 1999). Other dietary sources of SM include eggs and soybeans, but the amounts are relatively small and vary greatly (Vesper et al., 1999).

Whey protein, commonly the byproduct of cheese and dairy manufacturing, is a pure, natural, high quality protein from cow's milk. It is a rich source of the essential amino acids needed on a daily basis by the body, including cysteines. The purest form is as whey protein isolate. Whey protein concentrates (WPCs) appear to be particularly rich in SM (Houlihan and Goddard, 1991) and contain varying amounts of lipids. Processing methods, concentration of total protein, fat content, lactose content, and contaminants are some variables that may significantly influence SM content and the effects of whey products. Lipid levels in WPCs have been reported to range from 1.9-17.59% .

In addition to being a cysteine-rich protein source, whey is also rich in phospholipids. The two phospholipid-rich membrane systems in milk include the skim milk membrane (SMM) and the milk fat globule membrane (MFGM) (Houlihan and Goddard, 1991). SMM is found in the serum component of the milk and MFGM surrounds fat globules in the cream phase, suggesting that SMM may provide the majority of phospholipids found in WPC. Both membrane systems consist of lipoproteins bound to phospholipids and triglycerides. Lipid components of the milk membrane fraction represent 22% of the total WPC lipid, of which, approximately 40% are phospholipids (Houlihan and Goddard, 1991). Therefore, only 10% of the lipids contained in WPC represent phospholipids. Some WPCs are enriched with lipids, however, and may contain higher percentages of phospholipids provided the lipid used to enrich the WPCs contained a large amount of milk membrane material. Of the phospholipids found in whey powders, up to 4% has been reported to be SM (Boyd et al., 1999).

Dietary sphingolipids, especially SM, are associated with a decreased risk for colon cancer. Supplementation of the diet with SM in non-pharmacological amounts significantly reduced tumor formation in a study with mice (Lemonnier et al., 2003). SM has shown to have many of the benefits of many chemotherapeutic agents without killing non-transformed cells, a serious side effect of chemotherapeutic agents. It has also proven to be just as effective when administered prior to or after tumor initiation (Lemonnier et al., 2003).

The present research examines the sphingolipid content in whey protein concentrates and isolates under the assumption that whey protein products with higher

lipid contents should contain higher proportions of SM. An extraction procedure is outlined for isolating sphingolipids from whey protein concentrates and isolates. Qualitative analysis was executed using thin layer chromatography (TLC). Samples were plated and compared to pure sphingomyelin standards to create a standard curve and determine the relative retention value (R_x). The average SM in each sample was determined following the extraction, fractionation, and TLC procedures outlined belowed. Quantitative analysis was then completed to confirm the qualitative results. Densitometry was used to determine the amount of SM in each spot on the TLC plates.

MATERIALS AND METHODS

Reagents

Methanol certified A.C.S., acetone HPLC grade, ammonium acetate certified A.C.S., primuline, nitrogen and standards of sphingomyelin from 99% pure bovine serum were purchased from Fisher Scientific (Fairlawn, NJ). Isopropyl ether stabilized with 100-200ppm BHT 98+%, hexane HPLC grade, acetic acid c.p., and ethyl acetate 99.5+% were obtained from ACROS Organics (Morris Plains, NJ). Chloroform, AR® (ACS) was from Mallinckrodt chemicals (Phillipsburg, NJ). Calcium chloride dihydrate reagent A.C.S was purchased from Baker & Adamson Products, Specialty Chemicals Division, Allied Chemical Corporation (Morristown, NJ).

