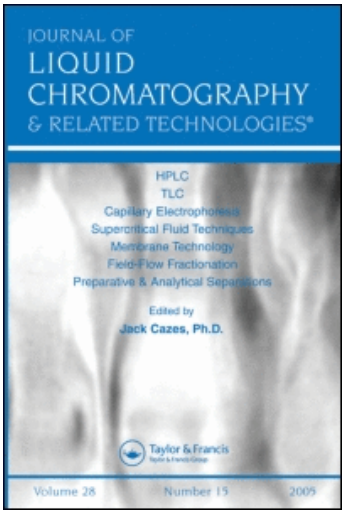


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# Assay of Analytes in Complex Matrices. II. Determination of Fat Content in IV Fat Emulsions by High Performance Liquid Chromatography

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# ASSAY OF ANALYTES IN COMPLEX MATRICES. II. DETERMINATION OF FAT CONTENT IN IV FAT EMULSIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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## ABSTRACT

Safflower oil and soybean oil, which are the major constituents of fat emulsions for intravenous use, are both complex mixtures of triglycerides. Separation of triglycerides from the other ingredients of IV fat emulsion and their elution together as a single peak have been achieved through the use of a highly retentive Polymer Reverse Phase (PRP) column in tandem with a strong mobile phase. Quantitation is performed against a standard preparation of soybean oil using refractive index detection. The procedure is based on the use of the composite response from a known amount of one complex mixture of triglycerides to quantitate another complex mixture of triglycerides, which may even differ from the former in composition. A validation strategy for this unique situation has been developed and implemented.

## INTRODUCTION

Commercially available fat emulsions are dispersions of soy bean and/or safflower oil. Soy lecithin or egg yolk phospholipids are used as the emulsifying agent. Glycerin is added to the fat emulsions to make them isotonic. Fat content values of IV Fat Emulsions need to be controlled within strict limits of 90% to 110% of label claims. This has necessitated a precise and accurate procedure for quantitation of fat in IV Fat emulsions. Described below is an HPLC procedure based on refractive index detection for the quantitation of fat in IV Fat Emulsions. Chromatography is performed on a Polymer Reverse Phase (PRP) column, using a mixture of isopropyl alcohol, ethyl acetate, and acetic acid as the mobile phase. Quantitation is performed against a standard preparation of soybean oil using refractive index detection.



## EXPERIMENTAL

### Equipment and Assay Conditions

a) The liquid chromatographic system consisted of a Model 510 pump and a Model 710B autosampler (both from Waters Associates, Milford, MA), a refractive index detector (Knauer Differential Refractometer, Rainin Instruments) and a Chromatopac CR3A integrator (Shimadzu, Kyoto, Japan).

b) Column: A PRP-1 column 25 cm x 4.5 mm (Hamilton Part # 79427) enclosed in a column oven (not heated) for thermal isolation.

c) Mobile Phase contained 0.5 % acetic acid in a mixture (90:10, v/v) of ethyl acetate and isopropyl alcohol (v/v). Mobile phase flow rate was adjusted between 0.8 to 1.2 mL so that the peak due to triglycerides appears between 5.5 and 7.5 minutes. Sample and standard injection volumes were 50 μL each.

### Materials

Certified analytical reagent grade glacial acetic acid, HPLC grade ethyl acetate, and HPLC grade isopropyl alcohol were used in the preparation of mobile phase. Egg yolk phospholipids, glycerin, safflower oil, and soybean oil used in these studies were tested and approved for use by Abbott Laboratories Quality Assurance Department against sets of FDA approved proprietary specifications and testing methodology.

One lot of soybean oil was extensively characterized and qualified for use as a reference standard. This material was filled into 1 mL ampoules (0.5 g/ ampoule) and sealed with a nitrogen headspace with each ampoule designated for one time use only.

### Standard, Sample, and System Suitability Preparations

The standard preparation contained 8 mg/mL of soybean oil reference standard in the mobile phase. A quantity of the sample equivalent to 200 ± 20 mg of fat was diluted to 25.0 mL with the mobile phase for the sample preparation. The system suitability preparation contained 8 mg/mL of soybean oil reference standard and 6 mg/mL of the egg yolk phospholipids dissolved in the mobile phase.



