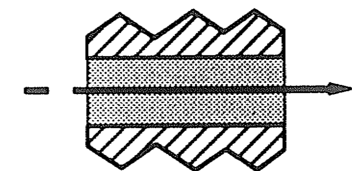
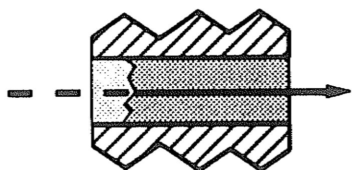
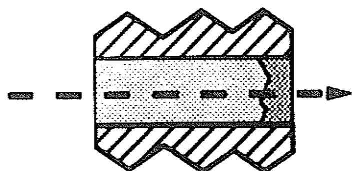
Fractionation of Egg Yolk Lipids Using Supercritical Carbon Dioxide and Entrainer



by
Lucia T. Labay





A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Department of Food Science
University of Manitoba
Winnipeg, Manitoba, Canada

© April 1991

A.M.D.G.

FRACTIONATION OF EGG YOLK LIPIDS USING

SUPERCRITICAL CARBON DIOXIDE AND ENTRAINER

BY

LUCIA T. LABAY

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

© 1991

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this thesis. to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film, and UNIVERSITY MICROFILMS to publish an abstract of this thesis.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

Abstract

The efficacy of egg yolk lipid fractionation with supercritical carbon dioxide and entrainers was studied. The principal aims were to study the feasibility of cholesterol removal and to determine the effects of extraction pressure and temperature and entrainer type and concentration upon extraction rate and lipid fractionation. Analysis for phosphatidylcholine (PC) was also performed.

A Superpressure Supercritical Screening unit was used to extract freeze-dried egg yolk samples. Extraction pressure was varied from 15 to 36 MPa at 40°C and extraction temperature was varied from 40°C to 75°C at 36 MPa. Ethanol at 3 wt % and methanol at 3 or 5 wt % concentration were tried as entrainers. Extracted samples were analyzed by HPLC for cholesterol content and FAME-GLC for fatty acid profiles. Phospholipid determinations were made using the enzymatic procedure outlined by Boehringer Mannheim.

Solubility of extracted lipids was found to be strongly dependent on extraction pressure. Maximum solubility (10 mg/g CO_2) at 36 MPa, 40°C dropped to a near negligible 0.67 mg/g CO_2 at 15 MPa, 40°C. Solubilities were found to decrease with increasing temperature, however, the difference in solubility between 40°C and 55°C was not significant. The inclusion of ethanol and methanol as entrainers also increased solubility significantly; however, at a 3% concentration these two entrainers had roughly equivalent effects on increasing solubility.

Lipid recovery, approximating 70% total extractable lipid, appeared to remain constant over the range of pressures and temperatures tested in this study. When an entrained system was run to exhaustion, higher percent recoveries of total lipid were obtained, more so, with entrained systems of higher concentrations. The rate of removal was a function of extraction temperature and pressure and was enhanced significantly by the addition of methanol or ethanol co-solvents.

The apparent solubility of cholesterol was found to be dependent on pressure and the addition of entrainer. An increase in CO_2 density increased the apparent solubility of cholesterol. The effect of a change in the temperature at constant pressure on the apparent solubility of cholesterol was insignificant over the temperatures studied. The apparent solubility of cholesterol was increased more by the presence of 3-5 % concentrations of entrainer than by changes in the pressure of several MPa. The relative concentration of cholesterol as a percent of egg yolk lipid was found to be affected by the extraction parameters. An increase in temperature increased the relative concentration of cholesterol in the extract. Likewise, a decrease in pressure increased the relative concentration of cholesterol in the extract. The fatty acid composition of the extractions was independent of temperature, pressure and presence of entrainer and remained constant throughout the extraction. When no entrainer was present, the CO_2 extracted lipid was essentially free from phospholipid content ($<$ 0.2%) at both early and late stages of an extraction trial. No appreciable quantities of phospholipids were detected in the extracts obtained at the onset of an entrained extraction run. However, significant phospholipid levels were found in the entrainer-enhanced SC-CO_2 lipid extracts collected in the latter part of the run.

Acknowledgements

My sincerest thanks is expressed to my two supervisors, Dr. N.R. Bulley and Dr. S. Arntfield. A thank-you to Dr. N.R. Bulley for his financial support and for his advice during the course of this research. Thank-you to Dr. S. Arntfield, your commitment to high quality and attention to detail challenged me and pushed me to achieve my personal best; your help was *most* encouraging at all times.

I am grateful to Dr. E.D. Murray and the Department of Food Science for providing me with testing facilities. Sincere appreciation is extended to Dr. E.D. Murray for his encouragement during our consultations and for his guidance of my M.Sc. program. Special thanks are due to J. Rogers and M. Szczygiel who kindly provided technical advice on many occasions.

Deepest appreciation is extended to W.J. Crerar who helped me throughout the course of this endeavour by providing both unfailing moral support and technical support.

I was fortunate, to say the least, to have my brother, Vladimir, as my closest companion and colleague during our years of study together at the University of Manitoba.

Heartfelt thanks are given to my husband, Kevin, for his enthusiasm and warm encouragement during my studies. Lastly, I would like to thank my parents, Rudolph and Dorothy Labay. You have brought me to this day and the greatest lesson I learned has not been from academia – most of all you have taught me that knowledge is not won by human effort but is a gift from God Almighty.

Ad Majorem Dei Gloriam!

Table of Contents

ABSTRACT ii

ACKNOWLEDGEMENTS iv

LIST OF TABLES viii

LIST OF FIGURES xi

1.0 INTRODUCTION **1**

2.0 LITERATURE REVIEW **8**

2.1 The chemical compostion and structure of eggs (Gallus domesticus) 8

2.1.1 Composition of egg yolk 8

2.1.1.1 *Egg yolk lipids* 10

2.2 The role of eggs in health 13

2.2.1 Nutritive value of eggs 15

2.2.2 Cholesterol 16

2.2.2.1 *Structure* 16

2.2.2.2 *Biomedical Importance* 16

2.2.2.3 *Diet and risk factors in cornary heart disease* 18

2.3 Lecithin 26

2.3.1 Structure, nomenclature, composition, sources 26

2.3.2 Commercial lecithin products and food use of soybean lecithin 29

2.3.3 Therapeutic and research applications of lecithin 29

2.4 Isolation of egg components 33

2.4.1 Conventional methods of extraction 33

2.4.2 Supercritical extraction 34

2.4.2.1 *Supercritical carbon dioxide as a solvent* 34

2.4.2.2 *Solubility of organic compounds in SC-CO_2* 38

2.4.2.3 *Supercrical extraction of lipids* 40

3.0 EXPERIMENTAL EQUIPMENT AND PROCEDURES **42**

3.1 Experimental Equipment 42

3.1.1 Extraction apparatus 42

3.1.1.1 *Extraction vessel, temperature & pressure regulation* 44

3.1.1.2 *Flow control* 44

3.1.1.3 *Flow rate* 44

3.1.1.4 *Sample Collection* 44

3.1.2 Experimental extraction procedures 46

3.1.2.1 *Operating conditions, pressure, temperature entrainer, flow rate* 46

3.1.2.2 *Vessel loading procedure* 47

3.1.2.3 *System start-up and pressurization* 47

3.1.2.4 *Extract sampling* 48

3.1.2.5 *Shut-down and post-extraction* 48

3.2 Materials 48

3.2.1 Egg Yolk 48

3.2.2 Extraction solvents 49

3.2.2.1 *Carbon dioxide* 49

3.2.2.2 *Entrained mixtures* 49

3.2.3 Analytical standards, chemicals, solvents 51

3.3 Extract analysis 51

3.3.1 Compositional analysis 51

3.3.2 Analysis of fatty acids 51

3.3.2.1 *Transesterification* 52

3.3.2.2 *G.C. identification and quantification* 52

3.3.3 Extraction and quantification of cholesterol by HPLC 53

3.3.4 Phospholipid analysis 56

3.4 Data calculations 58

3.4.1 Extraction data 58

3.4.1.1 *Mass Balance* 60

3.4.1.2 *Solubility determinations* 60

3.4.1.3 *Lipid Recovery* 65

3.4.2 Component analysis of extracts 66

3.4.2.1 *Fatty acid data standarization* 66

3.4.2.2 *Triglyceride solubility* 68

3.4.2.3 *Cholesterol* 68

4.0 RESULTS AND DISCUSSION **70**

4.1 Introduction 70

4.2 Lipid Solubility 70

4.2.1 Egg Lipid Solubility as a Function of Temperature and Pressure 71

4.2.2 Egg Lipid Solubility as a Function of Entrainer Type and Concentration 74

4.3 Lipid Recovery 78

4.3.1 Effect of Temperature, Pressure and Entrainer 78

4.4 Chemical Analysis of Extracts 81

4.4.1 Triglycerides 82

4.4.1.1 *Effect of temperature, pressure, and entrainer on solubility* 82

4.4.1.2 *Fatty acid composition of CO_2 extracts* 84

4.4.1.2.1 *Fatty acid composition of sequential CO_2 extracts* 84

4.4.1.2.2 *Effect of temperature, pressure and use of entrainer on fatty acid composition* 87

4.4.2 Cholesterol 89

4.4.2.1 *Effect of temperature, pressure, and entrainer on solubility and recovery* 89

4.4.2.2 *Effect of extraction time* 96

4.4.3 Phospholipid recovery 96

4.5 Chemical Analysis of Residue 99

5.0 CONCLUSIONS AND RECOMMENDATIONS **102**

5.1 Conclusions 102

5.2 Recommendations 105

REFERENCES **107**

APPENDIX A **117**

List of Tables

TABLE 2.1: *Constituents of egg yolk.*

9

TABLE 2.2: *Fatty acid compostiion of egg yolk lipid.*

14

TABLE 2.3: *Recommended diet modifications to lower blood cholesterol (ATP, 1988).*

25

TABLE 2.4: *Typical values of viscosity, density and diffusively for liquid, gaseous and supercritical carbon dioxide.*

37

TABLE 3.1: *Specifications of commercial siphon grade carbon dioxide as supplied by Canadian Liquid Air, Ltd.*

49

TABLE 3.2: *Typical Quattro Pro spreadsheet used to analyze, record and display data from the extraction of egg yolk lipids using supercritical carbon dioxide*

59

TABLE 3.3: *Typical Quattro Pro spreadsheet used to analyze, record and display fatty acid data from the extraction of.egg yolk lipids using supercritical carbon dioxide.*

67

TABLE 3.4: *Typical Quattro Pro spreadsheet used to analyze, record and display cholesterol data from the extraction of egg yolk lipids using supercritical carbon dioxide.*

69

TABLE 4.1: *Summary of lipid recoveries as affected by pressure,temperature and the use of entrainers during the supercritical CO_2 extraction of egg yolk.*

79

TABLE 4.2: *Solubility of triglycerides as affected by extraction pressure, temperature and use of entrainer*

83

TABLE 4.3: *Effect of temperature, pressure, and use of entrainer on fatty acid composition.*

88

TABLE 4.4: *Solubility and recovery of cholesterol as affected by extraction pressure, temperature and use of entrainer.*

90

TABLE 4.5: *Phospholipid content in lipids extracted from freeze-dried egg yolk with pure SC-CO_2 and with alcohol entrained SC-CO_2*

97

TABLE 4.6: *Proximate composition of untreated and residual egg yolk (dry basis) at different extraction conditions.*

100

List of Figures

FIGURE 2.1: *Part of a lipoprotein particle showing possible arrangement of neutral lipid, phospholipid and protein (Burley, 1975).*

11

FIGURE 2.2: *Compostion of the lipid portion of egg yolk (Parkinson, 1966).*

12

FIGURE 2.3: *Steroids are characterized by the tetracyclic ring system (a) Cholesterol contains this tetracyclic skeleton modified to include an alcohol function at C-3, a double-bond at C-5, methyl groups at C-10 and C-13, and a* C_8H_{17} *side chain at C-17 (b) (Carey, 1987).*

17

FIGURE 2.4: *Phosphatidylcholine. The polar head consists of a negatively charged phosphate group (pH 7.0) esterified to the head alcohol (shaded area). Two fatty acid molecules (nonpolar tail) are esterified to the first and second hydroxyl groups of the glycerol (Lehninger, 1982).*

27

FIGURE 2.5: *Polar lipids, especially phosphoglycerides, readily and spontaneously form very thin bilayers, separating two aqueous compartments (b). In their structures, the hydrocarbon tails of the lipid molecules extend inward from the two surfaces to form a continuous inner hydrocarbon core. The hydrophyllic heads face outward, extending into the aqueous phase. Such bilayers are readily formed by agitating phospholipids in aqueous suspensions at high frequencies, resulting in the formation of liposomes - closed vesicles surrounded by a continuous lipid bilayer (c). A phosphoglyceride monolayer is also shown (a) (Lehninger, 1982)*

31

FIGURE 2.6: *Phase diagram for carbon dioxide. The lines A-TP, TP-C and TP-B divide the diagram into three regions representing three phases: solid, liquid, and vapour. Along each of the lines two phases exist in equilibrium with each other while at point TP, the triple point, three phases co-exist. The line TP-C, which separates the liquid and vapour regions of the diagram, terminates at the critical point C. Beyond this point, CO_2, will no longer exist as a liquid or vapour and is referred to as supercritical CO_2 (Fattori, 1986).*

36

FIGURE 2.7: *Density of carbon dioxide as a function of pressure at different temperatures. The critical point (CP) of the CO_2 is indicated on the diagram (Newitt et al., 1956).*

37a

FIGURE 3.1: *Schematic diagram of supercritical fluid extraction system.*

43

FIGURE 3.2: *Design and specifications of high pressure extraction vessel used for supercritical fluid extraction.*

45

FIGURE 3.3: *Recovery of 3 wt % ethanol as a function of consumed CO_2.*

50

FIGURE 3.4: *Chromatogram of the fatty acid methyl esters in an esterified sample of a typical CO_2 extract of egg yolk lipid (36 MPa, 40 ℃, flowrate 2.0 g/minute). Extraction and chromatographic conditions are described in the text.*

54

FIGURE 3.5: *HPLC calibration curve for cholesterol where peak height is plotted as function of cholesterol concentration.*

57a

FIGURE 3.6: *A typical HPLC chromatogram showing the elution profile of cholesterol. Extraction and chromatographic conditions are described in the text.*

57b

FIGURE 3.7: *Typical extraction curve showing lipids recovered from egg yolk as a function of the CO_2 passed through the extractor (values given for a 43.01 g sample at 40 ℃ and 36 MPa); CO_2 = 2.0 g/minute.*

61

FIGURE 3.8: *Schematic diagram of lipid solubilization during extraction with supercritical carbon dioxide.*

62

FIGURE 4.1: *Extraction of egg yolk lipids as a function of extraction pressure 15, 20 and 36 MPa) at a constant temperature of 40 ℃. Data points represent a single supercritical run. Average solubilities, indicated in the line descriptions, represent duplicate, triplicate and 6-replicate determinations for 15, 20 and 36 MPa, respectively.*

72

FIGURE 4.2: *Extraction of egg yolk lipids as a function of extraction temperature (40, 55 and 75 ℃) at a constant temperature of 40 ℃. Data points represent a single supercritical run. Average solubilities, indicated in the line descriptions represent 6-replicate determinations for 40 ℃ and duplicate determinations for 55 ℃ and 75 ℃.*

73

FIGURE 4.3: *Effect of the entrainer methanol and ethanol on egg yolk lipid solubility in supercritical CO_2 at constant pressure and temperature; CO_2 flowrate - 2.0 g/minute. Average solubilites, indicated in the line descriptions, represent duplicate determinations.*

75

FIGURE 4.4: *Standardized extraction curve (36MPa, 40 ℃) for egg yolk lipids in which horizontal and vertical axes have been normalized by sample mass (~40g). The recovery is expressed as a percentage of theoretical lipid in egg yolk.*

80

FIGURE 4.5: *Extraction curve for 39.7 g sample of freeze-dried egg yolk. The extraction was carried out at 20 MPa, 40 ℃ and at a flowrate of 2.0 g/minute. The data points on the curve represent discrete intervals over which lipid samples were collected for fatty acid analysis. Seventeen consecutive extract fractions were collected.*

85

FIGURE 4.6: *Fatty acid composition (mass percent of extract) of sequential CO_2 extracts of freeze-dried egg yolk. Extract fractions correspond to those identified in Figure 4.5 except that the data values at the point of origin have been omitted.*

86

FIGURE 4.7: *Extraction curve (20 MPa, 40 ℃) for cholesterol in which horizontal and vertical axes have been normalized by sample mass (~40g). Cholesterol recovery is plotted as a function of consumed CO_2 and is expressed as a percentage of theoretical cholesterol content of egg yolk.*

94

FIGURE 4.8: *Extraction curve (36 MPa, 40 ℃) for cholesterol in which horizontal and vertical axes have been normalized by sample mass (~40g). Cholesterol recovery is plotted as a function of consumed CO_2 and is expressed as a percentage of theoretical cholesterol content of egg yolk.*

95

1. Introduction

From the beginnings of civilization man has tried to improve and adapt his surroundings to better suit his needs and wants. This has been especially so with foodstuffs. Cooking raw meat on an open fire was an early application of technology to increase the palatability and digestibility. Since that time many advances have been made both in food preparation and in scientific methods.

Today, the fields of food science and food engineering concern themselves with the application of scientific principles and modern technology to produce a plentiful, safe, varied and appetitizing food supply. In particular, much research activity and manufacturing effort have gone into the separation of compounds and modification of chemical properties to produce foods which satisfy specific needs. For example, artificial sweetners have been developed as low-calorie replacements for sugar. Sacchrin derived from coal, was first introduced in 1897. It was banned by the FDA in April 1977 as a food additive because of possible carcinogenic effects (Crapo, 1988). The flavor, although sweet, is easily distinguishable from sugar with its bitter aftertaste. Later sugar substitutes, such as aspartame, improved the "trueness" of the taste while avoiding side effects and health risks.

Other processes of interest involve either the concentration / isolation of valuable compounds (e.g. flavor essences) or the removal of a toxic / waste substances

(e.g. caffeine, vomitoxin). Commercial plants in Europe use liquid carbon dioxide to extract the flavor essence from hops.

The past decade saw the emergence of several noteworthy trends. Enhanced consumer concern for the quality and safety of foods resulted in more stringent government regulations for solvents and allowable residues. Health has increasingly become a more important aspect of the North American lifestyle. Marketing has translated this into terms such as "fresh," "natural, "low calorie," and "light." These and other health claims can now be found in virtually all food and beverage categories.

One major health concern has been hypercholesterolaemia and its role in cornary heart disease (Gotto, 1989). Although there is considerable controversy regarding the risk level involved there is a general perception, at least in the public view, that high dietary intake of cholesterol should be avoided. Eggs have traditionally been a valued component of the human diet because of their high nutrient content, low caloric value and ease of digestibility (Cook and Briggs, 1986). Egg protein is one of the highest quality proteins for human foods since it contains all the essential amino acids. Egg lipids are high in desireable unsaturated fatty acids and contain all the essential vitamins, except vitamin C (Mountney, 1976). However, egg yolks contain a relatively large amount of cholesterol. The average cholesterol content of a raw chicken egg yolk is 1602 mg per 100 gram compared with 71 mg per 100 gram for a choice grade T-bone steak (Hepburn *et al.*, 1986).

Consequently, there has been considerable interest in lowering the cholesterol content of eggs either by selective breeding of the laying hens (Hargis, 1988; Turk and Barnett, 1971; Washburn and Nix, 1974) or by a separation process for the actual yolk (Tokarska and Clandinin, 1985; Larsen and Froning, 1981). Cholesterol is not the only compound of interest in eggs. As mentioned previously, the egg white and yolk have very high quality protein while the fatty acid composition is highly desireable, for example, for infant formula (Tokarska and Clandinin, 1985).

Lecithin is a valuable component of egg yolks. Lecithin is used as a functional ingredient in the manufacture of food because of its emulsifying and re-wetting properties. Lecithin is composed of neutral lipids and phospholipids. One of these phospholipids, phosphotidylcholine (PC) is currently the subject of medical interest. PC has been indentified as a significant factor in the treatment of neurological disorders, such as Alzheimer's disease (Krishnan *et al.*, 1988). There is a strong research demand for high purity PC free of triglycerides and phospholipids such as phosphatidylethanolamine (PE) and sphingomyelin.

Phospholipids tend to be unstable with regard to oxidation, heat and light degradation. A cool extraction process with relatively inert solvents is, therefore, best suited for this type of separation. Extraction with supercritical fluids (SCF) is one such method. Supercritical fluid extraction (SFE) exploits the unique properties of solvents above their critical values to extract soluble components from a mixture (Rizvi *et al.*, 1986). Liquid carbon dioxide (LCO_2) is also used in a similar manner to extract certain food components (e.g. spice oleoresins) (Hubert and Vitzthum, 1978; Bethuel and Neige, 1989). Dense gas extraction is a term used to describe extraction processes using supercritical fluids or liquidified gases. Supercritical fluids are particularly attractive as extracting agents because the solvent power, that is the density, can be manipulated over a wide range of temperatures and pressures. With densities very close to those of liquids and viscosities lying between those of gases and liquids, the result is a highly efficient extracting solvent that is capable of dissolving many compounds which would have been sparingly soluble in the same fluid at gaseous conditions. A dramatic reduction in the solvent density with changes in temperature and or pressure allows for the recovery of the solute from the solvent.

Important characteristic features of dense gas extraction include:

1. Operating temperatures are close to the solvent critical temperature. Therefore, high boiling, heat-sensitive components may be extracted at relatively

low temperatures. Mild conditions are particularly suitable to the isolation of thermally labile substances as low temperature separation avoids thermal decomposition.

2. The selectivity and capacity of the solvent may be changed by varying the density and temperature (operating conditions) as well as the composition (choice of solvent and/or entrainer). These parameters vary the properties of the solvent over a wide range.