Whey protein concentrate

Salibra™ 700, a bioactive whey concentrate, was a spray dried powder obtained from Glanbia Nutritionals (Monroe, WI). It was produced using a unique selective membrane transfer system and had the following characteristics: 72% protein, 15% fat, ~5% lactose, 4.0% moisture, 3.5% minerals, 3% phospholipids, and 0.8% sphingomyelin. Proliant 6000 is a tentative concentrated whey protein that is processed by an ultra-low temperature microfiltration. It was derived from sweet whey, membrane filtered, and spray dried by indirect heat. It was supplied by Proliant Inc. (Ames, IA) and has the following characteristics: 58.6% protein, 14.50% fat, and 3.65% moisture. Cold filtered PL-60 was derived from sweet whey using cold-filtered technology and was supplied by Trega Foods (Luxemburg, WI). No chemicals or solvents were used in the protein extraction to obtain the powder with the following characteristics: 58.92% protein, 17.59% fat, 12% lactose, and 2.35% moisture.

Whey protein isolates

ALACEN™ 895 whey protein isolate was manufactured by ion exchange and ultrafiltration (NZMP Inc. is a division of Fonterra, Lemoyne, PA). ALACEN 895 was a specialized protein providing acid and heat stability throughout the pH range with the following characteristics: 98.2% protein, 5.1% moisture, 0.6% lactose, and 0.3% fat. ALACEN™ 894 was manufactured by cross flow microfiltration and ultrafiltration (NZMP Inc. is a division of Fonterra, Lemoyne, PA). Microfiltration naturally isolates undenatured proteins, providing a product which is low in fat and high in protein. The

undenatured, soluble whey protein has the following characteristics: 94.6% protein, 4.3% moisture, 0.8% lactose, and 0.3% fat.

Equipment and Instrumentation

A DYNAC II Centrifuge, Clay Adams Brand was obtained from BD Diagnostic Systems (Sparks, MD). The BUCHI rotary evaporator used was from Brinkman Instruments (Westbury, NY). Aminopropyl columns, Discovery DSC-NH_2 were purchased from SUPELCO (Bellefont, PA). High-performance thin-layer chromatography (HPTLC) plates, 10x20cm, silica gel 60 on glass, were purchased from Macherey-Nagel (Germany). The water used was purified using a US Filter PURELAB purification system (18 MΩ).

Methods

The techniques used in this research can be divided into three parts: extraction, fractionation, and analysis of lipids. Whey protein isolates and concentrates utilized in this study include: whey protein isolates (one prepared by ion exchange and microfiltration and one prepared by ultrafiltration and microfiltration) and whey protein concentrates (15% lipids and 18% lipids). Table 1 summarizes the characteristics and composition of the samples studied.

sample	brand	isolate/concentrate	total lipid content (%)	moisture (%)	protein	lactose
894	Alacen	isolate	0.3	4.3	94.6	0.8
895	Alacen	isolate	0.3	5.1	98.2	0.6
PL-60	Trega	concentrate	17.59	2.35	58.92	12
700	Salibra	concentrate	15	4	72	~5
6000	Proliant	concentrate	14.5	3.65	58.6	na

Table 1: Overview of product information and composition for whey protein concentrates and isolates.

Extraction

The solid phase extraction (SPE) technique has been described previously (Folch et al., 1956). Each sample (10 g) is mixed with 10 mL of ultra purified water (18 MΩ), followed by the addition of a chloroform-methanol (2:1) solution (170 mL). The samples are homogenized in a Waring blender and filtered using steel wool to remove proteins. Water (17 mL) is added and the lipid extract is allowed to separate. The upper phase contains a small amount of residual proteins and ions. The lower phase contains the lipids from each sample, thus the upper phase is discarded using a volumetric pipet and the lower phase is collected. Methanol (5-10 mL) is added to the lower phase. The mixture is then centrifuged for 5 minutes at 2500 rpm. The upper phase is again removed with a pipet and the lower phase is dried in a rotary evaporator. Each dried lipid sample is resolubilized in 12 mL chloroform.

Fractionation

Fractionation is achieved by ion exchange with solid phase aminopropyl columns using established procedures (Bodennec et al., 2000). The 500 mg columns are preconditions with 3 mL hexane before the lipid samples (2 mL), which are in

chloroform:methanol solution are loaded and allowed to absorb and run through the columns. Each fraction collected is dried under nitrogen, weighed, and stored at -70˚C.