### System Suitability

With the system components in place, and with a steady base line, the standard preparation was repeatedly injected into the chromatograph and the flow rate was adjusted between 0.8 and 1.2 mL/min until the peak due to fat eluted between 5.5 and 7.5 minutes. On injection of the system suitability preparation under these conditions the signal after the peak due to the egg phosphatide came to baseline about 1 minute prior to the start of the peak due to fat. Such a baseline resolution between the fat and the egg phosphatide peaks was set as a system suitability condition for the assay. The egg phosphatide, being a mixture primarily of phosphatidyl choline and phosphatidyl ethanolamine, often eluted as a pair of split peaks and sometimes as a single broad peak with a shoulder. The fat being a complex mixture of triglycerides of both saturated and variably unsaturated long chain fatty acids, with chain lengths varying from 16 to 18 carbon atoms, the peak due to the fat was broad (width at base of about 1.5 to 2 minutes). In addition to being broad, the peak due to fat was also sometimes slightly skewed either to the lead side or to the tail side of the peak. The integration parameters were adjusted so that the peak due to fat was integrated as a single peak.

### Linearity, Spike Addition/Recovery, and Short-Term Precision

Six sets of solutions of 2, 4, 8, 12, and 16 mg/mL were prepared using two lots of safflower oil, two lots of soybean oil, and two 50/50 (w/w) soybean oil/safflower oil mixtures. The six sets of peak area/concentration data were used to compute linear regression parameters for individual oils as well as a composite regression data for all the oils together. Inter-correlation coefficients of responses between pairs of oils were also computed from these data.

Addition/recovery samples, for establishing accuracy, were prepared by mixing known volumes of appropriate stock solutions of oil and/or mixtures of oils, egg phosphatide (both in mobile phase) and of glycerin (in water), and finally diluting to known volumes. The quantities were chosen such that at final dilutions, these solutions contained glycerin, water, and egg phosphatide at levels expected for a sample preparations that would result from 2%, 10%, 20%, and 30% IV fat emulsions and oil concentrations of 7, 8, 9, or 10 mg/mL. Both 100% soybean and 50/50 mixture of soybean and safflower oils were used in these studies. Precision data for the same three different lots of fat emulsions were generated over six different days.

### Procedure

Equal volumes (50 μL) of the standard and sample preparations were injected into the chromatograph and the respective peak area responses ($A_s$ and $A_u$) were obtained from the integrator. Average $A_s$ and $A_u$ values from duplicate injections of the standard and sample preparations were used in the calculation of the sample assay result.



### Regeneration of Columns

The column backpressure tended to increase with number of samples injected. The egg phosphatide used as the emulsifier in fat emulsions contains trace levels of electrolytes. The pH adjustment of fat emulsions during manufacturing also introduces trace levels of electrolytes into the emulsion. These salts which are not soluble in the 100% organic mobile phase, precipitate as fine particles and collect at the inlet end frit of the column and may even partially plug the inlet end frit. This results in the increase in column backpressure. After about 40 to 50 sample injections, or when the increase in the column back pressure was about 25 to 30% over the initial value, the column was back flushed with about 200 mL of a 5% solution of acetonitrile in water. The column was reequilibrated with the mobile phase prior to subsequent use.

## RESULTS AND DISCUSSION

Abbott currently markets a number of safflower and soybean oil based intravenous fat emulsions under the LIPOSYN registration mark. The marketed products are formulated at 10% and 20% fat content. The fat contents in these marketed products were for a long time determined by a gravimetric procedure in which a sample of lyophilized fat emulsion was subjected to column chromatography. Elution with chloroform and rotary evaporation of the eluate to constant weight provided a means to quantitate the fat content in the samples.

The procedure leads to precise and accurate results. However, it is labor intensive and requires the use of large quantities of chloroform, a known mutagen. The need to eliminate the use of a known mutagen from the work place itself was sufficient enough of an incentive to pursue an alternate methodology for this assay.

Chromatography based assay procedures make use of the responses from known concentrations of reference standard materials which are almost always well characterized single chemical entities. When multi-component samples are assayed, the corresponding standard preparation often will be a mixture of known composition of single entity chemical reference standard of each analyte of interest. On rare occasions, response relationships between each analyte of interest with a single surrogate standard are also used to assay individual components in a multi-component sample.