3. The recovery of solute is straightforward since relatively small changes in the conditions can result in considerable solubility changes. This behavior has a distinct advantage over liquid extraction. Solvent/solute separation may be accomplished by simple unit operations (isothermal decompressions or isobaric heating).

4. Solute fractionation is often possible during the solvent/solute separation.

5. Solvents vary considerably in polarity and introduce a wide range of potential extraction temperatures. The degree of solubility can be easily manipulated unlike normal liquid extraction with conventional organic solvents. Supercritical carbon dioxide holds an advantage over conventional liquid organic solvents and over other supercritical solvents because it is relatively chemically inert. In addition, it is inexpensive, nontoxic, nonflammable, and environmentally acceptable. The low critical temperature (31°C) allows extraction at moderate temperatures for minimal degradation of heat labile natural materials. Carbon dioxide's low boiling point permits ease of removal after processing.

6. The low viscosity of supercritcal gases allows excellent powers of penetration into the solid structure of typical organic matrices.

7. Dense gas extraction is particularly suitable for the processing of easily-oxidized materials, as pressure operation ensures that no oxygen enters the system.

The application of dense gas extraction as an alternative to distillation and conventional solvent extraction has been considered by a number of industries including the food, pharmaceutical, petroleum and synthetic fuel industries. Many possible food processing applications for supercritical extraction and fractionation have been identified. For a more detailed account of the food applications of SC CO_2 extraction, the reader is referred to the review article by Rizvi and co-workers (1986). These authors give examples of the types of unit operations that have been shown to be feasible as well as a partial list of specific foods that have been tested with those processes.

SCE has found particular application in the extraction and separation of lipid materials. Considerable work has been done on oilseed extraction. More recently, SCE has been applied to the purification of fish oils and the removal of cholesterol from dairy products. Levi and Sim (1988) have used multi-stage separation to selectively remove over 70% of the cholesterol from egg yolk products.

In the present investigation, supercritical CO_2 was used to establish the efficacy of egg yolk lipid fractionation. The possible removal of cholesterol, saturated fatty acids and phospholipids have been considered in this study. The following hypotheses have been proposed for testing during this investigation.

A. Effects on overall lipid solubility in supercritical CO_2

1. The overall solubility of egg lipids in SC CO_2 increases with increasing pressure from 15 to 36 MPa, at a constant temperature of 40°C.

2. The overall solubility of egg lipids in SC CO_2 decreases with increasing temperature from 40 to 75°C, at a constant pressure of 36 MPa.

3a. The overal solubility of egg lipids in SC CO_2 increases with the addition of a polar entrainer (e.g. 3% ethanol) to the SC CO_2.

3b. The overall solubility of egg lipids in SC CO_2 increases more with the addition of a more polar solvent such as 3% methanol than the addition of a less polar solvent such as 3% ethanol.

3c. The overall solubility of egg lipids in SC CO_2 increases with an increase in the entrainer concentration (e.g. from 3 - 5% methanol).

B. Lipid Recovery

1. The overall percentage of egg lipid that can be recovered from freeze-dried egg yolk is independent of extraction pressure, temperature and the addition of entrainers over the range of conditions reported in this study.

2. The relative concentration of triglycerides, cholesterol, phospholipids in egg lipid extract is independent of pressure and temperature.

C. Cholesterol

1. Cholesterol solubility is independent of pressure, temperature, or the addition of entrainers.

D. Triglycerides / Fatty acids

1. The triglyceride solubility increases with increasing pressures from 15 to 36 MPa, at a constant temperature of 40°C.

2. The trigylceride solubility decreases with increasing temperature from 40 to 75°C, at a constant pressure of 36 MPa.

3. The triglyceride solubility of egg lipids in SC CO_2 increases with an increase in the entrainer concentration (e.g. from 3 - 5% methanol).

4. The fatty acid composition of the extract varies with time during the extraction; the more soluble fatty acids (smaller ones or ones with double bonds) being selectively extracted during the early stages and the less soluble ones later.

5. The fatty acid composition of the extract will be independent of temperature, pressure and the addition of entrainers.

E. Phospholipids

1. Phospholipids are relatively insoluble in SC CO_2 at the temperatures and pressures studied.

2. Phospholipid solubility increases with the addition of polar entrainers. The solubility will increase with increasing entrainer polarity and concentration.

2. Literature Review

2.1 THE CHEMICAL COMPOSITION AND STRUCTURE OF EGGS (Gallus domesticus)

Liquid whole egg consists, on average, of 64% white and 36% yolk. The white contains approximately 12% of solid matter, which is predominately protein with small amounts of minerals and sugars and only a trace of fat. Yolk contains about 50% of solids, nearly two-thirds of which is fat and one-third protein (Parkinson, 1966). Because of this marked difference in composition, and because it is easy to separate whites and yolks physically, detailed experimental studies on the major constituents of eggs have hitherto been carried out on either white or yolk, and it is convenient to consider them separately. Egg yolk is the raw material of interest in this study, therefore, its chemical and physical properties are summarized in this section.

2.1.1 Composition of egg yolk

The constituents of egg yolk can be classified as in Table 2.1 Physically, egg yolk can be regarded as a mixture of particulate 'granules' and soluble plasma, the latter including low-density 'globules,' rich in fat. Lipoproteins are complexes of neutral lipid, phospholipid and protein with properties that differ from those of their constituents but are not stabilized by covalent bonds. Lipoproteins are 60% of the dry weight of yolk and contain 12% of protein (Burley *et al.*, 1985). It is speculated that the protein is at the

TABLE 2.1 CONSTITUENTS OF EGG YOLK

			Percent of egg yolk solids [1]
Proteins			
	livetins		4 - 10
	phosphoprotein	vitellin	4 - 15
		vitellenin	8 - 9
		phosvitin	5 - 6
Lipoproteins	lipovitellin		16 - 18
	lipovitellenin		12 - 13
Lipids	triglycerides		46
	phospholipids		20
	sterols		3
Carbohydrates			2
Mineral consitiuents			2
Vitamins			traces

[1]Values obtained from Fevold (1951) and Bernardi and Cook (1960a, 1960b) for proteins, Lea (1962) for lipids and Brooks and Taylor (1955) for other constituents.

surface of the particles. As well, a large proportion of charged lipid, i.e. phospholipid, is part of the surface layer (Burley and Kushner, 1963). The phospholipids, proteins and cholesterol interact at the surface, leaving those lipids that do not contain phosphorus, mainly the neutral lipids, to occupy the centre of the lipoprotein particles. The neutral lipids coalesce as an oily droplet, well protected from the outside by the protein-phospholipid cholesterol layer (Figure 2.1). The lipid in yolk is not free, but is bound in lipoprotein particles in some way at present obscure (Burley *et al.*, 1985). It is, therefore, incorrect to refer to 'free lipid' in yolk. Part of the lipid of yolk is easily extracted with ether, for example, but this is probably because organic solvents readily disrupt the lipoprotein particles.

The granules, representing about 19 - 23% of the yolk solids (Burley and Cook, 1961; Saari *et al.*, 1964; Saito *et al.*, 1965), consist of 70% α– and β–lipovitellins (high-density proteins), 16% phosvitin (a phosphoprotein), and 12% low-density lipoprotein (Burley and Cook, 1961). Little is known about the organization of the yolk granules, and their function is unknown. They contain all the iron in the yolk and much of the calcium (Burley, 1975).

2.1.1.1 Egg yolk lipids

The composition of the lipid portion of egg yolk is summarized in Figure 2.2. Approximately 50% of the yolk is solid matter consisting of 31.4 - 33.2% protein and 64 - 72% lipid. The composition of yolk lipid is 65.5% triglyceride, 28.3% phospholipid and 5.2% cholesterol. About 84% of cholesterol is present in the free state, and the remainder in an esterified form. The cholesterol content of the egg is very constant and varies only slightly among the eggs of different species of birds. In addition, modifying dietary lipid treatments fed to laying species does not influence the cholesterol deposition in the egg yolk (Privett *et al.*, 1962; Chung *et al.*, 1965). Polyunsaturated fatty acids make up 18, 18.6 and 28.6% of the trigycerides, phosphatidylcholine,

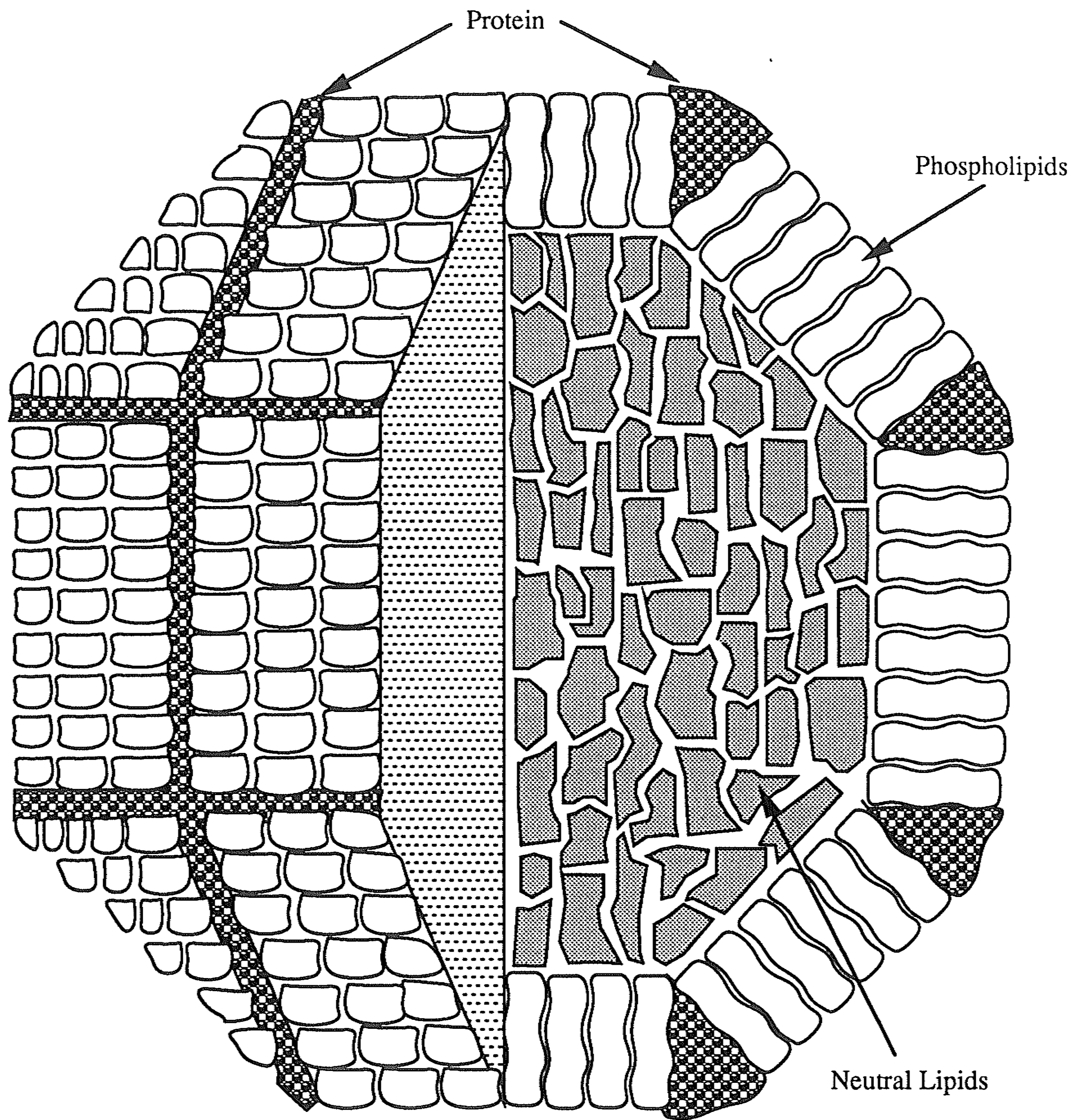


FIGURE 2.1: *Part of a lipoprotein particle showing possible arrangement of neutral lipid, phospholipid, and protein. (Burley, 1975)*

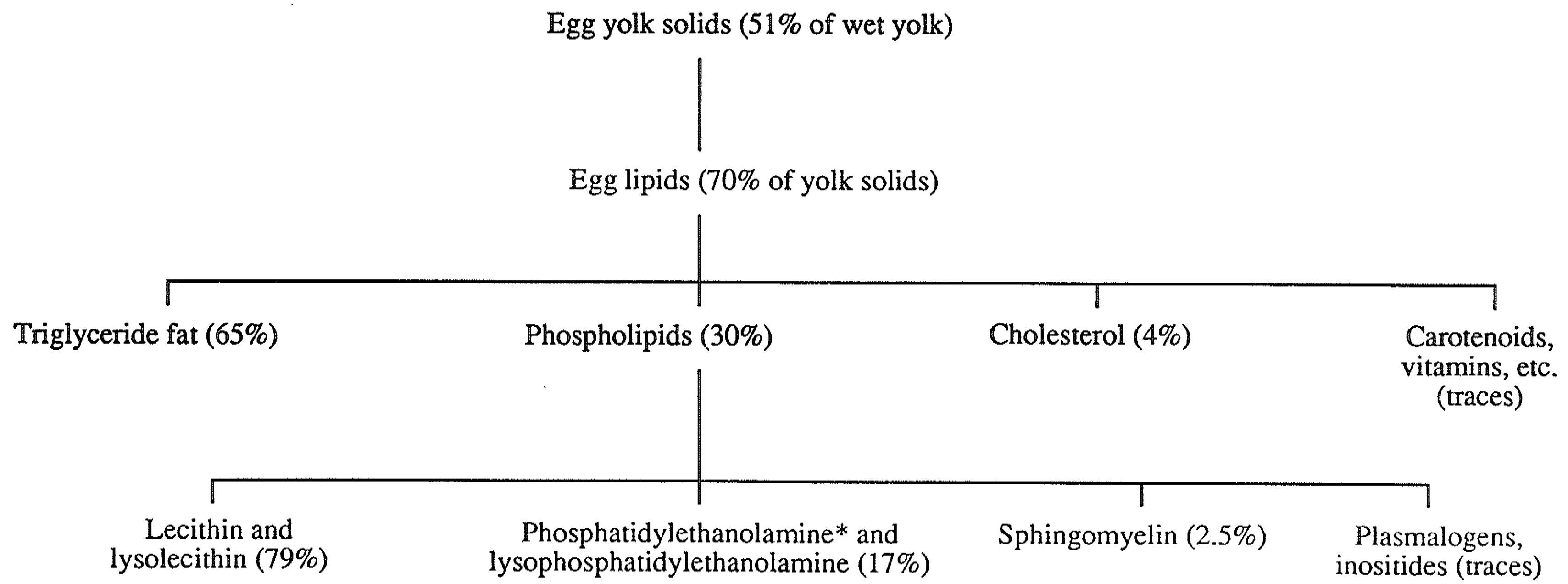


FIGURE 2.2: *Composition of the lipid portion of egg yolk. (Parkinson, 1966)*

phosphatidylethanolamine, respectively (Pike and Peng, 1985). The composition of yolk phospholipid approximates 73.0% phosphatidylcholine, 15.0% phosphatidylethanolamine, 5.8% lysophosphatidylcholine, 2.5% sphingomyelin, 2.1% lysophosphatidyl-ethanolamine, 0.9% phasmalogen and 0.6% inositol phospholipid (Rhodes and Lea, 1957; Parkinson, 1966). The various triglycerides that make up yolk lipid contain both saturated and unsaturated fatty acid moieties ranging in carbon atoms from 14 to 22.

Earlier work on egg lipids has been comprehensively reviewed by Privett and coworkers (1962), and later work has been summarized by Parkinson (1966). The latter lists the fatty acid compostion of egg lipids as shown in Table 2.2.

2.2 THE ROLE OF EGGS IN HEALTH

As consumers become increasingly health and diet-consious, the effects of many foods in nutrition and disease are being closely re-examined. Eggs have long been regarded as a staple in the Western diet. At one time a hearty breakfast of bacon and eggs was regarded as a nourishing, healthy start to the day. Nowadays, the general population has a more sedentary lifestyle. Portions are smaller, "light" and "low-cal" foods are popular and people are eating less red meat. Particularly with recent concerns about cholesterol, eggs have come to be avoided by the health conscious. Yet, eggs themselves are rich in essential nutrients, and do not deserve to be exiled to the dietary scrap heap.

TABLE 2.2. FATTY ACID COMPOSITION OF EGG YOLK LIPID[1]

		Saturated acids (%)			Unsaturated acids (%)				
	Total lipids %	14:0	16:0	18:0	16:1	18:1	18:2	18:3	20:4
Total lipids	100.0[2]	0.7	27.5	7.3	4.3	44.0	14.0	1.0	0.5
Triglycerides	62.0	0.5	27.5	5.5	6.0	48.0	11.5	0.5	trace
Free fatty acids	0.4	1.5	25.0	8.5	4.5	46.5	8.5	1.5	4.0
Sterol esters	0.34	4.0	32.5	10.5	6.5	35.0	11.5	trace	trace
Phospholipid	(30.5)	0.7	32.0	12.5	2.8	34.5	14.5	1.4	3.8
Lecithin	20.7	1.8	36.5	14.0	4.3	30.0	13.5	-	-
Cephalin	6.7	3.0	22.5	23.0	5.5	34.0	11.0	-	-

[1] From Parkinson (1966)

[2] Other constituents present were cholesterol (4.1% of total lipid), sphingomyelin (1.2%, lysolecithin (1.9%), and cerebrosides, etc. (2.6%)

2.2.1 Nutritive value of eggs

Eggs may be the most nutritious food available in the human diet. Eggs have been used as reference standards for biological food value (Sim, 1987). Egg protein, particularly, is of high quality and easily digestible. Eggs are a source of the vitamin A, B, (including B_{12}), D, E, and K. They are an important source of iron, phosphorus and trace minerals (Cook and Briggs, 1986).

Eggs are included in the meats section of the Daily Food Guide. Two or more servings per day are recommended from this group which also includes poultry, beef, veal, lamb, fish, cheese and as alternates, dry beans, dry peas, and nuts. One serving is defined as three eggs, 50-90 g lean boneless cooked meat, poultry or fish, 60 g cheese, 250 mL dry beans, dry peas or lentils or 60 mL of peanut butter. Egg's blandness, high nutritional value, and relatively low caloric value make them especially ideal for convalescents and the elderly. Furthermore, their low cost makes them well-suited for those planning a well-balanced diet on a limited budget.

Eggs (specifically egg yolks) are the richest potential source of cholesterol in a typical North American diet. The estimated cholesterol content of 274 mg/egg set by the Consumer and Food Economics Institute of the U.S. Department of Agriculture (1976) is used as the current standard by the medical community to determine the recommended daily intake of cholesterol. One egg yolk contains most of the cholesterol 'allowed' in a prudent diet. Currently, the suggested average target limit for cholesterol intake is less than 300 mg/day (section 2.2.2.3). With the heightened awareness among both the general public and physicans about the link between elevated plasma cholesterol levels and risk of atherosclerotic cardiovascular disease, many individuals limit their egg consumption. Since eggs are such an ideal food in other regards, and for economic reasons, the egg industry has been interested in reducing the cholesterol content of hens' eggs.

2.2.2 Cholesterol

2.2.2.1 Structure

Cholesterol (5-cholesten-3 β-ol) is the most widespread animal steroid and is found in almost all animal tissues. Steroids are characterized by the tetracyclic ring system shown in Figure 2.3a. Cholesterol contains this tetracylic skeleton modified to include an alcohol function at C-3, a double bond at C-5, methyl groups at C-10 and C-13, and a C_8H_{17} side chain at C-17 (Figure 2.3b).

2.2.2.2 Biomedical Importance

Cholesterol is present in tissues and in plasma lipoproteins either as free cholesterol or (combined with a long-chain fatty acid) as cholesteryl ester. It is synthesized in many tissues from acetyl-CoA and is ultimately eliminated from the body in the bile as cholesterol or bile salts. Cholesterol is a necessary intermediate in the biosynthesis of all other steroids in the body such as corticosteroids, sex hormones, bile acids, and vitamin D. However, since it can be synthesized from acetylcoenzyme A, it is not a dietary necessity. It is typically a product of animal metabolism and therefore occurs in foods of animal origin such as egg yolk, meat, liver, and brain (Mayes, 1988a).