The first fraction containing neutral lipids is eluted from the column using ethyl acetate-hexane (15:85, 2 mL). The second fraction of $CHCl_3$:MeOH (23:1, 3 mL) elutes free ceramides, monoacylglycerols and a portion of free sphingosine and sphinganine. The third wash, diisopropyl ether-acetic acid (98:5, 2 mL), elutes normal and α-hydroxy free fatty acids, and trace amounts of free sphingoid bases. Fraction four containing neutral glycospingolipids and the remaining free sphingoid bases, is eluted using acetone-methanol (9:1.35, 7 mL). The addition of the fifth fraction, $CHCl_3$:MeOH (2:1, 3 mL), elutes neutral phospholipids (sphingomyelin, SM). Finally, the sixth fraction containing sphingosine 1-phosphate, ceramide 1-phosphate, and sulfitides is eluted using $CHCl_3$:MeOH:6M ammonium acetate (30:60:8, 4 mL). A flowchart of the extraction is presented in Figure 2.

Once the six fractions are eluted, each fraction is dried and weighed. A stock solution is made by adding a chloroform:methanol solution (2:1) to the sample to achieve a concentration of 10 mg/mL. From this stock solution, 10 µL are added to 490 µL chloroform:methanol solution (2:1) to achieve a final concentration of 1µg/5µL. The TLC plates are spotted with 5 µL of the sample solution (Figure 3).