Tchapla and Heron have recently reported results of a systematic study on choice of stationary and mobile phases for the liquid chromatographic separation of triglycerides.[1] These authors recommended a polymeric column with a mixture of acetonitrile and methylene chloride as the mobile phase. The chromatographic procedures in the published literature for triglycerides can be grouped into three categories:

a) HPLC or GC based procedures for separation based on volatility and carbon number of the triglycerides.[2-5]

b) HPLC or GC based procedures for separation of fatty acids as their methyl esters and characterization of the oil based on the relative abundance of these fatty acids in the oil.[6]

c) HPLC based procedures using silver ion impregnated columns, where separation is based on the degree of unsaturation, stereochemistry about the double bond etc., of the component fatty acids.[7,8]

Published procedures are primarily for identification of the oil type through their fatty acid distribution profiles. Quantitation of triglycerides has been only a secondary consideration in many of these publications. Adaptations of methods based on the Hantsz reaction is routinely relied on for the determinations of serum triglycerides in clinical chemistry. The procedure involves saponification of the triglycerides, periodate oxidation of the resulting glycerin to formaldehyde, and the reaction of the formaldehyde with a 1,3-dicarbonyl compound such as acetylacetone in presence of ammonium ions. The concentration of the lutidine derivative that results is spectrophotometrically determined and correlated to the concentration of triglycerides in serum.

Alternatively, the glycerin from saponification of serum derived triglycerides are also estimated using enzymatic procedures in clinical laboratories. Serum is practically devoid of free glycerin and contains only trace levels of phospholipids or other glycerides. Hence procedures based on Hantsz reaction or alternate enzymatic procedures are acceptable for the serum triglycerides determinations in clinical chemistry. However, intravenous fat emulsions contain added glycerin for the control of tonicity and phospholipids as the emulsifier. Hydrolysis of phospholipids will result in the formation of glycerin. Therefore, prior separation of glycerin and phospholipid will have to occur if the clinical laboratory methods were to be adapted for fat content determination in fat emulsions. If the separation of glycerin and phospholipid from triglyceride could be achieved in an HPLC procedure, it was felt that a mass sensitive detection such as differential refractometry could be used for the quantitation of the triglyceride. Since the triglyceride itself is a complex mixture of many species, at the outset it was recognized that such an HPLC procedure will need to be one which suppresses separation based on degree of unsaturation, carbon number etc., but capable of separating mono, di, and triglycerides, phospholipids, and glycerin. In other words the separation conditions should achieve class separation based on large differences in polarity, partition coefficients etc., as appropriate, but avoid species differentiation where differences in polarity, partition coefficients, etc., are minimal.



Class separation without species differentiation can be achieved by using a stationary phase with very high retention for the analytes of interest, and using a very strong mobile phase. Instances of such separations are abundant in thin layer chromatography where classes of polar compounds are routinely separated on silica gel plates (very high capacity factors for polar compounds), using strong developing solvents (often mixtures of chloroform, methanol, water, and acids like formic acid or bases like ammonia). The packing material used in PRP-1 columns is a highly cross-linked copolymer of styrene and divinylbenzene. These columns have very high retention and in general behave as reverse phase columns with very high capacity factors for lipophylic analytes. These columns can also tolerate a wide range of aqueous, aqueous-organic and organic mobile phases and both acidic and basic pHs. It was felt that a PRP-1 column in tandem with a primarily organic mobile phase would effect the desired separation. Typical chromatograms of a sample and standard preparation, a glycerin preparation in water, and an egg phosphatide preparation in the mobile phase are shown in Figure 1.

As can be seen from Figure 1, the peaks due to glycerin, water, and components of egg phosphatide occur well before the peak due to fat. The separation profile validated our choices of the column and the mobile phase for this assay.