Cholesterol is an amphipathic lipid and as such is an essential structural component of membranes and of the outer layer of plasma lipoproteins. Additionally, lipoproteins transport free cholesterol in the circulation, where it readily equilibrates with cholesterol in other lipoproteins and in membranes. Cholesteryl ester is a storage form of cholesterol found in most tissues. It is transported as cargo in lipoproteins. Low density lipoprotein (LDL) is the mediator of cholesterol and cholesteryl ester uptake into many tissues. Free cholesterol is removed from tissues by high density lipoprotein (HDL) and transported to the liver for conversion to bile acids. Cholesterol is a major consitutent of gallstones. However, its chief role in pathologic processes is a factor in the genesis of

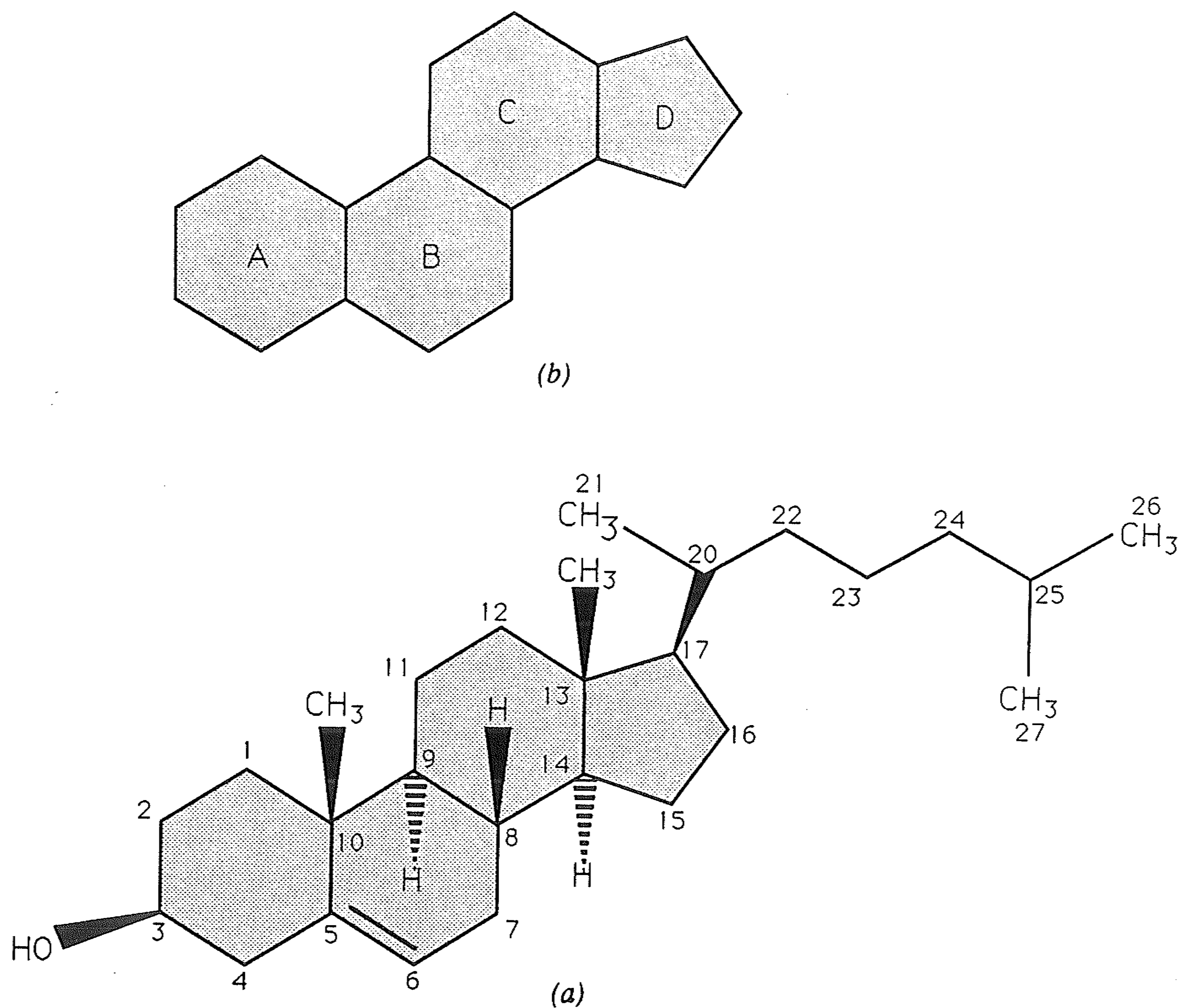


FIGURE 2.3: *Steroids are characterized by the tetracyclic ring system (a). Cholesterol contains this tetracyclic skeleton modified to include an alcohol function at C-3, a double-bond at C-5, methyl groups at C-10 and C-13, and a C_8H_{17} side chain at C-17 (b) (Carey, 1987).*

atherosclerosis of vital arteries, causing cerebrovascular, coronary, and peripheral vascular disease (Mayes, 1988a).

Approximately half the cholesterol of the body arises by synthesis (about 500 mg/d), and the remainder is provided by the average diet. The liver accounts for approximately 50% of total synthesis, the gut for about 15%, and the skin for a large proportion of the remainder (Mayes, 1988a).

2.2.2.3 *Diet and risk factors in cornary heart disease*

The role of cholesterol as a causitive factor in coronary heart disease (CHD) is controversial. Prior to discussing this subject, a short review of the terms used in cholesterol studies is in order.

Cholesterol is not water soluble. Serum cholesterol, the cholesterol carried in the blood, is combined with proteins to form lipoproteins. Lipoproteins are classed as low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very low-density lipoprotein (VLDL). The condition of hypercholesterolemia is defined as a blood cholesterol level greater than 240 mg/dL (The Expert Panel, 1988). However, the form in which the cholesterol is carried is also considered important.

The VLDL are composed largely of triglycerides and contain only 10 to 15% of the total serum cholesterol. The HDL carries about 20-25% of the serum cholesterol and the LDL about 60-70% (Stone and Van Horn, 1988).

CHD is characterized by atherosclerosis, 'hardening of the arteries', in which lipids are deposited on the walls of the blood vessels. The deposits impinge upon blood flow and weaken the vessel leading to a stroke or possibly an aneurysm (Sim, 1987). These deposits are referred to as plaque, while ulceration, calcification or hemorrhage of the vessel wall as a result of atherosclerosis is termed a lesion.

CHD is one of the leading causes of mortality and morbidity. It has been epidemiologically demonstrated that there is a high positive correlation between blood

cholesterol levels and heart disease (Sim, 1987). Population studies which have supported this finding include the NIH Pooling Project, the Israeli Perspective Study, the Framingham Heart Study and the Multiple Risk Factor Intervention Trial (Naito, 1988). The controversy arises when attempts are made to explain the relationships between diet, exercise, blood cholesterol level and composition and the risk and severity of CHD. It is generally held that the risk of coronary heart disease varies directly with LDL cholesterol level and inversely with HDL cholesterol level (Stone and Van Horn, 1988; The Expert Panel, 1988). However, the benignity of high HLD-cholesterol levels has been questioned (Havel, 1988). Total analysis of the cholesterol content of all blood lipoprotein groups is a costly and inconvenient procedure. An overnight fast of 12-16 hours is required (Sempos *et al.*, 1989). By comparison, measurement of total blood cholesterol is a simple procedure, requiring only a fingerstick sample from a resting subject (Shimamoto *et al.*, 1989). Since LDL-cholesterol level is roughly proportional to total serum cholesterol (TC), TC monitoring serves as a convenient method for identifying persons at risk of CHD. TC levels can be measured at any time of day in the nonfasting state as TC concentration is not appreciably changed following a fat-containing meal.

The National Cholesterol Education program in the US has established the Expert Panel (1988) on detection, evaluation and treatment of high blood cholesterol in adults (Adult Treatment Panel (ATP)). The ATP have established guidelines for initial classifications of CHD risk based on TC measurements:

$TC \leq 200$ mg/dL is considered a desirable blood cholesterol level

$200 < TC < 239$ mg/dL is considered a borderline range

$TC \geq 240$ mg/dL is designated as high blood cholesterol.

The 240 mg/dL cut-off is a value corresponding roughly to the 75th percentile for the adult US population. TC values above this level indicate a sharply rising risk of CHD (Sempos *et al.*, 1989; The Expert Panel, 1988). This has been supplemented by Multiple Risk Factor Intervention Trial (MRFIT), a six year mortality follow-up study of 361,662 men

aged 35-57 years. The results of the study reported a steady increase in CHD related death for men with TC levels above the 20th percentile (> 180 mg/dL) (Multiple Risk Factor Intervention Trial Research Group, 1982). The relative risk of CHD related death for men with TC levels above the 85th percentile (> 253 mg/dL) is 3.7 times greater than that of men with levels at or below 25th percentile. In addition to TC levels, the ATP (The Expert Panel, 1988) has identified several risk factors for CHD:

1. Male sex - considered a risk factor because the rates of CHD are three to four times higher in men than in women in the middle decades of life and roughly two times higher in the elderly. Hence, a man with one other CHD risk factor is considered to have a high-risk status, whereas, a woman is not so considered unless she has two other CHD risk factors.
2. Family history of CHD - where a parent or sibling has experienced myocardial infarction or sudden death before 55 years of age
3. Cigarette smoking
4. Hypertension - clinically diagnosed high blood pressure
5. Diabetes mellitus
6. History of definite cerebrovascular occlusive peripheral vascular disease
7. Marked obesity - > 130% of ideal weight as defined by the 1959 Metropolitan Life Insurance Company tables or by ratios of waist-to-hip circumference of greater than 0.8 for women and greater than 1.0 for men.
8. Low HDL-cholesterol level - < 0.91 mmol/L.

Assessment of risk and determination of appropriate intervention is based on TC measurements and the presence of these additional risk factors (The Expert Panel, 1988; American Heart Association, 1988).

On assessment of the TC levels and risk factors, the following intervention strategies are recommended:

1. For desireable blood cholesterol levels, general dietary and risk factor information should be provided. TC should be remeasured at least every 5 years for adults of age 20 and over.

2. For borderline TC, the presence of risk factors should be evaluated. If there is no personal history of CHD and none of the eight risk factors is present, dietary counselling is recommended. The patient's TC level should be measured annually to monitor patient's status.

3. If borderline TC level is combined with personnal CHD history or two risk factors or TC level is high (> 240mg/dL), a detailed lipoprotein analysis should be performed to determine relative amounts of HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol. The LDL-cholesterol determination should be repeated two or threee times, 1-8 weeks apart, to ensure repeatability (difference < 30mg/dL). Levels of LDL-cholesterol ≥ 160 mg/dL are classified as "high-risk LDL-cholesterol," and those 130 - 159 mg/dL as "borderline high risk LDL-cholesterol" (Naito, 1988). The ATP adds a third category: "desireable LDL-cholesterol of < 130 mg/dL. Others (Naito, 1988) consider only "borderline" and "high risk" LDL-cholesterol classifications once lipoprotein analysis has been prescribed. Once LDL-cholesterol has been established the level and risk factors are reassessed:

    1. If the LDL-cholesterol level is in the desireable range then the patient should be given dietary information and advised to have another serum cholesterol test within five years.

    2. For patients with"borderline high risk LDL-cholesterol" and no other CHD risk factors, dietary counselling and annual serum cholesterol retesting is recommended.

    3. Where CHD history or two CHD risk factors are combined with "borderline high risk LDL-cholesterol" or where LDL-cholesterol is

"high risk," a complete clinical evaluation is recommended which includes a complete history, physical examination, and basic laboratory tests. This workup will aim to determine whether the high LDL-cholesterol is secondary to another disease (hypothyroidism, diabetes mellitus, heptatic or renal dysfunction, familial lipid disorder). If the high LDL-cholesterol is due to the use of a drug (contraceptive pills, anabolic steroids, antihypertensive agents), the drug use must be reconsidered and possibly an alternative treatment prescribed.

Drug therapy, diet and exercise are the three main intervention strategies normally adopted for hypercholesterolaemia. The choice of intervention strategy must be a carefully weighed decision based on the severity of the condition. The first line of intervention is to consider appropriate non-pharmacological approaches including diet, weight control, exercise and lifestyle modifications such as quitting smoking (The Expert Panel, 1988). Drug therapy is normally a last resort treatment strategy used in cases where the patient does not respond to dietary / exercise therapy.

Drug therapy can not be considered a temporary or short-term treatment. A patient placed on a drug program will have to continue receiving medication for many years, if not for life (The Expert Panel, 1988). As well, there are definate health risks associated with the use of drugs to lower cholesterol (Havel, 1988). Normally, intensive dietary therapy will be carried out for at least six months to assess response before drug therapy is even considered. The exception is for individuals who have a definite CHD history, or whose LDL-cholesterol level is severely high (>225 mg/dL). For these patients there is an immediate life threatening risk and consequently a more urgent need to lower LDL-cholesterol. In such cases the initial period of dietary treatment may be shortened to as little as three months. An initial period is however required to establish a baseline from which to evaluate the efficacy of subsequent drug treatment.

Non-chemical means such as diet and exercise are preferrable methods for the reduction of bodily lipid levels. There is evidence that body fat is more easily reduced, and weight maintained when exercise is incorporated in a weight loss program. However, simple weight loss, by diet or exercise alone, does not significantly alter total- and LDL-cholesterol levels (Wood *et al.*, 1988), although HDL-cholesterol levels are increased. It would appear that a combination of diet and exercise will have optimal results in lowering LDL-cholesterol levels and reducing the risk of CHD and myocardial infarction (Schuler *et al.,* 1988).

The principle goal of dietary therapy is the reduction of low density lipoprotein. The cholesterol Adult Treatment Panel has developed a dietary regimen to reduce LDL and total serum cholesterol levels in patients where intervention has been deemed appropriate. The rational is to increase the rate of blood cholesterol catabolism (enhancing the number of hepatic LDL receptors) and to depress the synthesis of cholesterol in the liver. Two dietary factors have a major effect on LDL receptor activity, the amounts of cholesterol and saturated fats in the diet. Both cholesterol and saturated fat down-regulate LDL-cholesterol metabolism, and, thus, inhibit the removal of LDL from the plasma by the liver. Saturated fat particularly down-regulates the LDL receptor when cholesterol is concurrently present in the diet (Connor and Connor, 1989).

Saturated fats in the diet are directly linked to the formation of chylomicrons, cholesterol transporting fatty particles in the plasma (Connor and Connor, 1989). Saturated fats include all animal fats except those found in fish and shellfish, as well as, palm oil, palm kernel oil, and coconut oil. Monosaturated fatty acids, such as oleic acid (found in olive oil, rapeseed oil and sunflower oil) are generally considered neutral with regards to plasma cholesterol effects. However, recent research has shown that in Mediteranean countries where olive oil is a major dietary lipid, LDL and plasma cholesterol levels have been significantly reduced (Connor and Connor, 1989; The Expert Panel, 1988). Polyunsaturated fatty acids are essential in the diet as they are important in the

formation of cellular membranes and the hormone prostaglandin; they can not be synthesized by the body. The polyunsaturated fatty acids are divided into the omega-6 and omega-3 types. The most common, omega-6 fatty acid, is linoleic acid found in sunflower, safflower, soyabean and corn oil. Substitution of linoleic acid for dietary saturated fatty acids results in a decrease in plasma cholesterol levels (The Expert Panel, 1988). Omega-3 fatty acids are mainly found in fish oils; eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6) are the major acids in this class. Omega-3 fatty acids in the diet are believed to reduce LDL-cholesterol levels in the blood, although this is controversal (Connor and Connor, 1989; The Expert Panel, 1988, Mehta *et al.*, 1988).

The ATP (The Expert Panel, 1988) dietary guidelines attempt to incorporate these findings into a practical diet. Their recommendations are organized into two progressive steps. The step one diet is initiated for patients for whom dietary intervention has been prescribed. The step one diet attempts to reduce obvious and major sources of saturated fatty acids and cholesterol in the diet. Total fat should not exceed 30% of total calories. Saturated fatty acids should constitute less than 10% of total calories while monosaturated fatty acids should make up 10-15% of total calories. Polyunsaturated fatty acids may be increased to constitute up to 10% of calories. However, lack of information on the consequences of long-term consumption of linoleic acid means that the 10% ceiling should not be exceeded (The Expert Panel, 1988). Table 2.3 shows diet modifications designed to reduce blood cholesterol levels. Cholesterol intake is to be reduced to < 300 mg/day.

The minimum goal of diet therapy is to reduce LDL-cholesterol to less than 160 mg/dL (borderline threshold) if risk factors are present. If the desireable threshold is not achieved within three months the patient should be referred to a dietitian and should progress to the step two diet or to an additional trial period on the step one diet. The step two diet, by rigorous scrutiny of food intake, reduces saturated fatty acids and cholesterol

TABLE 2.3 RECOMMENDED DIET MODIFICATIONS TO LOWER BLOOD CHOLESTEROL (THE EXPERT PANEL, 1988)

	The Step-One Diet	
	Choose	Decrease
Fish, chicken, turkey, lean meats	Fish, poultry without skin, lean cuts of beef, lamb, pork, or veal, shellfish	Fatty cuts of beef, lamb, pork; spare ribs, organ meats, regular cold cuts, sausage, hot dogs, bacon, sardines, roe
Skim and low-fat milk, cheese, yogurt, and dairy subsitutes	Skim or 1% fat milk (liquid, powdered, evaporated), buttermilk	Whole milk (4% fat): regular, evaporated, condensed; cream, half and half, 2% milk, imitation milk products, most nondairy creamers, whipped toppings
	Nonfat (0% fat) or low-fat yogurt	Whole-milk yogurt
	Low-fat cottage cheese (1% or 2% fat)	Whole-milk cottage cheese (4% fat)
	Low-fat cheeses, farmer or pot cheeses (all of these should be labeled no more than 2 to 6 g fat per ounce)	All natural cheeses (e.g. blue, roquefort, camembert, cheddar, Swiss), low-fat or "light" cream cheese, low-fat or "light" sour cream, cream cheeses, sour cream
	Sherbet, sorbet	Ice cream
Eggs	Egg whites (2 whites equal 1 whole egg in recipes), cholesterol-free egg substitutes	Egg yolks
Fruits and vegetables	Fresh, frozen, canned, or dried fruits and vegetables	Vegetables prepared in butter, cream, or other sauces
Breads and cereals	Homemade baked goods using unsaturated oils sparingly, angel food cake, low-fat crakers, low-fat cookies	Commercial baked goods: pies, cakes, doughnuts, croissants, pastries, muffins, biscuits, high-fat crackers, high-fat cookies
	Rice, pasta	Egg noodles
	Whole-grain breads and cereals (oatmeal, whole wheat, rye, bran, multigrain, etc.)	Breads in which eggs are a major ingredient
Fats and oils	Baking cocoa	Chocolate
	Unsaturated vegetable oils: corn, olive, rapeseed (canola oil), safflower, sesame, soybean, sunflower	Butter, coconut oil, palm oil, palm kernel oil, lard, bacon fat
	Margarine or shortenings made from one of the unsaturated oils listed above, diet margarine	
	Mayonnaise, salad dressings made with unsaturated oils listed above, low-fat dressings	Dressings made with egg yolk
	Seeds and nuts	Coconut

to the minimum level commensurate with an acceptable and nutritious diet. Saturated fat is to be further reduced to comprise only 7% of the dietary calories and total fat is to be reduced to < 25% of calories. Cholesterol intake must be reduced to < 200 mg/day. After an additional three months, the patients response should again be re-evaluated. If the patient is still not responding to the step one diet, the step two diet should be initiated. If the pateint is adhering to the step two diet and has not reached desireable LDL-cholesterol levels, drug therapy should be considered.

2.3 LECITHIN

2.3.1 Structure, nomenclature, composition, sources

The term "lecithin" refers to a natural mixture of phospholipids commonly found in plant and animal membranes (Scholfield, 1985). These phospholipids combine highly polar phosphoric acid groups with nonpolar lipid groups (Lehninger, 1982). Thus, they possess useful properties as linkage and carrier compounds and as emulsifiers. The most common phospholipids in lecithin are phosphoglycerides, that is, they are based on a glycerol backbone (Figure 2.4). The first and second hydroxyl groups are esterified to fatty acid molecules, while the third hydroxyl group is esterified to phosphoric acid, which is in turn esterified to a second alcohol. The phosphoglycerides are named according to which alcohol is attached to their polar heads.

Choline and ethanolamine give their names to phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the two most common phosphoglycerides. Phosphatidylcholine is of particluar interest because choline is important in neural transmission, intermediary metabolism and cell membrane activity (Zeisel, 1985). Phosphatidylcholine was the first phospholipid isolated in egg yolk. In fact, the term "lecithin" (from the Greek "egg yolk") is often used in scientific literature to refer specifically to PC (Vance, 1985).

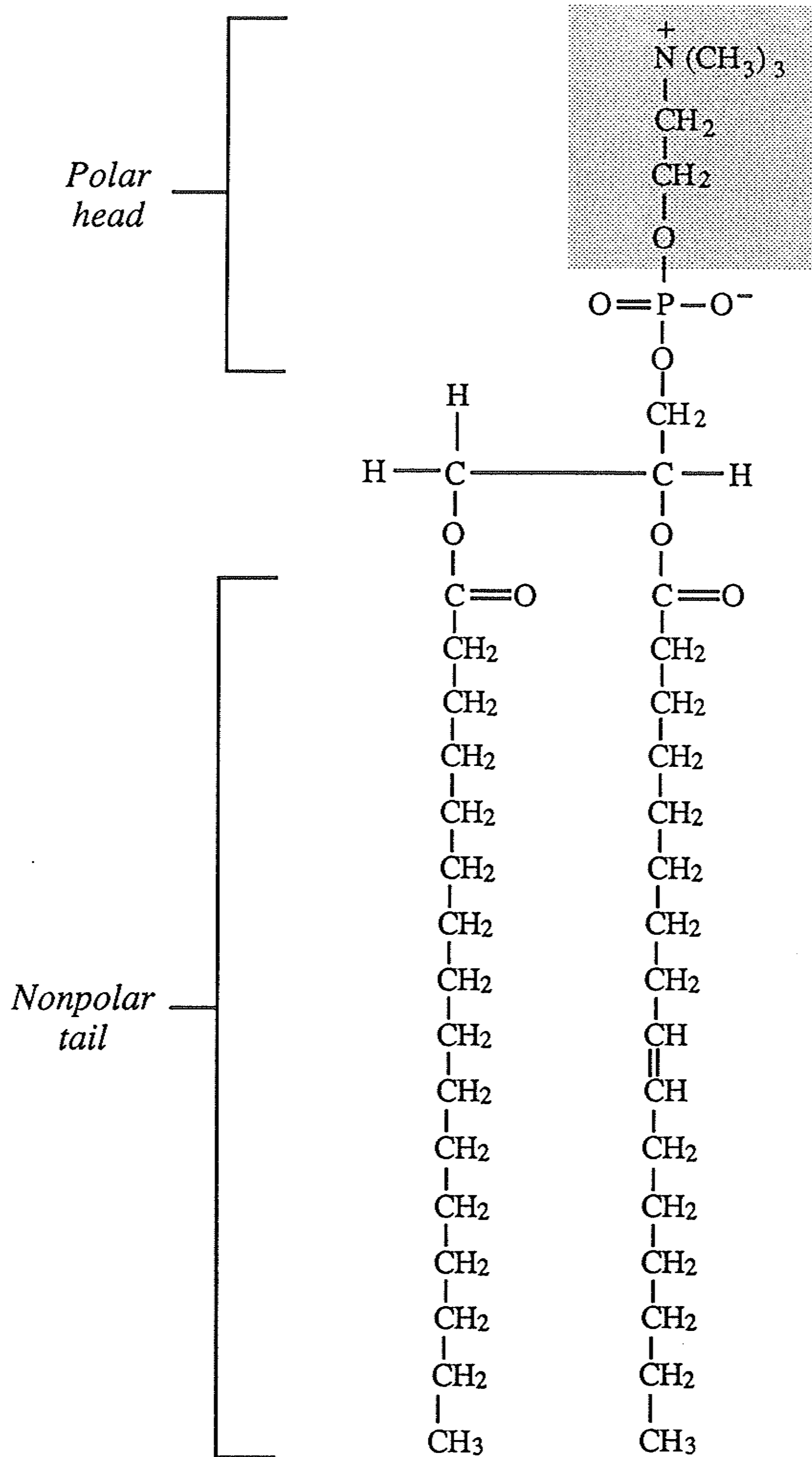


FIGURE 2.4: *Phosphatidylcholine. The polar head consists of a negatively charged phosphate group (pH 7.0) esterified to the head alcohol (shaded area). Two fatty acid molecules (nonpolar tail) are esterified to the first and second hydroxyl groups of the glycerol. (Lehninger, 1982)*

Other phosphoglycerides include phosphotidylserine, phosphotidylinositol and cardiolipin. A second class of membrane lipids is the sphingolipids. Some sphingolipids contain phosphorus and therefore, are also phospholipids; others do not. The sphingolipids consist of a long chain amino alcohol, sphingosine, attached to a fatty acid via an amide bond, and a polar head alcohol attached to the sphingosine hydroxyl group (Mayes, 1988b). Sphingolipids are subclassed as sphingomyelins, cerebrosides, and gangliosides. On hydrolysis, the sphingomyelins yield a fatty acid, phosphoric acid, choline and the amino alcohol sphingosine. Sphingomyelins are also common in aminal membrances, particularly in the myelin sheath surrounding certain nerve cells. Cerebrosides and gangliosides contain no phosphorus. They are glycolipids containing one or more sugar units and are found in brain cells and basal cell membranes (Mayes, 1988b).