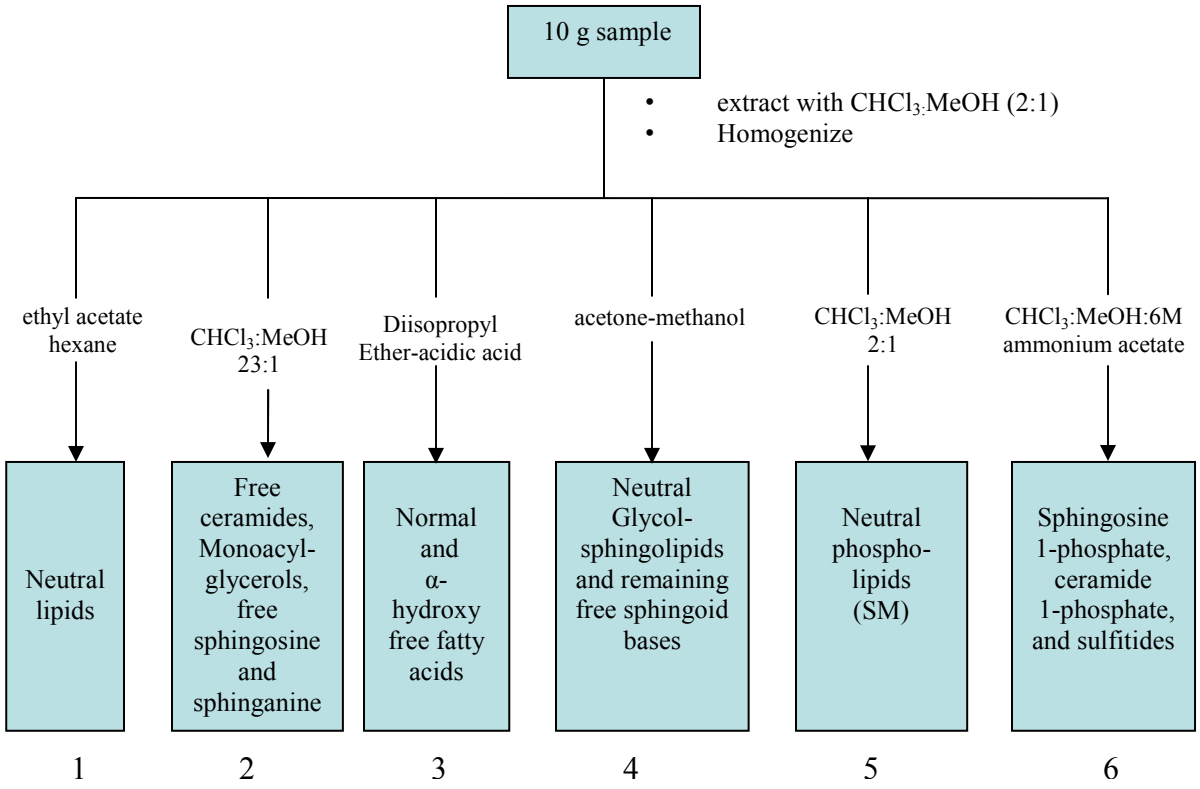

Figure 2: Flowchart of the extraction process used in the extraction step.

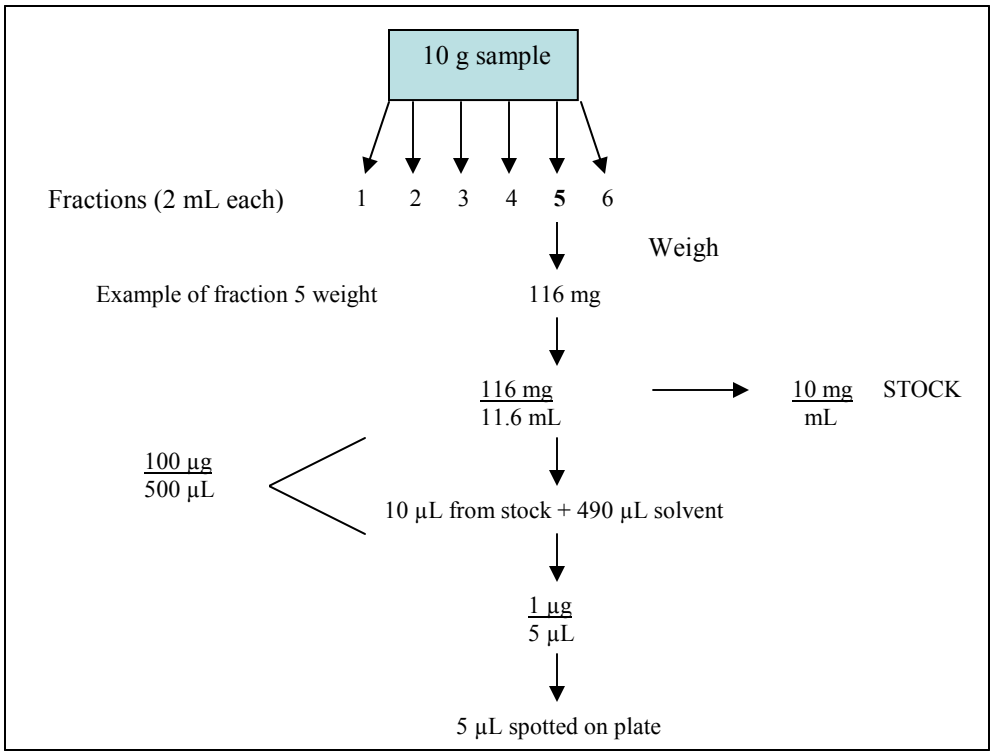

Figure 3: Typical dilution after SPE to prepare for TLC.

Analysis

The mobile phase, chloroform-methanol-calcium chloride (55:45:10), is poured into the glass TLC developing chamber such that the bottom of the chamber is covered, approximately 0.5 cm. Filter paper is placed inside the chamber to aid saturation of the developing chamber atmosphere. The tank is allowed to equilibrate for one hour. Fresh mobile phase is used after each plate development.