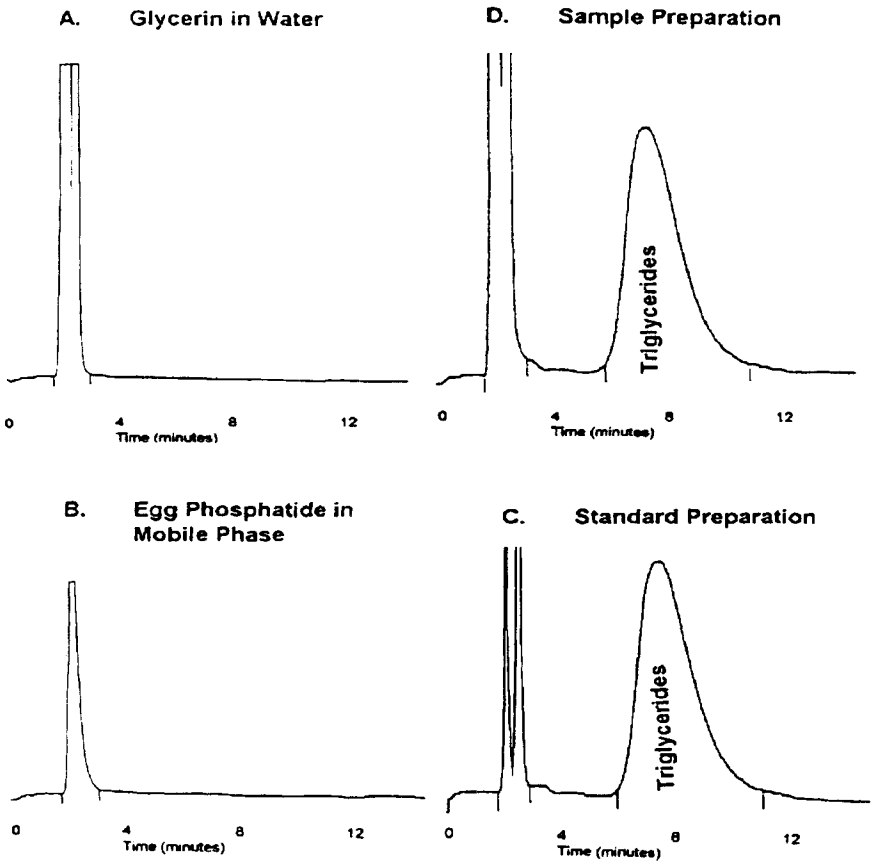


**Figure 1.** Typical chromatogram of a glycerin preparation in water (A), an egg phosphatide preparation in the mobile phase (B), an oil standard preparation (C) and a Liposyn sample preparation (D).

HPLC assay procedure predicates the use of a standard. In normal chromatographic assay procedures, the analyte is a single chemical entity for which a reference standard can be easily characterized and made available. Both the soybean oil and the safflower oil used in the IV fat emulsions are complex mixtures of mixed triglycerides of long chain fatty acids. The major fatty acids found in these two oils are palmitic, stearic, oleic, linoleic, and linolenic acids. The percentage range for individual acids will vary for the type of oil used and even for the same type of oil, from lot to lot, season to season, and even with the geographic location where produced. No two lots of the same vegetable oil material can be expected to be identical in every respect and test criterion.

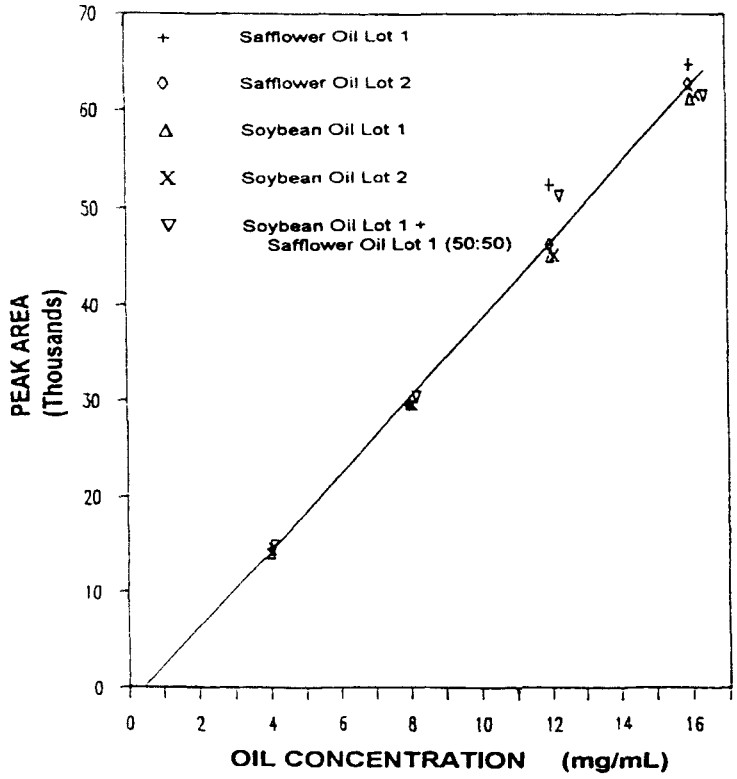


**Figure 2.** Plot of peak area versus concentration of oils: Composite regression line for six lots of oil.(Data for five individual oils are plotted by symbols only.)