In this investigation, the term lecithin will be used to refer to the commerial product containing a mixture of phospholipids. Phosphotidylcholine (PC) will be used to denote that specific compound. Lecithin can be extracted from a variety of plant and animal sources including soybean, corn, canola, sunflower, cottonseed, animal brain, and egg yolk. Commercial lecithin is derived principally from soybean because of the relatively high seed concentrations of phospholipids and the availability of the raw material.

However, for certain applications, lecithin from other sources may be preferred. For example, egg yolk lecithin is often used in the preparation of phosphatidycholine (PC) for medical research. Because the egg is essentially embryonic tissue, its components tend to be relatively innocuous and readily accepted by the human body. There are also definite differences in the phospholipid fatty acid composition from different sources. There are two positions available for fatty acid attachment, namely, the sn-1 and sn-2 positions. In most cases, the sn-1 position will be occupied by a saturated fatty acid and the sn-2 position by an unsaturated fatty acid (Kuksis, 1985). In egg yolk PC, the C16:0, C18:1 pairing predominates (approximately 40% of total PC) followed by

C16:0, C18:2 (approximately 20% of total PC) (Kuksis, 1985). For soybean PC, in comparison, the percentages suggest that there are almost equal amounts of the C16:0, C18:0, and C18:2, C18:2 isomers (approximately 30% each) and relatively little C18:1. It is known that an increase in the amount of dipalmityl PC (PC with two C16:0 fatty acids) will decrease the permeability of the membranes (Schmidt and Orthoefer, 1985). Therefore, for specific applications, certain lecithin sources, or even blends from several sources, may be dictated to achieve the desired final compostion.

2.3.2 Commercial lecithin products: food use of soybean lecithin

The combination of lipophillic and hydrophillic properties of phospholipid molecules make them "surface active" compounds or surfactants. That is, the phospholipid molecules can orient themselves between immiscible substances, reduce surface tension and allow easier mixing. Lecithins are commonly employed in the food industry for their unique surfactant properties. Their applications include emulsifying oil / water mixtures, solubilizing microemulsions, e.g. water-soluble dyes, solid particle dispersions, e.g. liquid chocolate and foaming agents, e.g. whipped topping. Lecithins are also utilized as wetting agents, e.g. for instant foods as release agents, e.g. in cooking and freezing applications. Perhaps the most widely known food application of lecithin is for crystallization control in food systems. The altered crystal sizes give desireable texture and viscosity to such foods as ice-cream and cookies (Prosise, 1985).

The industrial applications of lecithin are common knowledge and can be found in the literature (Prosise, 1985; Schmidt and Orthoefer, 1985).

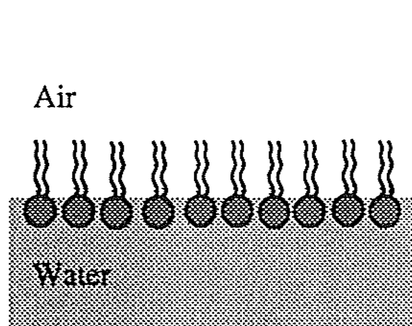
2.3.3 Therapeutic and research applications of lecithin

The principal interest of this paper is in the known and developing applications of lecithin in medical treatment and research. The physiochemical and biochemical properties of lecithin, in particular PC, have found applications in medical

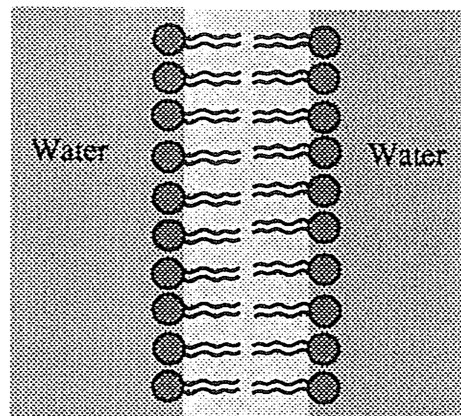
research and the treatment of several diseases involving the nervous and / or immune systems. The ability of lecithin to form semipermeable membranes and to act as surfactants is exploited in liposome formation, parenteral feeding solutions and lung therapy. Inconclusive clinical results have also been found when PC treatment is administered for the treatment of Alzheimer's disease (Leatherwood, 1986; Vida *et al.*, 1989; Eisai, 1989) and the aquired immune deficiency syndrome (AIDS) (Grierson *et al.*, 1988).

As previously mentioned, phospholipids have a polar "head" structure and nonpolar "tails" thus, in an aqueous medium, phospholipids will tend to orient themselves in such a way that their hydrophobic tails are hidden. The simplest such arrangement is for phospholipids to spread out with the tails exposed to the air. This is called a monolayer (Figure 2.5a). Stirring of such a solution may result in the formation of bilayers which are monolayers "doubled up" to separate two aqueous compartments. Agitation at very high frequencies (sonication) will result in the formation of liposomes. A liposome is a bilayer (Figure 2.5b) which has folded into itself to enclose a polar interior compartment - much like a jelly donut (Figure 2.5c) (Lehninger, 1985).

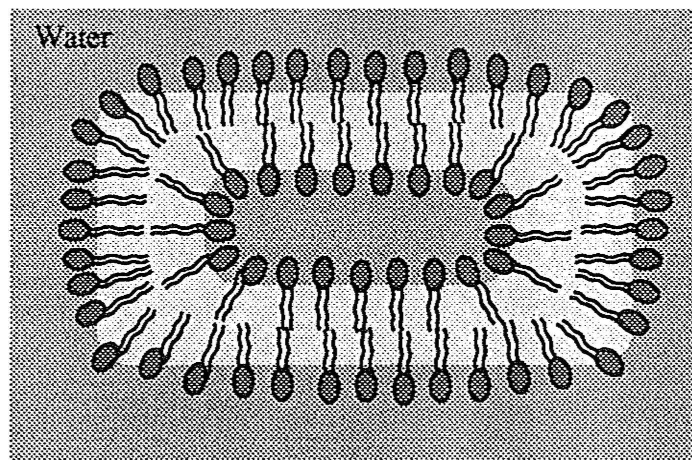
Liposomes are generally spherical in shape, approximately 25 - 2000 nm in diameter. Figure 2.5 shows a single aqueous inner compartment. Larger liposomes may have a series of concentric bilayers and aqueous cavities. These aqueous cavities can be used to carry drugs and other therapeutic agents (Schmidt and Orthoefer, 1985). The drug-loaded liposomes can then be injected or taken orally. This phospholipid envelope protects the drug from attack by body enzymes and the immune system (Lopezberes *et al.*, 1989). It also protects sensitive non-target tissues from the drug. For example, methrotrexate, which is toxic to bone marrow, has potential for the treatment of breast cancer (Anonymous, 1990). The liposomes tend to be stable until they are absorbed from the bloodstream by the cells of the reticuloendothelial system (RES) (Lehninger, 1985). The RES is made up of macrophages, the Kupffer cells of the liver and the reticulum cells of the lungs, bone marrow, spleen and lymph nodes. It's principal purpose is to dispose of cell



(a) A phosphoglyceride monolayer



(b) A phosphoglyceride bilayer



(c) A liposome

FIGURE 2.5: *Polar lipids, especially phosphoglycerides, readily and spontaneously form very thin bilayers, separating two aqueous compartments (b). In their structures, the hydrocarbon tails of the lipid molecules extend inward from the two surfaces to form a continuous inner hydrocarbon core. The hydrophyllic heads face outward, extending into the aqueous phase. Such bilayers are readily formed by agitating phospholipids in aqueous suspensions at high frequencies, resulting in the formation of liposomes - closed vesicles surrounded by a continuous lipid bilayer (c). A phosphoglyceride monolayrer is also shown (a). (Lehninger, 1982)*

breakdown products and to defend against infection (Mosby's Medical and Nursing Dictionary, 1986). The formation of liposomes from egg lecithin dispersed in water was first noted by Bangham in 1961 (Bangham *et al.*, 1965). The vesicles were applied as a model system to lyzosomes. It was thus that the idea of enclosing enzymes or pharmaceuticals in artificial phospholipid membranes originated (Bangham, 1989).

Numerous medical applications of liposomes have subsequently been discovered and or patented. Hormones such as insulin could potentially be administered orally - much more convenient for self medication (Schmidt and Orthoefer, 1985). Preliminary results with diabetic rats resulted in a decrease in blood glucose levels. Other hormones which have been given experimentally via liposomes include calcitonin (Deftos and Hostetler, 1989) and parathyroid hormone (PTH) (Hostetler and Deftos, 1987) for the treatment of hypercalcemia, Paget's disease and renal osteodystropy. The liposome acts to protect the hormones from the digestive enzymes of the small intestine and facilitates absorption of the hormones.

Similarly, antibiotics have been delivered into the blood using liposomes. Ampicillin or minocycilin have been used to treat listeriosis (Nippon Lederle, 1989). Encapsulations of mepartricin have been applied topically, orally, and parenterally for the treatment of disseminated fungal infections such as candidosis (Lopezberes *et al.*, 1989). The inventors note that the use of liposomes overcomes the poor solubility of mepartricin in aqueous solutions. Adriamycin can be used in a liposome preparation to treat malignant tumors with minimal cardiac toxicity (Yagi *et al.*, 1987). An example of enzyme encapsulation in liposomes is superoxidedismutase for the treatment of arterioschlerosis and cerebral thrombosis (Nippon Kayaku, 1989). Adminstering local anaesthetics and analgesics such as morphine using liposomes for epidural injection is said to increase anaesthetic effect and give longer-lasting effects (Legros and Ruysschaer, 1987).

Many liposome preparations use egg lecithin or PC purified from eggs to form a bilayer (Barenholz and Yechiel, 1989; Tremblay *et al.*, 1988; Mayer *et al.*, 1988).

Other PL sources include soy lecithin (Redziniak and Meybeck, 1985; Nippon Lederle, 1989) or syntheticly manuactured. Typical synthetic PC's include dimyristol-, dipalmitoyl-disteraryl-, and dioleyl-phosphatidycholine (Nippon Shinyaku, 1986; Lopezberes *et al.*, 1989) and diarachidonyl PC (Mayer *et al.*, 1988). Lopezberes and co-workers (1989), however, state that egg PC is especially preferable for these applications. A highly purified preparation of PC/lecithin from egg is therefore a desirable commodity. The potential for obtaining this commodity represents another reason for investigating the fractionation of egg yolk lipids.

2.4 ISOLATION OF EGG COMPONENTS

In addition to the separation of egg yolks and whites for use in specific food applications, other egg components can be isolated for non-food applications. The most important of these is lecithin which is used in pharmaceuticals. Phosvitin, livetins, biotin-binding protein and lysozyme are other bioactive components isolated from eggs (Hatta *et al.*, 1988; Proctor and Cunningham, 1988; Polson *et al.*, 1980). Egg oil is commercially produced in the United States for use in cosmetics (Blackwelder and Pike, 1990). Larsen and Froning (1981) extracted and refined an egg yolk oil and evaluated its use in foods such as infant formulations. Egg yolk immunoglobins have been isolated (Polson *et al.*, 1980) and projected as a source of antibodies for infant formulae. Hatta *et al.* (1988) investigated the separation of water soluble proteins from egg yolk before lipid extraction, a process designed to extract lecithin while still retaining protein functionality.

2.4.1 Conventional methods of extraction

The use of solvents to extract the lipid fraction of egg yolk is well documented (Tokarska and Clandinin, 1985; Warren *et al.*, 1988; Fletcher *et al.*, 1984; Larsen and Froning, 1981). Solvent extraction methods involve mixing the fresh yolks with a solvent, adjusting the pH and then centrifuging them at very high speeds (Tokarska

and Clandinin, 1985). Organic solvent extraction while effective for lipid removal, results in protein denaturation and loss of functional properties.

Silicic acid chromatography (SACC) used to fractionate and purify lipid samples (Hirsch and Ahrens, 1958; Sweeley, 1969), has been applied to egg yolk lipid fractionation (Blackwelder and Pike, 1990). Organic solvents are once again employed in the extraction procedure. Lipids were extracted from fresh egg yolks using hexane:isopropanol and separated on a silicic acid column into trigylceride, phospholipid, cholesteryl ester and cholesterol fractions using hexane, ethyl acetate and methanol as eluants, respectively (Blackwelder and Pike, 1990). Lipid extracted from the yolk was 35.5% of total yolk weight (98.5% recovery) yielding a cholesterol-free egg yolk oil. These authors claim high oxidative stability of their lipid fractions and the solvents used were in accordance with FDA regulations. Using a hexane-isopropanol solvent mixture, Larsen and Froning (1981) extracted an egg oil from yolk. The crude egg oil consisted of 80 to 95% lipid with most of the cholesterol originally present in the yolk. After the crude oil fraction was degummed, refined and bleached, the cholesterol content was reduced by 40%. The use of high temperatures for evaporation of solvent and bleaching causes decomposition of polyunsaturated C_{20} and C_{22} fatty acids desired in lipid sources intended for use in an infant formula.

Numerous attempts have been made to lower yolk cholesterol through the hen's diet or genetic selection and has been reviewed (Hargis, 1988; Turk and Barnett, 1971; Washburn and Nix, 1974).

2.4.2 Supercritical extraction

2.4.2.1 Supercritical carbon dioxide as a solvent

The central importance of solvents in chemical technology is well recognized. Indeed, scarcely a chemical process exists in which a solvent is not intimately

involved in the separation stages. In most separation processes, the choice of solvent is of pivitol importance. The search for new solvents having wider-ranging properties continues to be a significant part of separation research.

The design of biochemical and pharmaceutical separation processes is often constrained by the heat-sensitivity of the products and the organisms or enzymes involved. Many biochemical products are intended for use in foods and pharmaceuticals and are therefore subject to regulations which may limit the methods used in their production and recovery. The question of whether liquified / supercritical carbon dioxide has properties that make it suitable for use as a solvent in biochemical and pharmaceutical separation processes is a reasonable one. Carbon dioxide is readily available, inexpensive, nontoxic, nonflammable, chemically inert under many conditions, environmentally acceptable, and liquefiable at reasonable pressures.

The critical point of carbon dioxide is 31°C (304 K) and 7.38 MPa (Figure 2.6); its critical density is 0.468 g/cm^3. Below this point, liquid CO_2 can be maintained under relatively modest pressure (about 950 psi at 25°C) (Hyatt, 1984). Above 31°C, no amount of pressure will serve to liquify CO_2—there exists only the supercritical fluid phase that behaves as a gas, although when highly compressed, this fluid is denser that liquid CO_2 (ca 0.47 g cm^{-1}). The subcritical liquid phase of CO_2 behaves like any other liquid. The supercritical fluid phase can also act as a solvent, but it has higher diffusivity, lower viscosity, and lower surface tension than does the liquid phase (Table 2.4). The properties of the supercritical extraction medium can be varied within wide limits by means of pressure and temperature changes. Separation of extracted substances from the supercritical fluid can be accomplished, in whole or in part, by altering the pressure and/or the temperature of the fluid.

The solubility of a solute in a supercritical fluid has been shown to be largely a density-driven phenomenon (Chrastil, 1982). Solubility increases dramatically with increasing pressure above the critical point (Hyatt, 1984). This is especially true near

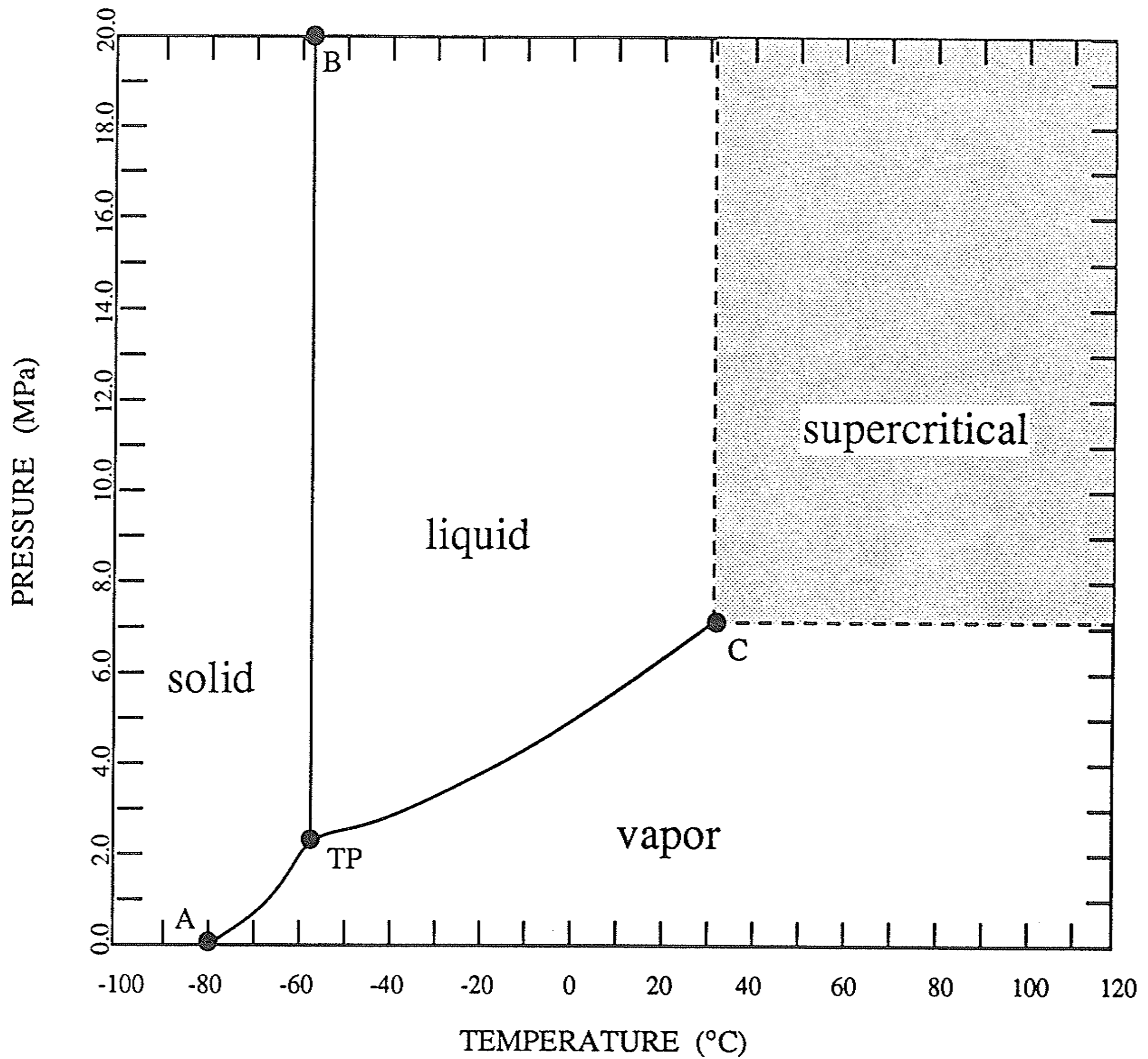


FIGURE 2.6: *Phase diagram for carbon dioxide. The lines A-TP, TP-C, and TP-B divide the diagram into three regions representing three phases: solid, liquid, and vapor. Along each of the lines, two phases exist in equilibrium with each other while at the triple point (TP) the three phases co-exist. The line TP-C, which separates the liquid and vapor reqions of the diagram, terminates at the critical point (C). Beyond this point, CO_2 will no longer exist as liquid or vapor and is referred to as supercritical CO_2. (Fattori, 1986)*

the critical point where the fluid is highly compressible and a rather small increase in pressure can induce a large increase in fluid density. The ability to vary the density is an integral feature of the supercritical fluid extraction process. Consequently, the process is most often carried out near the critical point, where the density variation is the greatest. Figure 2.7 shows the density of carbon dioxide as a function of pressure at different temperatures. The dependence of solubility on temperature is somewhat more complex (Nilsson *et al.*, 1988). At pressures near the critical point, a moderate temperature increase can cause a large decrease in fluid density resulting in a decrease in solute solubility (retrograde behavior). An increase in temperature causes an increase in vapor pressure of the solute which means an increase of solute solubility in solvent. The temperature increase will also cause a decrease in CO_2 density which means a decrease of its solvent capacity (Peter and Brunner, 1978; Brogle, 1982; Fattori, 1986). However, at much higher pressures, the fluid becomes less compressible and an increase in temperature induces a much less dramatic decrease in density. Thus, at higher pressures, with the vapor pressure effect predominating, an increase in temperature can cause an increase in solubility, i.e, a nonretrograde behavior.