The high-performance thin-layer chromatography (HPTLC) plates are scored 6 cm from the origin to ensure a standard distance for mobile phase migration. Samples are spotted 2 cm from the bottom of the plate. First, standards of sphingomyelin from 99% pure bovine serum in concentrations of 0.15, 0.25, 0.5, and 1.0 µg/µL (chloroform:methanol solution (2:1)) were made and spotted on TLC plates and developed.

The plates are then spotted with 5 μL of each extraction and compared to standards. The sample is allowed to evaporate for a few minutes before being placed in the chamber for development. Once the solvent has migrated 6 cm, the plate is removed from the chamber and allowed to dry before being dipped in primuline, a non-specific visualization reagent. Again, the plate is dried and an ultra-violet (UV) photograph is taken. After each sample was extracted and plated, it became apparent that Fraction 5 was the only fraction that had the same R_x value as the standards. Thus, Fraction 5 is the SM sample and will be referred to as such for the remainder of the paper.

With the aid of the visualization reagent, a UV camera was used to make the spots visible. Densitometry was then used to convert the spots into a chromatogram consisting of a series of peaks. The peak positions are related to the migration distance and the identity of the component. The areas are related to the concentrations of the substances in the spots (Fried and Sherma, 1986). Peak areas were measured using Scion Image software.

Due to a great deal of variation when using TLC, standards are spotted on each plate with the set of samples. The peak areas were then used to develop a calibration curve. By using the equation of the line, the samples are compared to the standards. Back calculations were performed to find the amount (mg and percent) or SM present in each sample. From the curve, the coefficient of determionation (R^2) was also determined to predict the significance of the model. R^2 is a measure of how well all the points (for x and y values) fit on a straight-line graph ranging from -1.0 to 1.0. It is simply the square of the correlation coefficient (r), or the proportion of variance in y attributable to variance in x. Thus, it is not until a correlation of greater than 0.7 is obtained that a majority of the

variation of *y* is explained by variation in *x*. Since the R^2 value indicates the strength of the relationship between the samples and the standards, the high R^2 achieved indicates that the value is fairly accurate (O'Mahony, 1986).

RESULTS AND DISCUSSION

Sphinogmyelin is the predominant membrane sphingolipid that may have many biological advantages. It can be hydrolyzed to form ceremides which are proposed as messengers for events as diverse as differentiation, senescence, proliferation, cell cycle arrest and apotosis. SM has also been associated with a decreased risk for colon cancer (Lemonnier er al., 2003). The present research analyzes whey protein concentrates and isolates to determine the amount of SM present. Three steps, extraction, fractionation and analysis, were completed to make this determination.

Fractionation of Sphingolipids

After the extraction procedure (as described above), ion exchange columns were used to separate the remaining solids from the whey protein. All fractions were weighed, but Fraction 5 was the only one that contained SM. The triplicate Fraction 5 values were averaged and reported in Table 2.

Thin Layer Chromatography (TLC)

The purpose of the TLC procedure was to serve three functions, to separate, isolate, and identify the components of the whey protein extraction.

Once the sample spots have migrated, the plates were removed from the chamber, allowed to evaporate and the UV photographs were taken. They then underwent

densitometric analysis. A chromatograph was created consisting of a series of peaks (Figure 4). The area under each peak is related to the concentration of the SM present in each spot. This was measured using Scion Image software.