Fatty acid profiles of soybean and safflower oil used in the emulsion can at best be considered comparable within broad limits. Use of one lot of oil (whether soybean or safflower) as a standard would mean that the composite response from a known amount of one complex mixture of triglycerides will be used to quantitate another complex mixture of triglycerides, which may even differ in composition (i.e. fatty acid distribution) and type from the material used as the standard.

A logical sequence to establish the validity of this approach will be to show that:

1. Peak area versus concentration curves for various oils are superimposeable with in experimental error.

**Table 1**

**Linear Regression Parameters for Peak Area Versus Concentration Data for Different Oils***

| Oil Type | Corr. Coeff. | Slope | Intercept |
|---|---|---|---|
| Safflower oil Lot 1 | 0.9878 | 4315 | -2723 |
| Safflower oil Lot 2 | 0.9966 | 4044 | -2121 |
| Soybean oil Lot 1 | 0.9999 | 3899 | -1487 |
| Soybean oil Lot 2 | 0.996 | 3876 | -1365 |
| Mixture A** | 0.9849 | 3912 | -558 |
| Mixture B*** | 0.9826 | 4401 | -5410 |
| Composite for all six | 0.9882 | 4007 | -1633 |

*All data generated in a single uninterrupted HPLC run using the same instrumentation and same batch of mobile phase.
** (50/50 (w/w)) Safflower oil Lot 1 and Soybean oil Lot 1.
*** (50/50 (w/w)) Safflower oil Lot 2 and Soybean oil Lot 2.



**Table 2**

**Inter Correlation of Peak Area Data at Five Concentration Levels for Six Different Samples of Oil**

| | Safflower Oil Lot 1 | Safflower Oil Lot 2 | Soybean Oil Lot 1 | Soybean Oil Lot 2 | Mixture |
|---|---|---|---|---|---|
| Safflower oil Lot 2 | 0.996 | | | | |
| Soybean oil Lot 1 | 0.996 | 0.999 | | | |
| Soybean oil Lot 2 | 0.995 | 0.999 | 1.000 | | |
| Mixture A | 0.999 | 0.994 | 0.995 | 0.994 | |
| Mixture B | 0.984 | 0.994 | 0.994 | 0.995 | 0.979 |

2. Results obtained for the same sample using different lots of oil as standard are statistically indistinguishable from each other.

3. Respective sets of assay results from HPLC, and a known triglyceride specific non-chromatographic procedure for multiple lots of samples are identical with in experimental error.

**Table 3**

**Fat Content Values Obtained for Three Lots of IV Fat Emulsions Using Different Lots of Oil in the Standard Preparations**

| Type of Oil Used as Standard | Fat Content Found (mg/mL) Sample A (Liposyn 10%) | Sample B (Liposyn 20%) | Sample C (Liposyn 30%) |
|---|---|---|---|
| Soybean oil Lot 1 | | 199.0 | 308.4 |
| Soybean oil Lot 2 | 99.7 | 197.7 | 309.2 |
| Safflower oil Lot 1 | 98.6 | 194.2 | 308.4 |
| Safflower oil Lot 2 | 98.8 | 195.0 | 309.3 |
| Mixture A* | 99.0 | 198.7 | 307.2 |
| Mixture B** | 99.7 | 199.6 | 309.9 |
| Mean | 99.2 | 197.4 | 308.7 |
| Standard Deviation | ±0.5 | ±2.2 | ±0.9 |
| Relative Standard Deviation | ±0.5% | ±1.1% | ±0.3% |

* Mixture A: 50/50 mixture of Soybean Oil Lot 1 and Safflower Oil Lot 1.
** Mixture B: 50/50 mixture of Soybean Oil Lot 2 and Safflower Oil Lot 2.