TABLE 2.4. TYPICAL VALUES OF VISCOSITY, DENSITY AND DIFFUSIVELY FOR LIQUID, GASEOUS AND SUPERCRITICAL CARBON DIOXIDE

CO_2 state	viscosity g/cm s	density g/cm^3	diffusion coefficient cm^2/s
liquid	1.5×10^{-2} [1]	0.9 [2]	10^{-5} [3,4]
gas	1.4×10^{-4} [1]	0.002 [2]	10^{-1} [3,4]
supercritical fluid	9.1×10^{-4} [1]	0.9 [2]	10^{-3} - 10^{-4} [3,4]

[1] Newitt *et al.*, 1956
[2] Vukalovich and Altunin, 1968
[3] Randall, 1982
[4] Gere, 1983

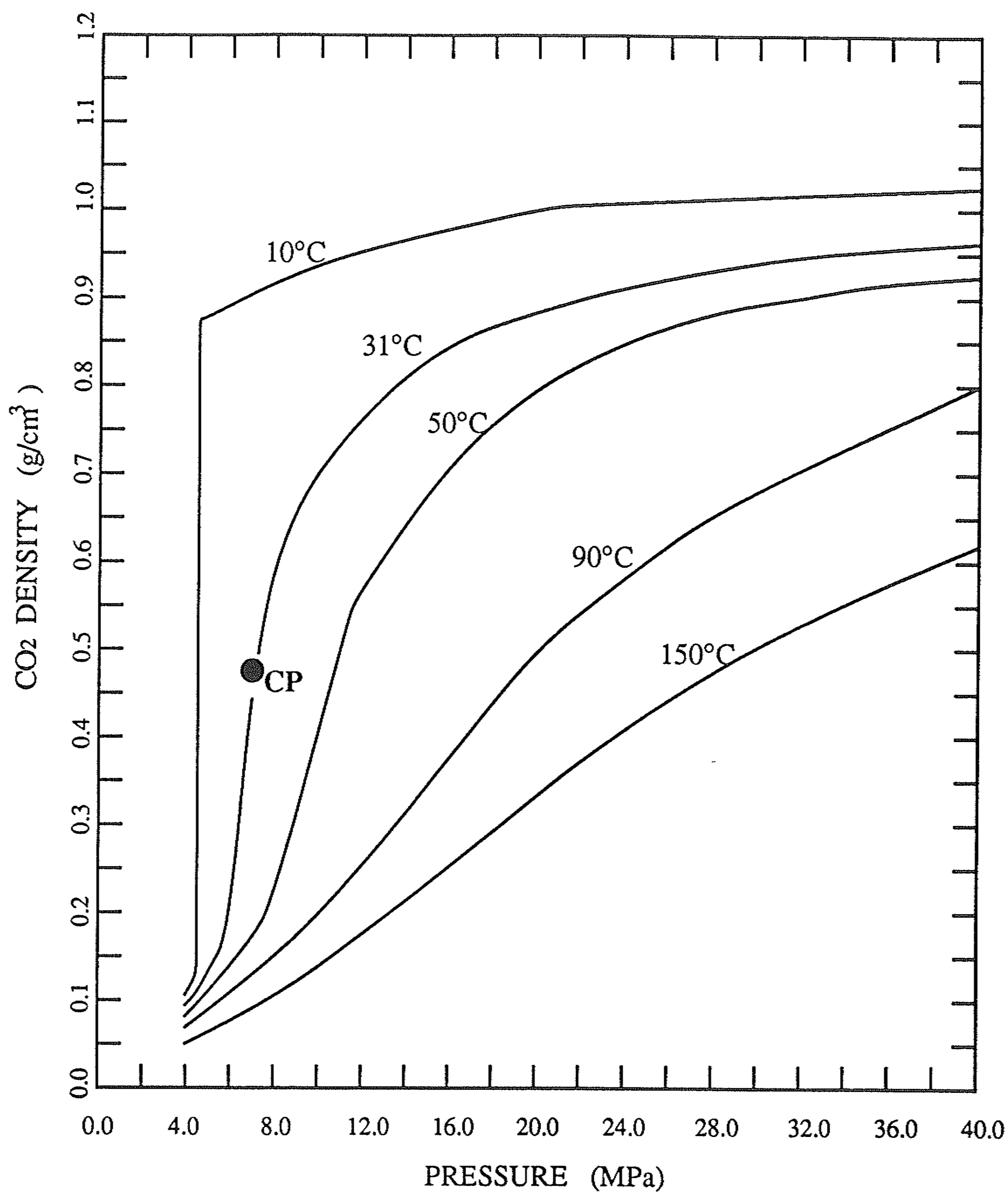


FIGURE 2.7: *Density as a function of pressure at various temperatures. The critical point (CP) of the CO_2 is indicated on the diagram. (Newitt et al., 1956)*

2.4.2.2 *Solubility of organic compounds in supercrical CO_2*

Literature data on the solubility of organic compounds for a wide range of organic structural types in subcritical (liquid) CO_2 have been augmented and summarized by Hyatt (1984). Far fewer data are available for solubilites under supercritical conditions, but the data for subcritical solutions can be used to give an idea of the supercritical solubilites. In general, for a given solute the difference in solubilites between subcritcal and supercritical CO_2 are a matter of degree—often an order of magnitude or so. Seldom is a material found to be completely insoluble under subcritical conditions yet soluble in the supercritcal phase (Hyatt, 1984). Some generalizations can be made from the solubility data of organic compounds in liquid and supercritical CO_2 (Francis, 1954; Hyatt, 1984; Willson, 1985):

1. Liquid CO_2 behaves like a hydrocarbon solvent, with a few notable points of difference (e.g. methanol miscibility).
2. Ionized compounds are essentially insoluble in CO_2. This includes compounds possessing dissociated acid groups or protonated basic groups, as well as inorganic salts. Other compounds possessing ionized groups, including amino acids, proteins and nucleic acids exhibit poor solubility in CO_2.
3. Highly polar compounds, such as amides, ureas, polysaccharides and sugars are not soluble in CO_2.
4. Hydrocarbons and lipid-soluble compounds of moderate polarity are, in general, readily soluble in supercritical CO_2. Such compounds include paraffins, ethers, lactones, glycerides (Williams, 1981). Esters, ketones, halocarbons, aldehydes, low alcohols (› C10) are freely soluble in CO_2.
5. Materials of many structural types with molecular weights around 500 such as chemotherapeutic drugs, caffeine, steroids, alkaloids are soluble in CO_2 (Willson, 1985).

The observance of solubility data alone cannot give a complete picture of the nature of CO_2 as a solvent. The effect of a given solvent on the course of a chemical or physical change, is usually rationalized in terms of the solvent's "polarity," where polarity includes the sum of all the solvent-solute interactions: coulomb, inductive, charge transfer, and hydrogen bonding (Hyatt, 1984). Hyatt (1984) used various empirical methods to determine solvent polarity of liquid and supercritical CO_2 and reports that CO_2 exhibits properties typical of hydrocarbon solvents, such as toluene, but has very low polarizability. No significant differences in polarity were detected between the liquid and supercritical phases.

The strength of supercritical carbon dioxide, can be enhanced by adding a co-solvent. The objective is to enhance considerably the polarizability by adding low concentrations of co-solvents, while maintaining the sensitivity of the solute's solubility with respect to temperature and pressure. The solubilites of many substances in supercritical fluids have been found to be increased by the addition of small quantities of a component intermediate in volatility between the supercritical fluid and the compound to be extracted (Dobbs *et al.*, 1986; Larson and King, 1986; McNally and Wheeler, 1988; Ikushima *et al.*, 1986). Such compounds are known as 'modifiers' or 'entrainers' after their ability to 'entrain' the desired substance into the supercritical phase. The extraction of polar compounds may be especially improved by the addition of substances able to form hydrogen, Lewis acid-base or other intermolecular bonds with the solute. An example of the use of entrainers is given by Shimshick (1983) who describes the enhancement of the extractability of the carboxylic acids, acetic, propanoic and butanoic acid with carbon dioxide from 1.0 M sodium salt solutions at 90°C and 220 bar by addition of 12 % dimethyl ether to the supercritical extractant. The supercritical fluid phase concentration of acetic acid, for example, was 0.05 wt % without dimethyl ether and 0.17% in the presence of the entainer. Similar solubility increases were exhibited by the other test carboxylic

acids. In addition to the effect of the entrainer, the influence of the increased size of the non-polar fraction of the molecule on extractability was also evident.

2.4.2.3 *Supercrical extraction of lipids*

Supercritical fluids offer alternative means of selectively extracting lipid components from food. The studies of Stahl *et al.* (1980, 1984), Friedrich and Pryde (1984), Snyder *et al.* (1984), Christianson *et al.* (1984), Bulley *et al.* (1984), Taniguchi *et al.* (1985), List *et al.* (1984a) and Lee *et al.* (1986) showed that SC-CO_2 can be used to extract oil from oilseeds. Pressure, temperature, size and physical structure of the seed particles, and moisture were reported to be key extraction parameters. The efficiency of the extraction also depended on contact time between the extracting fluid and the oil-bearing oil material, the ability of the fluid to penetrate the oil-bearing material and the solubility of the oil in the extracting fluid. Downward solvent flow was found to be most effective. Goodrum and Kilgo (1989) observed that upward or hoizontal solvent flow in the extraction cylinder tended to channel rather than flow uniformly through the bed. Oil yields by SC-CO_2 extraction were comparable to those of hexane extracted oil (Friedrich *et al.*, 1982). The oils are obtained in yields ranging from 95 to 98.5% and are virtually free of phospholipids, whereas those obtained with hexane contain between 1 and 3% of these polar lipids. The SC-CO_2 extracted oil also had a significantly lower refining loss (Friedrich *et al.*, 1982; List *et al.*, 1984a) and phosphorous content (Fattori *et al.*, 1987; List *et al.*, 1984b), was light-colored (List *et al.*, 1984b) and essentially degummed.

The process has also been used to to remove lipids and cholesterol from fish muscle (Hardardottir and Kinsella, 1988; Ikushima *et al.*, 1986; Yamaguchi *et al.*, 1986). Supercritical carbon dioxide extraction of antarctic krill yielded oils that were composed solely of nonpolar lipids, largely triglycerides, without phospholipids (Yamaguchi *et al.*, 1986). SC-CO_2 solvent entrained with ethanol enhanced lipid extraction from fish muscle

significantly (Hardardottir and Kinsella, 1988). The authors noted that pure CO_2 preferentially extracted the triglycerides, whereas, the SC-CO_2/ethanol solvent extracted both triglycerides and phospholipids.

To date there have been only a few citations in the literature concerned with the extraction and recovery of cholesterol from egg yolk by SC CO_2 (Levi and Sim, 1988; Froning *et al.*, 1990; Leiner, 1986). Levi and Sim (1988) extracted fresh and dried egg powder with supercritical carbon dioxide between 13.8 - 27.6 MPa (2000 - 4000 psi) and 32 - 54 °C. It was reported that more than 70% cholesterol was selectively removed from the egg material. Froning and co-workers (1990) used SC CO_2 to extract cholesterol from spray dried egg yolk. When extracted at 306 atm at 45°C or 374 atm at 55°C two-thirds of the cholesterol was removed. Extracting egg yolk powder with SC CO_2 at 300 bar and at 40°C resulted in an extract rich in cholesterol, triglycerides, free fatty acids, and waxes. The extract was free of phospholipid as they were left in the residue.

Wong and Johnston (1986) have investigated the solubility of cholesterol and other sterols in CO_2 under various conditions. The recovery and solubility of minor constituents, such as cholesterol, found in natural fats and oils have also been determined. Butterfat has been fractionated using supercritical carbon dioxide, with evidence that cholesterol can be concentrated into selected fractions (Kaufmann *et al.*, 1982; Arul *et al.*, 1987). Swientek (1987) demonstrated that up to 90% of cholesterol can be removed from milkfat. In another study, a continuous SC CO_2 process developed by reseachers D.K. Bandler and S.S.H. Rizvi at the Cornell University (Cornell University News Service, 1989) has been reported to remove up to 90% of cholesterol in butterfat. Using a multistage system, cholesterol is preferentially extracted. The first stage extracts cholesterol and some triglycerides from the butterfat sample; the second stage precipitates cholesterol. The third stage recovers low-melting triglycerides. Eighty-five percent of decholesterolized butterfat is recovered.

3. *Experimental Equipment and Procedures*

3.1 EXPERIMENTAL EQUIPMENT

3.1.1 Extraction apparatus

The schematic diagram of the extraction apparatus used in this study is presented in Figure 3.1. Carbon dioxide was pumped under high pressure by a single-end diaphragm-type compressor (Newport Scientific, Supperpressure Div., Jessup, MD). A 7 µm prefilter was placed between the supply cylinder and the pump inlet to prevent suspended particles from damaging the pump. The diaphram compressor delivered CO_2 under liquid or supercritical conditions to the temperature controlled vessel at pressures up to 70 MPa (10,000 p.s.i.). The sample was placed in the extraction vessel. Carbon dioxide flowing through the extractor vessel dissolved the components of interest and carried them to the let-down valve. As the CO_2 depressurized across the valve, it gasified and precipitated the dissolved compounds, trapping the components in the sample collector. The let down valve was heated to counteract Joule cooling, and was maintained at a constant temperature to promote a constant flow. After the solute was collected, the CO_2 was subsequently directed through a dry-test gas-meter, a rotameter, a cooled U-tube trap, and finally vented to the atmosphere.

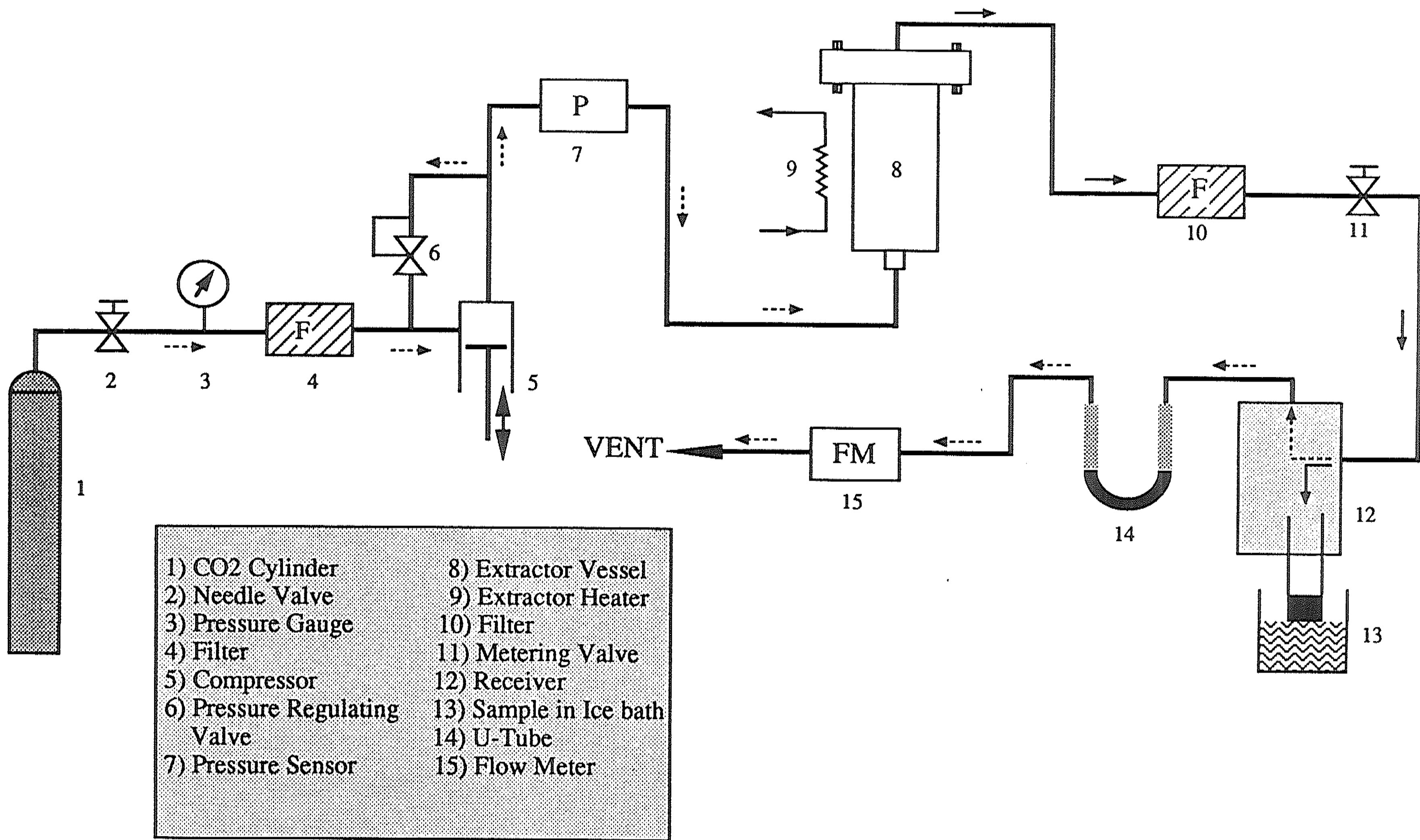


FIGURE 3.1: *Schematic diagram of supercritical fluid extraction system.*

3.1.1.1 Extraction vessel, temperature and pressure regulation

The extractor was equipped with a 300-mL capacity extraction vessel (Superpressure). The vessel was constructed of 316 stainless steel, pressure rated to 66 MPa at 100 °C, with dimensions of 38.1 mm i.d. x 254 mm (Figure 3.2). A thermocouple supplied with the vessel was inserted into the extraction vessel to monitor its internal temperature. The vessel temperature was maintained by heating with flexible electrical heating elements attached to the exterior of the extraction vessel and controlled by a digital temperature controller. The pressure in the extraction vessel was controlled by means of a manually adjusted front-pressure regulator (Tescom Corp., Elkriver, MN), and monitored with a diaphram pressure sensor (Data Intruments Inc., Lexington, MA).

3.1.1.2 Flow control

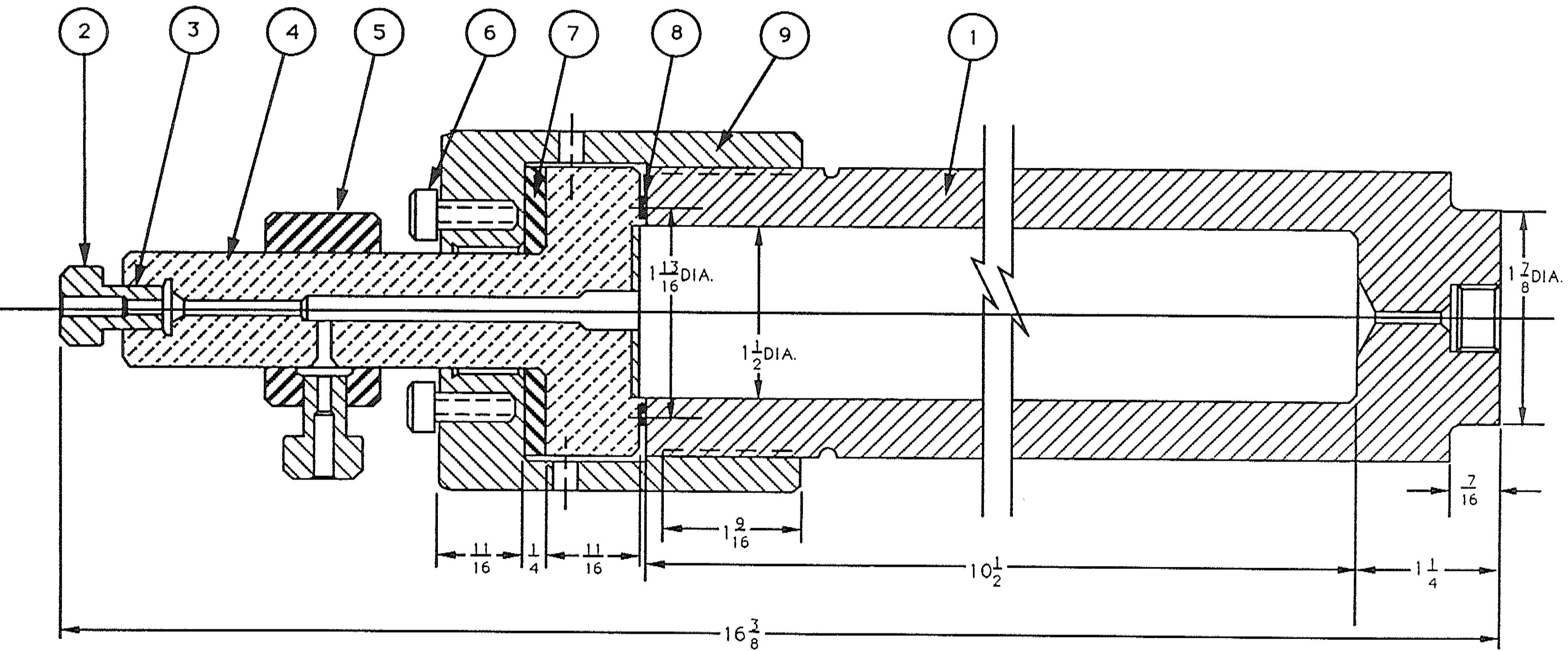
Flow control was by means of a manually adjusted needle valve supplied with the extractor. A heating block, as previously mentioned, prevented valve freeze-up and irregular flow due to the cooling effect of the expanding gas.

3.1.1.3 Flow rate

The instantaneous flow rate of CO_2 through the extractor was indicated with a rotameter (Fischer and Porter, Warminster, PA), while total flow was measured with a dry test meter (American Meter Div., Singer Co., Philadelphia, PA).