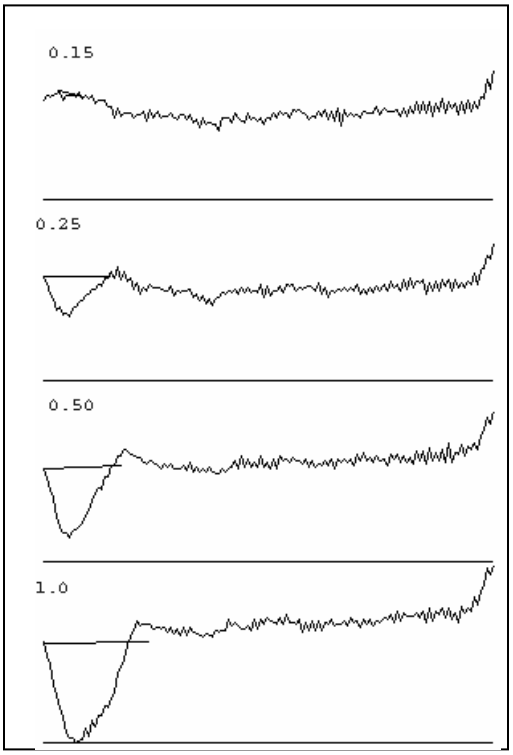

Figure 4: Typical plots of standard SM using densitometry for quantitative analysis.

Once the concentration of SM in each sample was determined, a calibration curve was developed. Figure 5A shows the standard spots increasing in size and intensity with the increasing concentrations. It also shows an equal migration distance of all the spots, indicating they are all SM.

By using densitometry, a calibration curve was developed (Figure 5B). As shown, the R^2 value is very close to one, indicating the concentrations of the standards

are accurate and the UV photograph provides an accurate image the densitometry software can properly measure.

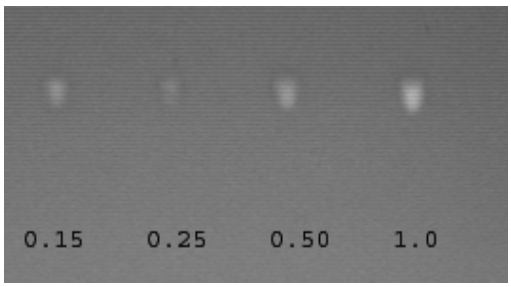

Figure 5A: Standards of SM from 99% pure bovine serum in concentrations of 0.15, 0.25, 0.5, and 1.0 µg/µL on HPTLC plate used to develop calibration curve.

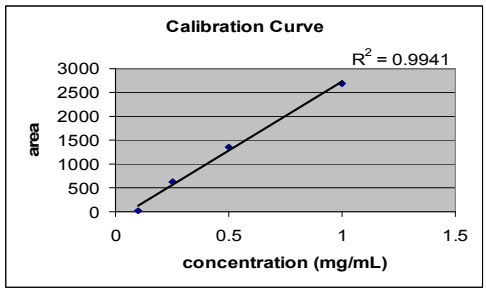

Figure 5B: Typical calibration curve for thin layer chromatography plate achieved by densitometry.

Qualitative Analysis

Once a standard curve was developed, SM samples from the extraction were plated and compared to the standards. From these plates, qualitative analysis could be performed. The R_f value is a convenient way to express the position of a substance on a developed chromatograph and is defined by

$$R_f = \frac{\text{distance of compound from origin}}{\text{distance of solvent front from origin}}$$

R_f values range from 0 to 0.99 and are unitless. Distance is typically measured to the center of the sample spot. The reproducibility of R_f values can be affected by many factors, including quality of sorbent, humidity, development distance, and ambient temperature (Touchstone, JC, 1992).

In order to express positions relative to the position of another substance, x, the relative retention value (R_x) is preferred. R_x values are more reproducible than absolute R_f values and values can be greater than 1.0. The R_x value is determined by the equation

$$R_x = \frac{\text{distance of compound from origin}}{\text{distance of reference compound x from origin}}$$

Since the SM samples are being compared to the SM standards, the R_x values are used to express the position of the samples in a more accurate way. Figure 6 shows a typical TLC plate with SM and standards. Each sample spot was measured and averaged to determine the R_x value. As shown in Table 2, the R_x values measured from all samples were very close to 1.0, which indicates that the identification of the component as SM is accurate.