A composite plot of peak area versus oil concentrations for two lots each of soybean and safflower oil and two mixtures of the two oil types are shown in Figure 2. Linear regression parameters for peak area versus concentration for each of the individual oil are shown in Table 1. Each parameter set was generated from duplicate injections of solutions of these oil or oil mixtures at 2, 4, 8, 12 and 16 mg/mL respectively. Table 1 also contains regression parameters for the composite linear curve from all injections of all oils. Peak areas from duplicate injections were normalized for each level of each of the five concentration levels. To get the normalized peak areas for each oil, for example, at 2 mg/mL level, the observed peak area was multiplied by 2 and divided by the actual known concentration. This process is repeated for each of the six oils and at each of the five concentration levels. The inter-correlation for pairs of oils were calculated from the regression of the paired sets of these normalized peak areas. Table 2 shows the inter-correlation data. Each inter correlation coefficient in Table 2 represent the slope of the normalized peak area versus the corresponding normalized peak area curve for the respective pairs of oils. A value of unity should be expected for an exact correlation since peak responses for identical concentrations will be expected to be identical.

**Table 4**

**Addition/Recovery of Soybean and Safflower/Soybean Oils in IV Fat Emulsions**

| % Level of Addition | Analyst | mg/mL Added | mg/mL Recovered | % Recovery |
|---|---|---|---|---|
| **10% and 20% Soybean Oil Emulsion** | | | | |
| 87.5 | A | 7.00 | 6.89 | 98.4 |
| | B | 7.00 | 6.81 | 97.3 |
| 100.0 | A | 8.00 | 8.02 | 100.2 |
| | B | 8.00 | 7.88 | 98.5 |
| 112.5 | A | 9.00 | 9.01 | 100.1 |
| | B | 9.00 | 8.80 | 97.8 |
| 125.0 | A | 10.00 | 10.19 | 101.9 |
| | B | 10.01 | 9.83 | 98.3 |
| **10% and 20% Soybean Oil/Safflower Oil Emulsion** | | | | |
| 87.5 | A | 7.05 | 7.22 | 102.4 |
| | B | 7.01 | 6.96 | 99.3 |
| 100.0 | A | 8.06 | 8.15 | 101.1 |
| | B | 8.02 | 7.94 | 99.0 |
| 112.5 | A | 9.06 | 9.23 | 101.9 |
| | B | 9.02 | 8.93 | 99.0 |
| 125.0 | A | 10.07 | 10.45 | 103.8 |
| | B | 10.02 | 10.03 | 100.1 |
| **30% Emuslion Soybean Oil/Safflower Oil (50/50 (w/w))** | | | | |
| 77.8 | A | 7.02 | 6.88 | 98.0 |
| | B | 7.02 | 7113 | 101.6 |
| 88.9 | A | 7.02 | 7.09 | 101.0 |
| | B | 8.02 | 8.21 | 102.4 |
| 100.0 | A | 9.02 | 9.08 | 100.7 |
| | B | 9.02 | 9.33 | 103.4 |
| 111.1 | A | 10.03 | 10.12 | 100.9 |
| | B | 10.02 | 10.32 | 103.0 |

(continued)

**Table 4 (continued)**

| % Level of Addition | Analyst | mg/mL Added | mg/mL Recovered | % Recovery |
|---|---|---|---|---|
| **2% Emulsion Soybean Oil/Safflower Oil (50/50(w/w))** | | | | |
| 87.5 | A | 7.05 | 7.18 | 101.8 |
| | B | 7.01 | 6.97 | 99.4 |
| 100.0 | A | 8.06 | 8.03 | 99.6 |
| | B | 8.02 | 7.97 | 99.4 |
| 112.5 | A | 9.06 | 9.13 | 100.8 |
| | B | 9.02 | 8.99 | 99.7 |
| 125.0 | A | 10.07 | 10.01 | 99.4 |
| | B | 10.02 | 9.98 | 99.6 |
| | | | Mean Recovery | 100.3% |
| | | | Standard Deviation | ±1.7 |
| | | | Relative Standard Deviation | ±1.7% |



As can be seen from Tables 1 and 2, Mixture B had the worst correlation coefficient for linear regression and also had the worst inter correlation with other oils. The closeness to unity of all values in Table 2 strongly suggests that standard preparation from any lot of oil can be used to accurately determine the fat content in emulsions made with other lots, types or even mixtures of oils.