3.1.1.4 Sample collection

Upon passing through the restricter, the carbon dioxide within the system changes from the supercritical to the gaseous state. Coincident with this change of state is a large decrease in the solvation capacity of the carbon dioxide. Compounds such as oils,



GENERAL SPECIFICATIONS

DESCRIPTION	SPECIFICATION
Catalog Number	41-12156
Materials of Construction	316 Stainless Steel
Weight (approx.)	18.5 lb.
Pressure rating	9,200 psi @ 100°C
Type of Seal	Flat Gasket
Outside Diameter	2-9/16"
Inside Diameter	1-1/2"
Inside Depth	10-1/2"
Volume (approx.)	0.300 liter

LIST OF MATERIALS

ITEM	QTY.	DESCRIPTION	MATERIAL
1	1	BODY	316 ST. STL.
2	3	GLAND NUT	416 ST. STL.
3	4	INNER SLEEVE	416 ST. STL.
4	1	HEAD	316 ST. STL.
5	1	COLLECTOR; RING	18-8 ST. STL.
6	8	CAP SCREW	STEEL
7	1	THRUST RING	420 ST. STL.
8	1	GASKET	316 ST. STL.
9	1	CAP	CARBON STL.

FIGURE 3.2: *Extraction vessel design, general specifications, and list of materials.*

soluble in CO_2 on the upstream side of the restricter, precipitate downstream of the restricter.

A 100 mm long section of 2.1 mm I.D. stainless steel tubing was connected on the down-side of the restricter valve to the sampling unit. A sampling head threaded for the sampler tubes enabled ease of tube exchange and sequential lipid samples to be collected. Immersing the sampling tube in a methanol bath during collection, maintained at approximately -10 °C with an attached refrigeration unit provided a nonreactive environment for the heat-, temperature- and light-sensitive lipids. The extracts were flushed with nitrogron then stored at approximately -40°C until required for analysis.

Considerable organic solvent was carried over with the lipid sample and subsequently collected with the extract when an entrainer was used. To remove residual solvent, the lipid extract was evaporated under a stream of nitrogen in a 0 ± 1°C ice bath. It was noted that the alcohol entrainers were intimately mixed with the lipid mixture, and even more so with the ethanol entrainer. Difficulties were encountered in removing all traces of entrainer.

3.1.2 Experimental extraction procedures

3.1.2.1 Operating conditions - pressure, temperature, flow rate, entrainer

Forty to forty-five gram subsamples from the lot of freeze-dried egg yolk were placed in the 300 mL vessel and extracted under three different combinations of pressure, including 15 MPa, 20 MPa, and 36 MPa at 40°C. Calculated densities for carbon dioxide under these conditions are 0.625, 0.84 and 0.94 g/mL, respectively. Three different combinations of temperature, including 40°C, 55°C and 75°C at 36 MPa. Densities for carbon dioxide under these conditions are 0.94, 0.89, and 0.81, g/mL, respectively, as calculated according to the IUPAC equation of state for CO_2 (Angus, 1976).

Co-solvent extractions using ethanol at a 3 wt % concentration and methanol at both 3 and 5 wt % concentrations in CO_2 were also carried out on the freeze-dried egg material. All solvent-modified trials were tested at the operating conditions of 40°C and 36 MPa. Pressures in the extraction vessel were regulated to ± 0.5 MPa, and temperatures were regulated to ± 2°C.

It has been shown that the equilibruim solubility of oil in CO_2 is independent of the flowrate of CO_2 passing through the extraction bed (Fattori, 1986; Stahl *et al.*, 1980). Hence, the flowrate of CO_2 passing through the pump head was set at 2.0 g/minute for all experiments in this work. Flow was continued until a minimum of 60 g carbon dioxide for each gram of sample had passed through the extractor. The material extracted was collected at discrete intervals over an extended time. All extracts were analyzed afterward for cholesterol, fatty acid and phospholipid contents. All treatments were performed at least in duplicate.

3.1.2.2 Vessel loading procedure

First, fine spun glass wool was packed into the bottom of the vessel. A weighed amount of freeze dried egg yolk (about 40 - 45 g to the nearest 0.001 g) was placed in the vessel. Before closing the vessel, another plug of glass wool was inserted and the sealing surfaces were wiped with a chloroform-methanol (2:1) solution. All glass fibers protruding from the vessel were cut and removed. The 'perculating' action of the solvent is facilitated with the inclusion of glass wool plugs; the inert glass material also retains the sample particles preventing the fine particulates from being carried downstream.

3.1.2.3 System start-up and pressurization

Once the vessel was in place, the CO_2 supply was cracked with the flow metering valve closed. The vessel and valve heaters were turned on, and the compressor

started. After the system was allowed to equilibrate for approximately 30 minutes, the metering valve was cracked and adjusted to the desired flowrate.

3.1.2.4 Extract sampling

The sampling procedure consisted of fitting, pre-weighed, 20 mL glass test vials on the sampling outlet for varying amounts of time for the duration of the extraction. From the 'before' and 'after' weights of each vial, the oil collected during that period could be determined. The volume of gaseous carbon dioxide that passed through each vial during the sampling period was determined using the dry test meter. The corresponding mass of CO_2 was calculated from the ideal gas law using the temperature and pressure in the dry test meter.

3.1.2.5 Shut-down and post-extraction cleaning

At the conclusion of a run, the shutoff valve at the extractor inlet was closed to prevent backflow. The compressor was then shut off, as was the extractor heater. The sample vial was changed, so that a final 'blowdown' sample was obtained. The system was allowed to depressurize, and the extractor vessel removed after approximately 1 hour. The extractor vessel was opened and the residue removed for analysis. The extractor was rinsed with 2:1 cholorform / methanol and the residue dried and weighed. The system was then flushed with 2:1 chloroform / methanol, and the residue dried and weighed.

3.2 MATERIALS

3.2.1 Egg Yolk

Frozen commercial egg yolk (Lot 05129, Export Packers Co., Ltd, Winnipeg, Canada) was freeze-dried to a constant mass. The dry, coarsely powdered

(passed 2 mm seive, retained by 850 μm aperature) egg material was flushed with nitrogen gas, sealed and stored at -40°C until needed.

3.2.2 Extraction solvents

3.2.2.1 Carbon dioxide

The carbon dioxide used was commercial grade, supplied in siphon cylinders by Canadian Liquid Air Ltd. (Winnipeg, Canada). Specifications of the carbon dioxide are listed in Table 3.1.

TABLE 3.1 SPECIFICATIONS OF COMMERCIAL SIPHON GRADE CARBON DIOXIDE AS SUPPLIED BY CANADIAN LIQUID AIR, LTD.

purity	99.5%
cylinder pressure at 20°C	5.7 MPa (830 psig)
typical impurities	< 300 ppm CO
	< 100 ppm H_2
	< 0.5% Air
	< 30 ppm CH_4
	< 200 ppm H_2O

3.2.2.2 Entrained mixtures

The co-solvent and the carbon dioxide were pre-mixed to specified concentrations by Matheson Gas Products (Whitby, Ontario). Entrained solvents included 3 wt % ethanol, 3 and 5 % by weight methanol.

Ethanol recovery as a function of CO_2 passed through the extractor is plotted in Figure 3.3. Discrepancies in the co-solvent concentration during a run were not

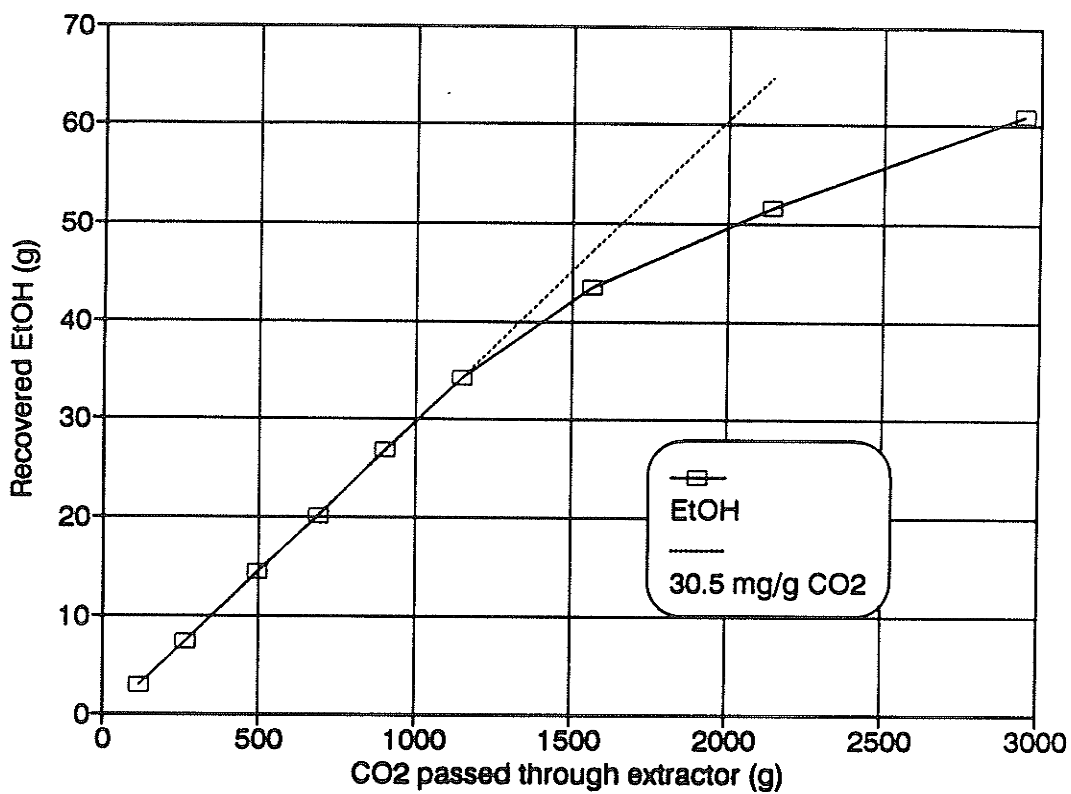


FIGURE 3.3: *Recovery of 3 wt % ethanol as a function of consumed CO_2*

found when full cylinders of the entrained mixtures were used. Variations in the amount of methanol or ethanol delivered from the tank was noted during a run when the cylinder's supply became depleted. Homogeneity of the mixture as it leaves the tank over a period of time is suspect. If during any run, the entrainer mass collected did not balance with the set concentration, the run was rejected.

3.2.3 Analytical standards, chemicals, solvents

Cholesterol standard (99%+ purity), methyl palmitate, methyl oleate, methyl stearate, methyl linoleate and methyl linolenate (all 99% purity) were obtained from Sigma Chemical Company (St. Louis, MO). Solvents used as eluants in the HPLC analysis were of LC grade purchased from Caledon Laboratories (Georgetown, Canada). All other reagents and solvents were analytical grade obtained from Canlab (Mississauga, Canada).

3.3 EXTRACT ANALYSIS

3.3.1 Compositional analysis

Total lipids in egg yolk and CO_2 extracted samples were determined by solvent extraction with chloroform / methanol (17.014 - 17.016, AOAC, 1984) and percent crude lipid was calculated. Moisture was determined by a vacuum oven drying procedure (16.002, AOAC, 1984), while protein was measured by the Kjeldahl method (17.008, AOAC, 1984). Extraction of freeze-dried egg yolk lipid was performed by the method of Folch *et al.* (1956); the lipid extract was used in further component analysis.

3.3.2 Analysis of fatty acids

The fatty acid analysis was performed in two steps. In the first step the fatty acid moieties were cleaved from the triglycerides and simultaneously converted to their

methyl esters. In the second step, the methyl esters were identified and quantified using gas chromatography.

3.3.2.1 Transesterification

The methylation of the total lipid fractions from control and CO_2 extracted dried egg yolk was done according to the boron-trifluoride-methanol procedure outlined by Metcalf and co-workers (1966).

Lipid material (150 mg) was accurately weighed in a 20 mL screw capped vial, to which 4 mL 0.5 N methanolic NaOH solution was added. The samples were immersed in a water bath (80°C) for about 5 minutes until the fat globules went into solution. Boron-trifluoride-methanol (BF_3-MeOH) (5 mL) was added to the solution, then shaken vigorously for 2 minutes. After cooling to room temperature, 4 mL saturated NaCl and 5 mL hexane were added to the solution. The tubes were shaken, then allowed to stand until a clear supernatent layer separated. The hexane layer (top), containing the mixed methyl esters, was transferred carefully with a disposable pipet to another vial, then sealed. Hexane (3 mL) was added to the aqueous layer; the extraction was repeated. The sample was flushed with nitrogen and stored (-40°C) for gas chromatographic analysis.

3.3.2.2 Gas chromatographic identification and quantification

The methylated samples were analyzed for their fatty acid composition using a Varian gas chromatograph (Model 3700) equipped with a flame ionization detector (FID) and a Hewlett Packard 3390 Integrator. The stainless steel column (12 m x 0.3 mm i.d.) was packed with 10% diethylene glycol succinate (DEGS) on 80-100 Chromosorb WHP (Supelco Canada Ltd., Oakville, ON). Operating conditions were as follows: Nitrogen was used as the carrier gas and the flow rate was set 20 mL/minute; the injection port

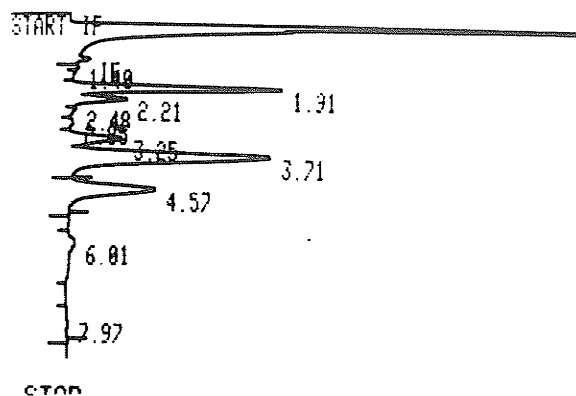
temperature was 220°C; the detector temperature was set at 220°C; column oven temperature was 180°C (isothermal); injection volume 1 μL.

Identification of the fatty acid peaks were made by comparison to the retention times of analytical standard fatty acids assayed under identical conditions. Quantitative data were then obtained by injecting known concentrations of fatty acid standards. The amounts of the fatty acids were determined from the areas given by the integrator using the response factor of the standards. A standard peak-area normalization method was built into the integrator to calculate the concentration. All lipid analyses were performed in triplicate. A typical chromatogram showing the elution sequence of each fatty acid ester and the peak resolution is given in Figure 3.4.

3.3.3 Extraction and quantification of cholesterol by HPLC

Cholesterol was extracted from the samples by a modification of the method of Beyer *et al.* (1989). The method uses alcoholic potassium hydroxide saponification, petroleum ether extraction of cholesterol, followed by quantitation by high pressure liquid chromatography.

A 0.125 g sample of SC CO_2 extracted lipid was weighed into a 50 mL glass centrifuge tube provided with a teflon-lined screw cap (Fisher Scientific, Edmonton, AB). After the addition of 5 mL ethanolic KOH solution (2.14 M), the samples were capped tightly, vortex-mixed for 20 seconds, then held in a water bath (70°C ± 1°C) for 1 hour. Samples were agitated every 15 minutes on a vortex. Upon cooling to room temperature, 5 ml petroleum ether, 2.5 mL saturated NaCl and 2.5 mL distilled water were added, then vortex-mixed for 20 seconds. The samples were centrifuged (0.15×10^2 g) for 10 minutes. The emulsion was broken and two clear layers with a distinct interface



RUN #90
ESTD

RT	Area	Type	Calibration #	PK Name
1.91	450500	PV	1	C16:0
3.25	158420	PV	2	C18:0
3.71	834920	BB	3	C18:1
4.57	375330	BB	4	C18:2
6.01	845	BP	5	C20:0

TOTAL AREA = 1820000
MUL FACTOR = 1.0000E+00

FIGURE 3.4: *Chromatogram of the fatty acid methyl esters in an esterified sample of a typical CO_2 extract of egg yolk lipid (36 MPa, 40 °C, flowrate 2.0 g/minute). Extraction and chromatographic conditions are described in the text.*

were formed. The upper layer, petroleum ether, was carefully transferred with a pasteur pipette to an empty vial. The aqueous layer was re-extracted with 5 mL petroleum ether, centrifuged, removed and combined with the previous ether layer; the aqueous layer was re-extracted a third time as previous. The 3 combined extracts were evaporated under a stream of nitrogen in a 50 ± 1°C water bath, resuspended in 1.0 mL hexane, and filtered (Millipore, Bedford, MA) (0.45 μm) prior to injection into the HPLC.

Cholesterol was separated from other sample components and quantitated by HPLC. The chromatographic system consisted of a Waters 510 pump (Waters Associates, Milford, MA), a Rheodyne (Model 7125) sample injection valve equipped with a 20 μL sample loop, and a Waters Model 484 ultraviolet absorption detector. The detector was equipped with an extended wavelength module that permitted detection at 206 nm by use of a tungsten lamp. Chromatograms were recorded and analyzed with a 820 Maxima work station running on IBM PC software.

Cholesterol was separated isocratically on a 3.9 mm i.d. x 30 cm stainless steel packed with a 10 μm diameter silica stationary phase (μPorasil, Waters Associate). The mobile phase consisted of 0.75 % 2-propanol (IPA) in hexane, and was pumped at a flow rate of 2.0 mL / minute. All solvents were of HPLC grade and were vacuum-filtered through a 0.45 μm Durapore filter and degassed prior to use. The detector sensitivity was set at 0.01 absorbance units full scale (AUFS). All samples were analyzed for cholesterol content at least in duplicate.

Identification and quantification of the eluted cholesterol standards and samples were done by comparing retention times and integration of the area under the peak. An external cholesterol standard (section 3.2.3) was used for quantitation. Four concentrations from 1.0 to 3.0 mg/mL were used to determine a linear relationship between cholesterol concentration and peak area of cholesterol. The detector response for peak area was linear ($r^2 > 0.999$) in this concentration range under the chromatographic conditions

given.. The calibration curve for cholesterol concentrations is plotted in Figure 3.5. A typical chromatogram of cholesterol in freeze-dried egg yolk is also shown (Figure 3.6).

Percent recovery of cholesterol was determined by adding cholesterol standard to the yolk material prior to the sample extraction. The percent recovery was determined by subtracting the average peak area of cholesterol present in unspiked counterparts from that in spiked samples. A 97.3 ± 1.1 % total recovery was obtained.

3.3.4 Phospholipid analysis

An enzymatic test kit designed for foodstuffs (Boehringer Mannheim Montreal, Canada) was used to determine lecithin (L - phosphotidylcholine) content in the egg material. L-α-lecithin was hydrolyzed by the enzyme phospholipase C to a diglyceride and phosphorylcholine at pH 8.0. The phosphorylcholine formed was hydrolyzed by alkaline phosphatase to choline and inorganic phosphate. The alkaline phosphatase was inactivated by immersing the assay solution into a boiling water bath and the formed choline was phosphorylated in the presence of adenosine-5'-triphosphate (ATP) by the enzyme choline kinase to phosphorylcholine. The adenosine-5'-diphosphate formed was then converted by pyruvate kinase with phosphoenolpyruvate into ATP with the formation of pyruvate. In the presence of the enzyme lactate dehydrogenase (LDH) pyruvate is reduced to lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the oxidation of NADH to NAD. The amount of NADH consumed in the reaction was stoichiometric with the amount of lecithin. NADH was determined by means of its absorbance at 365 nm. A description of the assay, sample preparation, the test solutions, and calculations are detailed in Appendix I.