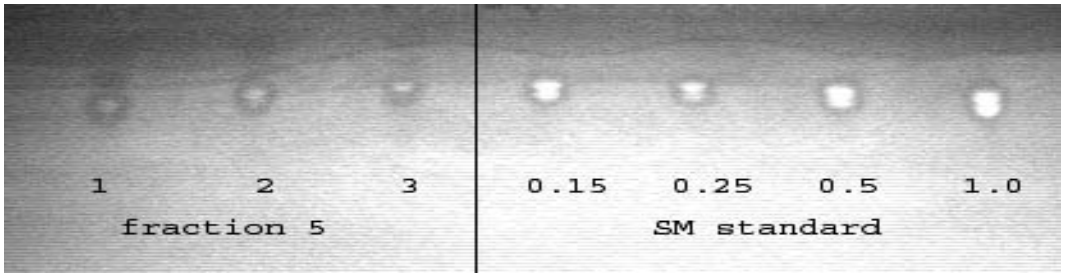

Figure 6: Typical TLC Plate. After SPE, fraction five is spotted (5 µL) in triplicate for each sample. SM standards are spotted (5 µL) in concentrations of 0.15, 0.25, 0.5, and 1.0 µg/µL. Densitometry was used to measure the concentration of SM in order to generate a calibration curve (Figure 5B).

sample	average of fraction 5 (mg)	R_x value
894	3.6667± 4.62	0.98
895	10.667± 6.66	0.9688
60	130.5 ± 11	1.07857
700	115.33 ± 19.01	0.981
6000	213.67± 42	0.9809

Table 2: Average weight extracted from fraction 5 and R_x value obtained through qualitative analysis.

Quantitative Analysis

Quantitative analysis can also be performed on the samples once the calibration curve is developed. Densitometry is again used to determine the amount of SM in each sample spot. The Scion Image software gives an area which can then be plugged into the equation from the standard curve. The equation that corresponds to that calibration curve in Figure 5B is $y = 2901.8x - 173.58$. Using the equation generated from the standards on each individual plates, the areas from the samples generated from the peaks can be plugged in for the y value to determine x, or the amount of SM present in each extraction. Using this number, back calculations were done to account for the fact that the procedure began with a ten gram sample of each whey protein. Table 3 shows how much SM was present (mg and percent) in each sample compared to the total lipid content.

sample	total lipid content (%)	mg SM	% SM
894	0.3	0.00564	0.001538
895	0.3	0.203	0.019
60	17.59	1.482	0.01136
700	15	2.223	0.019275
6000	14.5	2.917	0.01365

Table 3: Amount of SM (mg and %) found in samples compared to the total lipid content (%).

It was thought that samples of whey protein isolate and concentrate with higher fat contents would contain higher amounts of SM. After careful qualitative and quantitative analysis, that does not appear to be the case. When the lipid content of the sample is compared to the SM percentage, it is clear that the expected trend is not observed.

Error may have occurred in several different areas of the procedure. First, during the extraction, the sample is transferred several times before finally being resolubilized

and prepared for fractionation. After being absorbed through the ion-exchange columns, the fractions are again transferred before finally being dried and weighed. There are several instances where sample loss may have occurred

Another area to be considered is the different properties of each of the whey protein samples. Since each sample was prepared differently (selective membrane transfer, ultra-low temperature microfiltration), as discussed in the previous section, the interactions between SM and the reagents and packing material in the ion exchange columns may have effected the yield values.

It was found that the processing method of the whey protein plays a large role in the recovery of the lipids. Although both isolates have the same lipid content (0.3%), the isolate manufactured by cross flow microfiltration (ALACEN 894) should have more free lipids than the isolate manufactured by ion exchange (ALACEN 895). Since this was not the case, further study is needed to determine the discrepancy.

Humidity can greatly affect the migration of compounds during TLC. Because the experiments were conducted in a laboratory that does not have a controlled environment, changes in humidity may have affected the results. Further study is needed to determine if whey proteins with higher lipid contents actually contain higher concentrations of SM.

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