Three different lots of fat emulsions were assayed, each a total of six times, each time against a standard preparation from a different lot and/or type of oil. The assay results are summarized in Table 3. The three lots of emulsions assayed at mean fat contents of 99.2 ± 0.5, 197.4 ± 2.2 and 308.7 ± 0.9 mg/mL respectively. (The relative standard deviation ranged from ±0.3% to ±1.1%). The data in Table 3 showed that the standard preparation from any lot or type of oil or even mixtures of oil can be used to accurately determine the fat content in emulsions made with other lots, types or even mixtures of oils as long as the fatty acid profile of the standard and the oil used in the emulsion are comparable within broad limits.

## Accuracy and Precision

The results of addition/recovery studies are summarized in Table 4. The assay procedure is adjusted such that typical sample preparations of emulsions will nominally contain 8 mg/mL of oil. Addition sample were prepared by

**Table 5**

**Precision Data Obtained for Three Lots of IV Fat Emulsion**

| | Fat Content Found (% of Theory) | | |
|---|---|---|---|
| **Day** | **Lot A** | **Lot B** | **Lot C** |
| 1 | 100.7 | 97.4 | 100.9 |
| 2 | 101.2 | 97.7 | 101.4 |
| 3 | 101.4 | 97.6 | 100.7 |
| 4 | 103.1 | 99.5 | 103.1 |
| 5 | 102.2 | 99.3 | 101.9 |
| 6 | 98.6 | 97.1 | 102.8 |
| Mean | 101.2 | 98.1 | 101.8 |
| Standard Deviation | ±1.5 | ±1.0 | ±1.0 |
| Relative Standard Deviation | ±1.5% | ±1.0% | ±1.0% |



mixing known volumes of appropriate stock solutions of oil and/or mixtures of oils, egg phosphatide, (both in mobile phase) and of glycerin (in water), and finally diluting to known volumes. The quantities were chosen such that at final dilutions, these solutions contained glycerin, water, and egg phosphatide at levels expected for sample preparations corresponding to 2%, 10%, 20%, or 30% IV fat emulsion and oil at 7, 8, 9, or 10 mg/mL. As can be seen from Table 4, the mean recovery is 100.3% (RSD ±1.7%). Precision data for three different lots of fat emulsion are summarized in Table 5. The precision data were generated over a period of six days by two analysts, using six different standard preparations of the same reference standard lot of soybean oil and three different sets of six sample preparations, each set representing a single lot of fat emulsion. A relative standard deviation of 1.0 to 1.5 percent can be considered excellent for this assay.

Fat content results of a number of lots IV fat emulsion determined both by the HPLC procedure discussed here and by a gravimetric procedure are shown in Table 6. The gravimetric results were generated by chromatographing a lyophilized sample of emulsion on a column packed with silica gel. The triglycerides were isolated by elution with a mixture of chloroform and methanol (9:1, v/v) and rotary evaporation of the eluate to constant weight. The last column in Table 6, which shows the ratios of assay results by HPLC and gravimetry is an indicator of the correlation between the two vastly different procedures.

**Table 6**

**Comparison of Fat Content Assay Results by HPLC and Gravimetry for a Few Lots of IV Fat Emulsions**

| Fat Emulsion Lot # | Fat Content (% of Theory) HPLC | Gravimetry | HPLC/ Gravimetry |
|---|---|---|---|
| 1 | 99 | 100 | 0.990 |
| 2 | 99 | 100 | 0.990 |
| 3 | 102 | 98 | 1.041 |
| 4 | 101 | 99 | 1.020 |
| 5 | 101 | 99 | 1.020 |
| 6 | 101 | 98 | 1.031 |
| 7 | 101 | 99 | 1.020 |
| 8 | 102 | 99 | 1.030 |
| | | Mean | 1.018 |
| | | Standard Deviation | ±0.019 |
| | | Relative Standard Deviation | ±1.9% |



## SUMMARY AND CONCLUSIONS

Data presented here show that a simple sample dilution in the mobile phase and HPLC can be used to advantage for the assay of a complex analyte such as a triglyceride mixture in a complex matrix such as an intravenous fat emulsion. The chromatographic conditions employed assured that all triglycerides elute together without species differentiation as a single peak. Statistically indistinguishable results are obtained for the same sample when multiple assays, each against separate oil standard, were employed. Inter-correlation studies demonstrated that the composite response from a known amount of one complex mixture of triglycerides could be used to quantitate another complex mixture of triglycerides, which may even differ in composition.

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