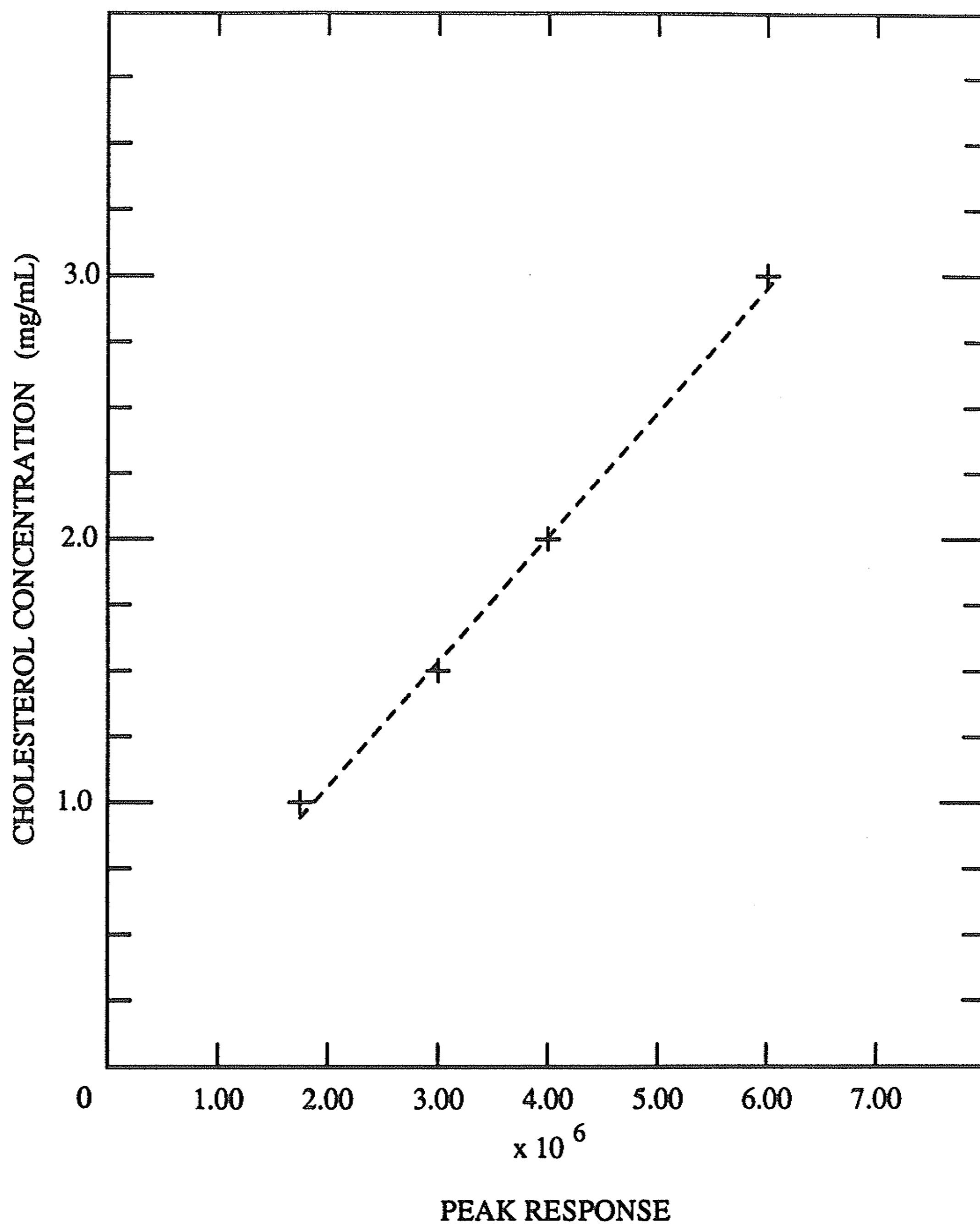


FIGURE 3.5: *Linear regression of cholesterol concentration as a function of HPLC peak response.*

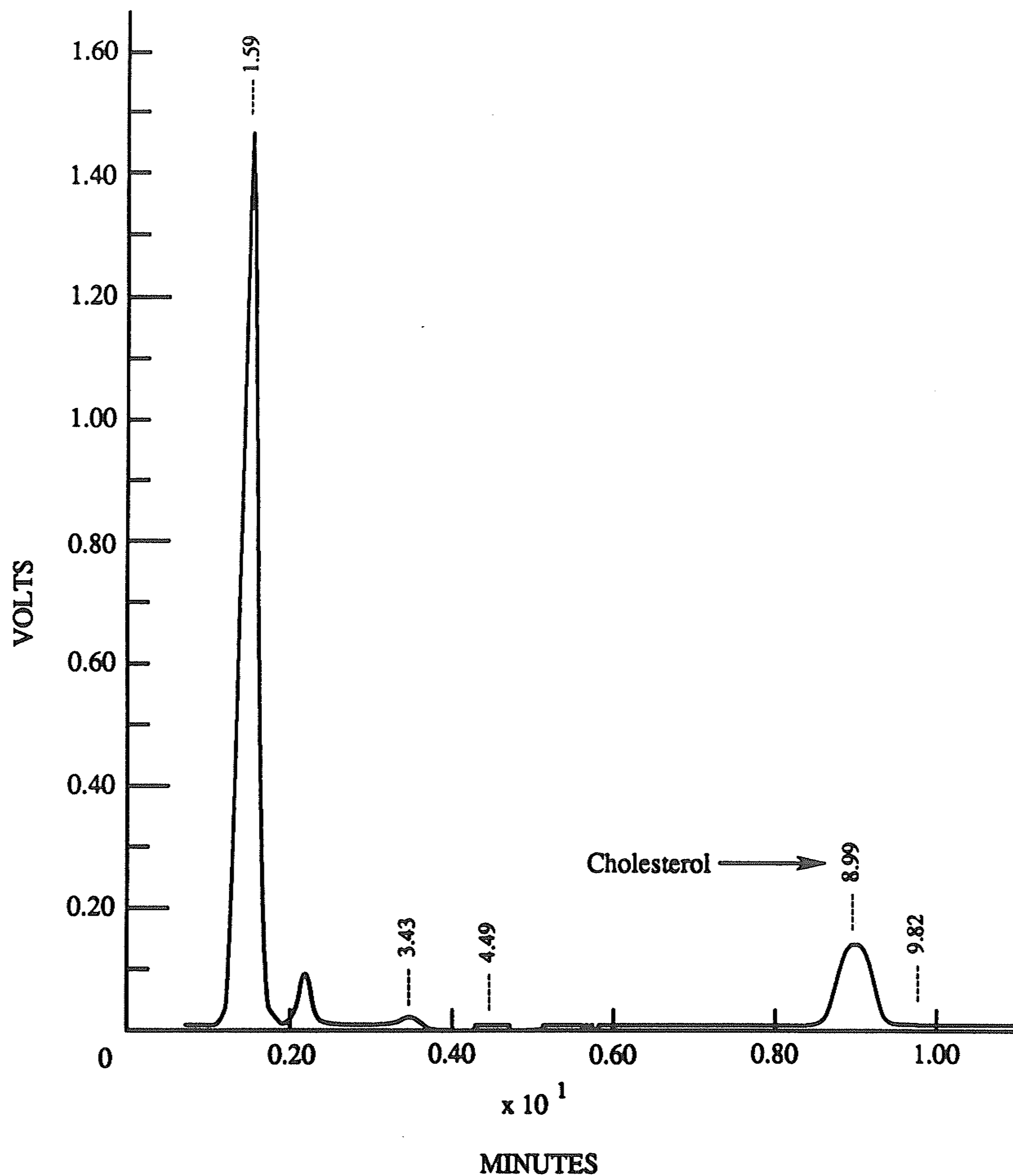


FIGURE 3.6: *A typical chromatogram showing the elution profile of cholesterol. Extraction and chromatographic conditions are described in the text.*

3.4 DATA CALCULATIONS

3.4.1 Extraction data

The data were analyzed using the Statistical Analysis System Package (SAS, 1982). Duncan's multiple range test and paired t-test were used to determine the significant of the differences between treatments. Treatments were considered significantly different when $p < 0.05$ (Duncan, 1955).

A Quattro Pro (Borlund, Scotts Valley, CA) spreadsheet was used to analyze, record and display data as obtained from an extraction trial (Table 3.2). At the top of the spreadsheet, the date and extraction conditions are recorded, as well as the mass of the sample placed in the extractor. The temperature and pressure of the volumetric flowmeter are also recorded; the density and, therefore, the mass of CO_2 exiting the extractor can be calculated. In the spreadsheet itself, columns 1, 5, 7, and 8 are raw data for each sample collected, denoting the volume of CO_2 passed through the extractor, the sample change time, and the initial and final collection tube masses, respectively. Using the density information from the flowmeter conditions, the CO_2 data is converted to a mass basis, and a cummulative total for the extraction obtained in column 3. This can be combined with the elapsed time data in column 6 to check on the CO_2 flowrate during the extraction. Column 4 shows the accumulated weight of CO_2 that has passed through the extractor CO_2 normalized by sample weight. Normalization standardizes run to run sample size variation, thereby making specific CO_2 a useful parameter to compare sample recoveries Recovered sample (9) is determined as the difference between final and initial masses of the collection vessel. A running summation of sample recovered is reported in column 10. Accumulated sample recovered is normalized to percent of original sample in column 11. Solubility is determined as the slope of a line describing the linear portion of

TABLE 3.2 TYPICAL QUATTRO PRO SPREADSHEET USED TO ANALYZE, RECORD AND DISPLAY DATA FROM THE EXTRACTION OF EGG YOLK LIPIDS USING SUPERCRITICAL CARBON DIOXIDE

January 14, 1990

Mass, freeze-dried egg yolk:	43.01 g
Extraction temperature:	40.0 °C
Valve temperature:	55.0 °C
Extractor Pressure	36.0 MPa
Flow meter temperature:	26.0 °C
Flow meter pressure:	0.1013 kPa
CO2 Density:	0.0018 g/mL
Initial mass of U-tube	43.118 g
Final mass of U-tube	43.130 g
	———
Trapped	0.012 g

1	2	3	4	5	6	7	8	9	10	11	12
Indicated CO_2 (L)	CO_2 Flow Interval (g)	Accumulated CO_2 (g)	Specific CO2 (g/g sample)	Time (hh mm)	Elapsed Time (s)	Mass of Collector Initial (g)	Mass of Collector Final (g)	Collected Sample (g)	Accumulated Sample (g)	% Recovered	Fit Curve
35467.8	0	0	0	18 25	0	-	-	-	-	-	-
35505.1	66.8	66.8	1.55	18 35	600	19.582	20.140	0.558	0.558	1.30	1.2126
35584.4	142.1	209.0	4.86	19 15	2400	18.692	20.108	1.416	1.974	4.59	4.7746
35680.5	172.2	381.2	8.86	19 54	2340	19.008	20.865	1.857	3.831	8.91	9.0912
35766.3	153.8	534.9	12.44	20 23	1740	19.156	20.896	1.740	5.571	12.95	12.945
35833.8	121.0	655.9	15.25	20 44	1260	18.471	19.849	1.378	6.949	16.16	15.977
35914.6	144.8	800.7	18.62	21 08	1440	18.712	20.350	1.638	8.587	19.97	19.607
35990.0	135.1	935.8	21.76	21 31	1380	20.043	21.404	1.361	9.948	23.13	22.993
36075.8	153.8	1089.6	25.33	21 56	1500	18.092	19.520	1.428	11.376	26.45	26.847
36263.5	336.4	1426.0	33.15	22 55	3504	18.245	20.771	2.526	13.902	32.32	35.278
36308.0	79.7	1828.0	42.50	23 43	2880	18.842	20.231	1.389	15.291	35.55	45.354
36618.7	556.8	2384.8	55.45	25 06	4980	17.400	18.774	1.374	16.665	38.75	NA
37470.5	1508.6	3893.4	90.52	33 15	29340	17.717	18.749	1.032	17.697	41.15	
37989.7	948.4	4841.8	112.57	36 55	13200	18.339	18.482	0.143	17.840	41.48	
39042.0	1885.8	6727.6	156.42	47 00	36300	18.518	18.539	0.021	17.861	41.53	
	blowdown		0.00			18.966	19.005	0.039	17.900	41.62	

13 Mass Balance

Residure recovered from vessel	25.10 g
Captured in vials:	17.90 g
Trapped in U-tube:	0.01 g
Washed from vessel:	0.34 g
Flushed from lines:	0.35 g
	———
Total recovered:	43.70
Loss:	- 1.61 %

14 Regression Output

Constant	0.199
Std Err of Y Est	0.1128
R squared	0.9993
No. of observations	8
Degrees of Freedom	6
X Coefficient	0.0108
Std Err of Coef	0.0001

the plot of accumulated sample (10) as a function of accumulated CO_2 (3). The regression results are shown in area 14.

3.4.1.1 *Mass Balance*

Area 13 (Table 3.2) represents the mass balance performed on inputs and recoveries to the extraction system for each run to ensure the integrity of the results. The results shown are typical. As can be seen, the great majority of the sample originally placed in the vessel was either recovered as sample or remained as residue in the vessel. A negligble amount (0.02%) by passed the collector tube and was trapped in the U-tube and small amounts (0.81%) were trapped in the lines. Mass balances indicate an error of only 1.6% when accounting for all material recovered.

3.4.1.2 *Solubility determinations*

Solubility of egg lipids in SC CO_2 was determined as the slope of the initial linear portion of the recovery curve. A typical extraction curve is shown in Figure 3.7. Use of the extraction curve shape can be explained by a consideration of the transfer behavior of lipid materials from the yolk matrix to the carbon dioxide stream on the extraction bed (Figure 3.8). The extraction bed is composed of semiporous granules (Figure 3.8a) in which lipid deposits are distributed in the nonextractable protein matrix. Material close to the surface of the granule is readily dissolved into the CO_2, while less accessible lipids must migrate through the matrix to a exposed surface. Generally, the rate of migration is slower than the rate of lipid uptake (Fattori, 1986) so that after the original surface deposits are depleted, the overall rate of lipid uptake is determined by the amount of lipid available at the surface of the granules at a given time (Figure 3.8b). For this reason, CO_2 entering at the bottom of the bed dissolves available lipids, and gradually becomes saturated as it moves through the bed, providing that residency time is adequate, and that

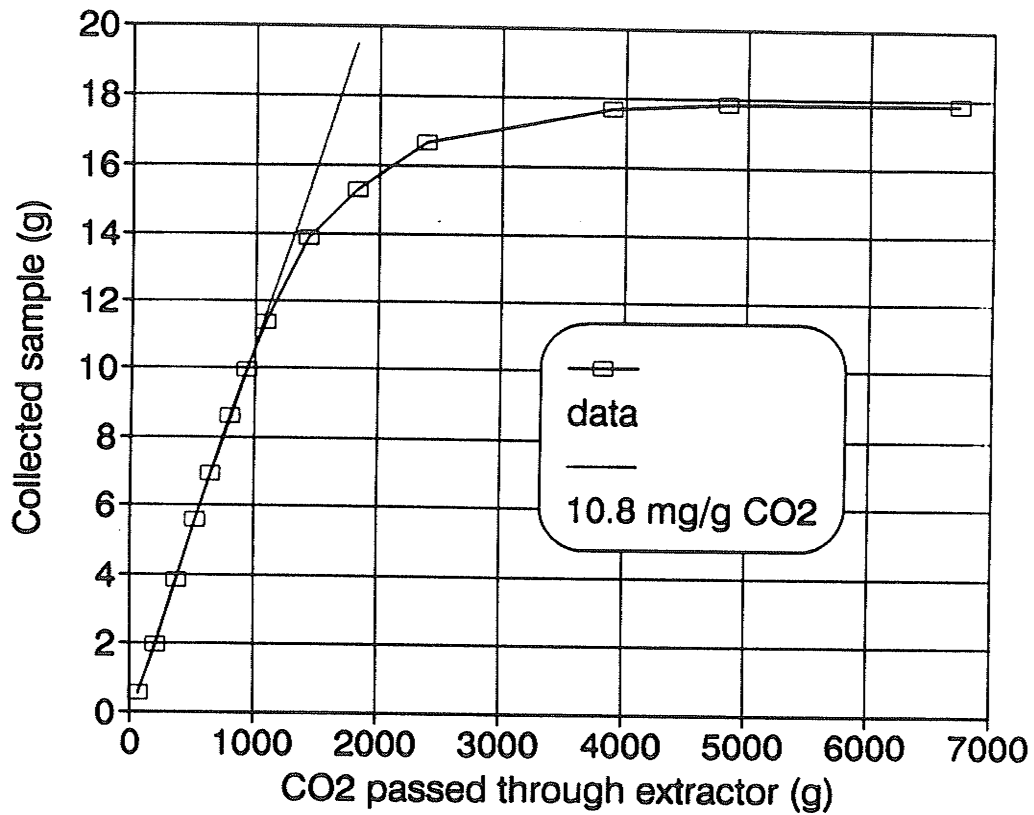


FIGURE 3.7: *Typical extraction curve showing lipids recovered from egg yolk as a function of the CO_2 passed through the extractor (values given for a 43.01 g sample at 40 ℃ and 36 MPa); CO_2 flowrate = 2.0 g/minute.*

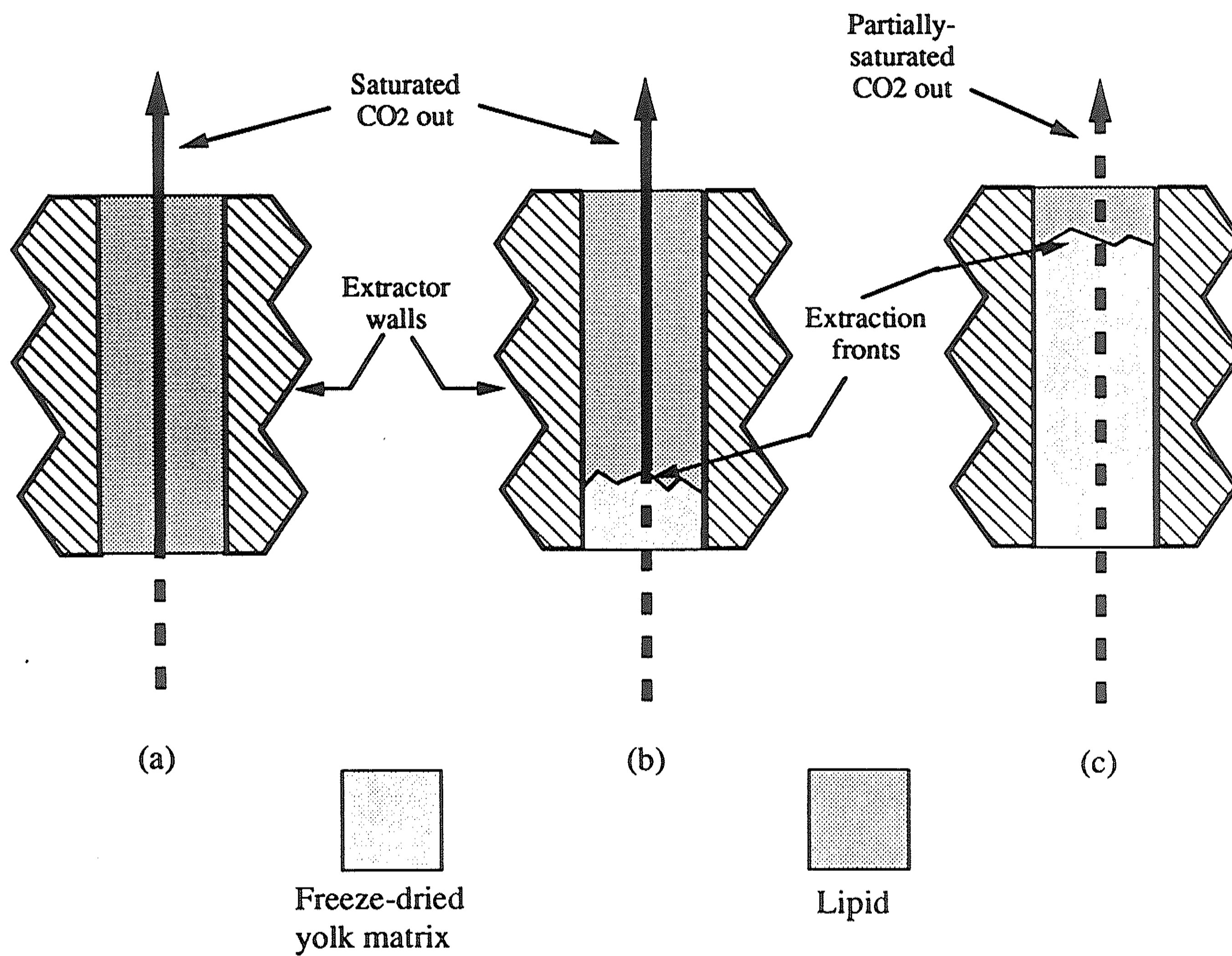


FIGURE 3.8: *Schematic diagram of lipid solubilization during extraction using supercritical CO_2.*

intimate contact between the granules and CO_2 has been achieved. Once saturated, the CO_2 simply moves through the bed so that the CO_2 exiting the system is saturated with lipid. As the extraction progresses, the lipids in the lower portion of the bed move to the surface of the granules and are progressively extracted by unsaturated CO_2, as described above. However, as long as there is sufficient bed length above the "extraction front," the CO_2 passing through the bed will be saturated with lipid, and the curve will be linear. Hence, the linear portion of the curve represents a system that is in an "equilibrium" state. The level of saturation reflects the solubility, and, hence, this slope represents a meaningful solubility value.

Eventually, however, the extraction front will reach a level such that there is insufficient bed length where free surface dissolving of lipid is taking place, and the CO_2 exiting the top of the extractor is no longer saturated. As seen in Figure 3.6 the slope of the extraction curve consequently decreases. Finally, as the extraction continues, the curve becomes asymptotic to a line equivalent to the theoretical total extractable lipid. All extraction runs were terminated at a point where the CO_2 was no longer saturated (i.e. no longer in the equilibrium state) and to a point where there was a significant reduction in lipid recovery for equivalent sampling periods (usually < 0.1g in 30 minutes). Exhaustive extraction runs were terminated when < 0.01 g of lipid was recovered during a collection period of a minimum of 2 hours.

Other empirical methods for measuring the solubility of substrates in a supercritical fluid are described in the literature (Krukonis and Kurnik, 1985; Van Leer and Paulaitis, 1980; McHugh *et al.*, 1984), whereby, solubility determinations in compressed gases correspond to the conventional determinations of saturated vapor pressures of substrates. Difficulty is introduced, though, by the fact that the system has to be examined at high pressures. As with vapor pressure determinations, they have been divided into two main catagories, static and dynamic systems. In static systems, equilibrium is established

between the phases in a closed vessel. The compositions of the phases are then determined either *in situ*, by such techniques as spectroscopy or radioactive counters, or by carefully withdrawing small samples of the equilibrium phases. In dynamic systems, gas is continuously passed over the substrate, at a rate slow enough to reach equilibrium, and the gas is then analyzed. The analysis is often carried out by decompressing the gas to atmospheric pressure, when the dissolved material precipitates and is collected. With both these systems, static and dynamic, equilibrium must be established to monitor solubility, something which is both time consuming and not practical in terms of supercritical investigations. In this study, a simpler approach has been used in which equilibrium solubility is determined by the plotting of the accumulative mass of egg lipid against the corresponding mass of CO_2 during a typical extraction run. The average lipid concentration in the CO_2 at the extractor outlet, at any interval during the extraction, can be determined from the mass of oil collected during the interval and the mass of CO_2 passed. As described earlier, if the solute concentration measurement is made during the initial linear portion of the extraction curve, the measurement will represent the solubility of the solute in the CO_2 at that pressure and temperature. Other researchers (Fattori *et al.*, 1987; Cheok, 1984; Ikushima *et al.*, 1986; Taniguchi *et al.*, 1985) have experimentally supported this method as applied to lipid solubility and recovery in SC CO_2. Fattori (1986) tested two methods to ensure that this concentration measurement did in fact represent the oil solubility. In the first procedure, two separate extractions were performed on identical samples of canola seed under identical conditions of pressure, temperature and carbon dioxide flowrate. The only difference between the two extractions was that in the second extraction the length of the seed bed was increased. If the slope of the linear part of the second extraction curve was identical to the first, it provided evidence that the increased bed length did not increase the concentration of the oil in the CO_2 phase. With the resultant identical extraction curves, it suggested that the CO_2 was saturated and that the slope of the

curves could be used to calculate the oil solubility. In the second assessment, the extraction was stopped during the 'linear phase' and the bed of seed was divided into sections; each bed section was analyzed for its oil content. It was found that the oil content of the section of seed nearest the extractor outlet was still equal to its original concentration, indicating that the carbon dioxide was saturated prior to reaching this segment. This in turn provided evidence that the slope of the linear portion of the extraction curve represented the oil solubility.

In the present study, two separate extractions were performed on freeze-dried egg yolk samples under identical conditions of pressure, temperature and CO_2 flowrate. A grinding pretreatment to reduce particle size was applied to one sample. The grinding pretreatment increased exposed surface area by reducing particle size, and hence, diffusion distance of the solute through the meal substrate. It was found that although the increased surface area and decreased particle size should result in an increase in the rate of transfer of the oil into the CO_2, solubility values remained similar to the untreated egg samples. Since the slope of the linear part of the extraction curve was independent of particle size, it was felt that these values reflected the solubility of the lipid in the CO_2 at the specific pressure and temperature conditions used rather than difference in migration of lipids through the granules.

3.4.1.3 Lipid Recovery

Lipid recovery calculations were based on percent theoretical extractable material. The major extractable components of yolk lipids were assumed to consist of triglycerides (65.5%), phospholipid (28.3%) and cholesterol (5.2%) (Privett *et al.*, 1962). Assuming lipid content of fresh yolk is 34% and the average total solids content of the fresh yolk is 52%, then freeze-dried egg yolk can theoretically amount to 64.4% by-weight lipid. Actual recovered lipid was expressed as a percent of the theoretical lipid content.

3.4.2 Component analysis of extracts

3.4.2.1 Fatty acid data standarization

Fatty acid data as recorded on a Quattro Pro worksheet is typified in Table 3.3. The grams of CO_2 passed through the extractor during the collection of sample *n* is recorded in column 1. The accumulated CO_2 passed through extractor and the undifferentiated sample collected during interval *n* are logged in columns 2 and 3, respectively. Analytically determined fatty acid content of sample *n* (column 4) is used to determine triglyceride equivalent concentrations (mg/g sample), given by

$$TG = 1/3\ [(3 \times FAME) + Glycerol\ MW \times (FAME\ /\ MD) - (3 \times MeOH\ MW) \times (FAME\ /\ MD)]$$

where, TG is triglyceride equivalent; FAME is the given mass of a fatty acid methyl ester as obtained by GLC (mg/g sample); molecular weight of glycerol is 92.044 (g/mole) the molecular weight of methanol is 32.042 (g/mole); MD, the molar divisor, is the molecular weight of respective FAME (g/mole).

A given mass of a FAME was converted to the mass of triglyceride theoretically present (column 5) in the extract. Based on the assumption that the fatty acid distribution in the extract is not different to that found in fresh egg yolk, triglyceride equivalent concentrations for C16:1 and C18:3 were estimated (column 6) and included in the sum of triglycerides (column 7). These two fatty acids were identified in the gas chromatographic profile, as described in section 3.3.2.2; their quantities estimated by their area count. The extracts consist exclusively of SC-CO_2 soluble material, that is, nearly all triglycerides, a little cholesterol and a trace of phospholipids (columns 7, 8 and 9, respectively). The total lipid content (mg/g) in the extracts is cumulated in column 11. Percent cholesterol and percent fatty acid concentration (as equivalent triglycerides) of the recovered sample is also recorded (column 12).

TABLE 3.3 TYPICAL QUATTRO PRO SPREADSHEET USED TO ANALYZE, RECORD AND DISPLAY FATTY ACID DATA FROM THE EXTRACTION OF EGG YOLK LIPIDS USING SUPERCRITICAL CARBON DIOXIDE

May 05, 1990
57°C, 36 MPa
52.413 g sample 19.101 g residue

sample no.	Recovere CO_2 (g)	Accumulate CO_2 (g)	Specific CO_2 (g/g sample	Sample collected (g)	Fatty acid methyl ester concentration mg/g					Triglyceride equivalent concentration mg/g					Estimated TG's mg/g		Total Equiv. T mg/g	Cholesterol mg/g	Phospholip mg/g	Estimate Protein mg/g	Total Sample mg/g	(% of recovered sample) Fatty acid concentration as equiv. TG					Cholesterol
					C16:0	C18:0	C18:1	C18:2	C20:0	C16:0	C18:0	C18:1	C18:2	C20:0	C16:1	C18:3						C16:0	C18:0	C18:1	C18:2	C20:0	
1	135.7	135.7	2.6	1.196	217.3	45.5	350.8	149.6	15.5	216.2	45.3	349.2	148.9	15.4	45.3	12.4	832.7	44.6	0.0	0.0	877.2	24.7	5.2	39.8	17.0	1.8	NA
2	153.4	289.1	5.5	1.601	221.1	43.8	362.8	144.4	16.3	220.0	43.6	361.2	143.7	16.2	43.6	12.0	840.3	46.75	0.0	0.0	887.1	24.8	4.9	40.7	16.2	1.8	4.7
3	221.5	510.6	9.7	2.209	224.4	44.3	360.0	142.2	14.9	223.3	44.0	358.4	141.6	14.9	44.0	11.8	838.0	51.23	0.0	0.0	889.2	25.1	5.0	40.3	15.9	1.7	5.1
4	199.5	710.0	13.5	2.214	222.0	42.9	359.2	143.8	15.1	220.8	42.7	357.6	143.2	15.1	42.7	11.9	833.9	52.38	0.0	0.0	886.3	24.9	4.8	40.3	16.2	1.7	5.2
5	204.5	914.5	17.4	1.717	220.2	41.7	350.0	140.8	14.5	219.1	41.5	348.4	140.2	14.5	41.5	11.7	816.9	50.67	0.0	0.0	867.5	25.3	4.8	40.2	16.2	1.7	5.1
6	247.1	1161.6	22.2	1.693	222.1	41.2	361.6	144.7	15.3	221.0	41.0	360.0	144.1	15.3	41.0	12.0	834.4	46.90	0.0	0.0	881.3	25.1	4.7	40.8	16.3	1.7	4.7
7	194.8	1356.5	25.9	1.492	221.2	41.4	358.4	143.3	14.8	220.0	41.2	356.8	142.6	14.8	41.2	11.9	828.5	46.24	0.0	0.0	874.7	25.2	4.7	40.8	16.3	1.7	4.6
8	551.4	1907.9	36.4	2.769	224.7	43.0	359.7	143.5	14.0	223.5	42.8	358.1	142.8	13.9	42.8	11.9	835.8	43.26	0.0	0.0	879.1	25.4	4.9	40.7	16.2	1.6	4.3
mean																						25.04903	4.859013	40.46121	16.28708	1.703709	4.820428571
blowdow				1.04	NA	NA	NA	NA	NA									NA		0.0	NA	NA	NA	NA	NA	NA	NA
residue				19.101	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	40.110	NA		NA	NA	NA	NA	NA	NA	4.0
egg 1.0				52.413	121.9	50.4	189.0	238.2	0.6	121.3	50.1	188.1	237.1	0.6	13.9	19.8	630.8	30.973	153.8	329.0	1144.6	10.6	4.4	16.4	20.7	0.0	3.1
egg 2.0				52.413	121.5	49.5	186.7	228.0	0.6	120.9	49.3	185.9	226.9	0.6	13.7	18.9	616.3	28.080	153.8	329.0	1127.2	10.7	4.4	16.5	20.1	0.1	2.8

3.4.2.2 Triglyceride solubility

The above GLC procedure yields only total fatty acid methyl ester content of the samples. In order to calculate the amount of trigylceride present, the fatty acid methyl esters were converted to their mass trigylceride equivalents (section 3.4.2.1). Triglyceride solubility in SC-CO_2 was determined from the slope of the plot of triglyceride yield to total amount of CO_2 consumed.

3.4.2.3 Cholesterol

The cholesterol content in the SC CO_2 extracted egg samples was recorded and calculated (Table 3.4) in a similar manner to the fatty acid data. The data contained within columns 1 to 3 have been previously explained (section 3.4.2.1). The analytically determined cholesterol concentration of sample *n* (mg/g sample) is recorded in column 4. The product of collected sample (g) and cholesterol concentration of the extract (mg/g) yields the cholesterol content of sample *n* (mg) as accounted in column 5. Total cholesterol recovered (mg) since start of the extraction (column 6) was recorded. The efficiency of cholesterol recovery at interval *n* , that is, the cholesterol recovered per gram CO_2 passed through the extractor (column 7), was calculated by dividing the cholesterol content in the sample (mg) by the total recovered CO_2 (g). Percent theoretical recovery (column 8) was computed by normalizing accumulated cholesterol by total cholesterol originally present in sample; the original sample was estimated to have a cholesterol content of 1173.15 mg as determined by the procedure outlined previously (section 3.3.3). Finally, fitting a curve to the linear portion of the recovery curve provided an estimated solubility of cholesterol in SC CO_2 (column 9). The regression results are shown in area 10.

TABLE 3.4 TYPICAL QUATTRO PRO SPREADSHEET USED TO ANALYZE, RECORD AND DISPLAY CHOLESTEROL DATA FROM THE EXTRACTION OF EGG YOLK LIPIDS USING SUPERCRITICAL CARBON DIOXIDE

February 5, 1990

Mass, freeze-dried egg yolk:	39.7 g
Extraction temperature:	40.0 °C
Extractor Pressure	20.0 MPa
mean residual cholesterol after extracion	1.67 mg
Estimated cholesterol content in original egg material	1173.2 mg

	1	2	3	4	5	6	7	8	9
Sample no.	CO_2 Flow Interval (g)	Accum. CO_2 (g)	Collected Sample (g)	Cholesterol Conc. mg/g sample	Sample Cholesterol (mg)	Accum. Cholesterol (mg)	Efficiency g chol / g CO2	Recovery (% theoret)	Fit Curve
1	85.8	85.8	0.167	NA	NA	NA	NA	NA	NA
2	203.0	288.9	0.705	63.93	45.07	45.07	0.22	3.84	3.69
3	259.7	548.6	0.778	55.31	43.03	88.10	0.17	7.51	7.28
4	302.9	851.4	0.630	55.21	34.78	122.89	0.11	10.47	11.47
5	286.2	1137.6	1.185	54.84	64.99	187.88	0.23	16.01	15.43
6	593.4	1731.0	1.705	53.71	91.58	279.45	0.15	23.82	23.63
7	226.5	1957.5	0.643	50.60	32.54	311.99	0.14	26.59	26.76
8	977.8	2935.3	2.147	49.99	107.3	419.31	0.11	35.74	40.28
9	806.3	3741.6	1.676	49.26	82.56	501.87	0.10	42.78	51.42
10	437.5	4179.0	0.788	47.03	37.06	538.93	0.08	45.94	57.47
11	712.2	4891.2	1.930	45.48	87.78	626.71	0.12	53.42	67.32
12	812.5	5703.8	1.385	44.11	61.09	687.80	0.08	58.63	NA
13	1500.0	7203.8	1.240	43.98	54.53	742.33	0.04	63.28	NA
14	1300.7	8504.5	0.433	46.15	19.98	762.31	0.02	64.98	NA
15	1908.6	10413	0.266	NA	NA	NA	NA	NA	NA

10

Regression output

Accumulated cholesterol vs accumulated CO_2

Constant	-3.5439
Std Err of Y Est	7.1219
R squared	0.9964
No. of Observations	6
Degrees of Freedom	4
X Coefficient (s)	0.1622
Std. Err of Coef.	0.004849

4. *Results and Discussion*

4.1 INTRODUCTION

The results presented in this section and the pertaining discussions have been grouped into three major sections. Initially there is a macroscopic study of the effects of extraction conditions on gross lipid solubility and recovery in supercritical CO_2. This is followed by an examination of the chemical analysis of the extracts revealing information about the extraction behavior of the individual triglycerides, cholesterol, and phospholipids. The final section describes the interrelation of these three components during the extraction. This section culminates in an assessment of the use of SC CO_2 potential for lipid fractionation or enrichment.

4.2 LIPID SOLUBILITY

The application of the supercritical processes is intimately coupled with the high pressure behavior and the physical chemistry of the system. The solubility of a solute is readily influenced by small variations in pressure or temperature in supercritical fluids. In both cases, the density of the supercritical fluid also changes. The solubility of a given compound may be influenced in several ways. Both vapor pressure and intermolecular forces determine solubilities. The following results and discussion focuses on the effects of temperature, pressure and added entrainer on the solubility of egg lipids in a supercritical fluid.

4.2.1 Egg Lipid Solubility as a Function of Temperature & Pressure

In this study, both temperature and pressure can affect the equilibrium solubilities of egg lipids in CO_2. At a constant temperature of 40°C, the solubility of egg lipid in SC CO_2 increases quite dramatically as the pressure is increased from 15 MPa to 36 MPa (Figure 4.1). At pressures below 10 MPa, egg lipid solubility is extremely low (< 0.01 mg/g CO_2), as would be expected for the solubility of a solid in a gas. With a pressure increase to 36 MPa, the solubility of the lipid reaches a value of 10.46 mg/ g CO_2. The solvation capacity of a supercritical fluid at constant temperature is strongly dependent on its density which in turn is proportional to the external pressure applied to the fluid (Hyatt, 1984). It follows, therefore, that the solvent power of a supercritical fluid can be controlled by varying the pressure. This phenomenon is illustrated in this study in which a exponential relationship exists between lipid solubility and CO_2 density. The solubility of egg yolk lipid in CO_2 at 40°C, 20 MPa is about 2.46 mg/g CO_2 ($\rho = 0.84$ g/cm3). As the pressure of the carbon dioxide is increased, the lipid mole fraction rises. At 36 MPa ($\rho =$ 0.94 g/cm3) the solubility of the egg lipid approximates 10 mg/g CO_2. This represents a 4-fold increase in solubility with an increase in pressure of only 16 MPa. Ikushima and co-workers (1986) reported similar pressure effects when extracting lipid-bearing freeze-dried mackerel with SC CO_2 at pressures ranging from 4.9 - 24.5 MPa with a fixed temperature of 313 K. The solubility at 24.5 MPa was about 4.6 times that at 9.8 MPa.

The extraction curves of egg yolk lipids as a function of extraction temperature (40°C, 55°C and 75°C) are shown in Figure 4.2. The effect of temperature on solubility is somewhat complex because of two competing effects. As temperature increases, vapor pressure of the solute increases which tends to increase solubility. Concurrently, CO_2 density decreases which tends to decrease solubility (Nilsson *et al.*, 1988). For egg lipids at 40°C and 55°C and at a constant pressure of 36 MPa, no significant difference in solubility was evident. This suggests that the decrease in CO_2

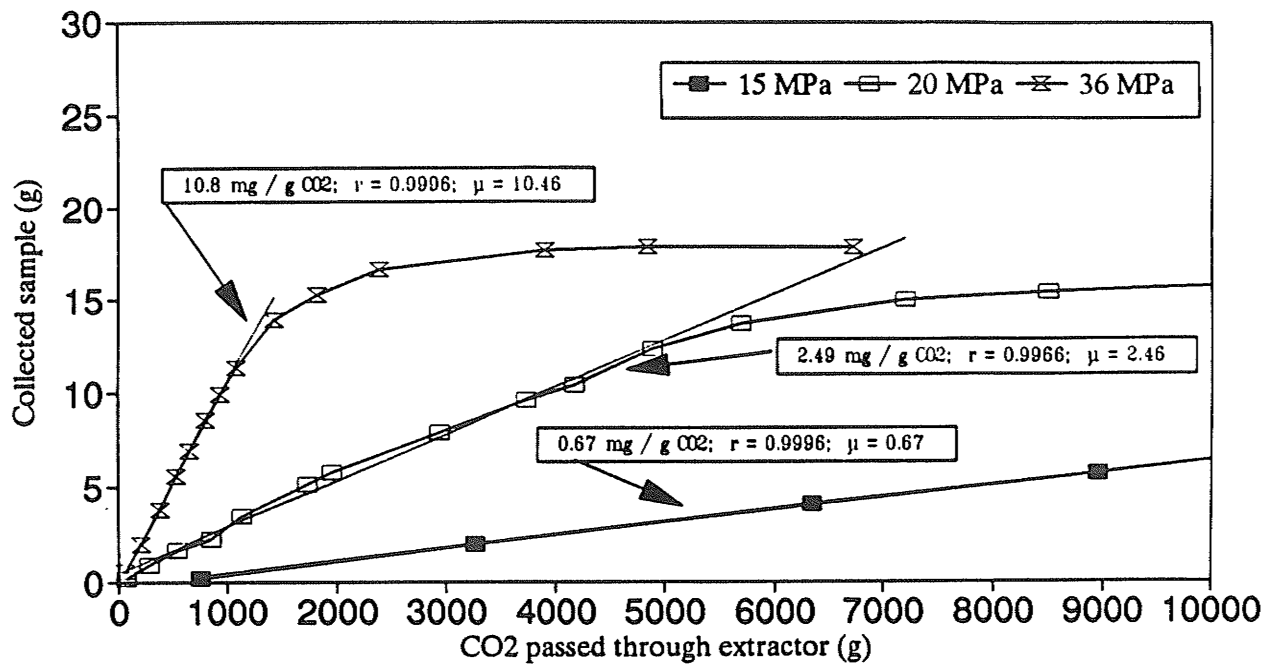


FIGURE 4.1: *Extraction of egg yolk lipids as a function of extraction pressure (15, 20 and 36 MPa) at a constant temperature of 40 °C. Data points represent a single supercritical run. Average solubilities, indicated in the line descriptions, represent duplicate, triplicate and 6-replicate determinations for 15, 20 and 36 MPa, respectively.*

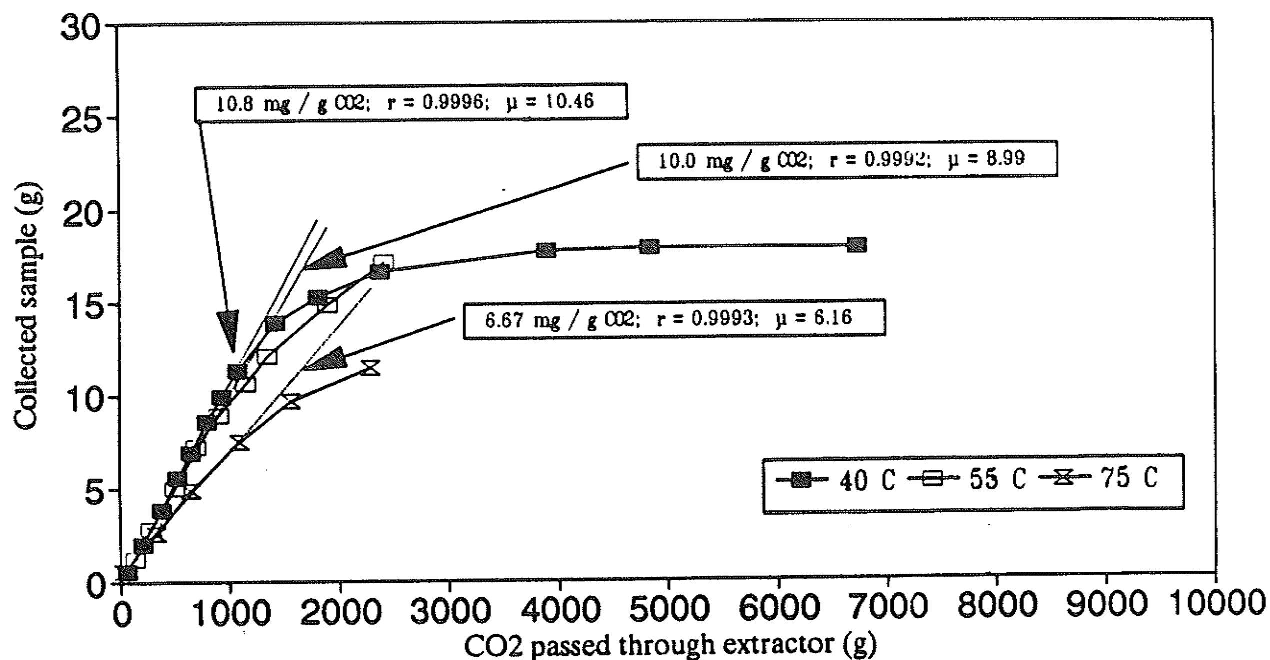


FIGURE 4.2: *Extraction curves of egg yolk lipids as a function of extraction temperature (40, 55 and 75 °C) at a constant pressure of 36 MPa and at a constant CO_2 flowrate of 2.0 g/minute. Data points represent a single supercritical run. Average solubilities, indicated in the line descriptions, represent 6-replicate determinations for 40 °C and duplicate determinations for 55 and 75 °C.*

density and increase in the vapor pressure of the lipid cancel out and the resulting solubility is therefore unchanged (Figure 4.2). At 75°C, a solubility decrease was noted as the influence of temperature on CO_2 density at this higher temperature was greater than the solubility increase due to increased vapor pressure.

4.2.2 Egg Lipid Solubility as a Function of Entrainer Type and Concentration

The effect of entrained SC CO_2 on the overall solubility of egg lipids is shown in Figure 4.3. Using 3% methanol or 3% ethanol more than doubled the solubility compared to its unentrained equilivalent. Solubility is doubled again with the use of 5 % methanol resulting in a solubility value of 44.4 mg/g CO_2. Overall solubility appeared to be independent of entrainer type at the same concentration. For 3 wt % methanol and 3 wt % ethanol, the solubility values were 19.7 mg/g CO_2 and 22.1 mg/g CO_2, respectively. The results suggest that egg lipid components are more soluble in the more polar fluid system compared to its unentrained counterpart. Carbon dioxide has a small polarizability and no dipole moment, so additives (polar entrainers) increase the polarizability of the solvent and the dielectric constant. Polar cosolvent molecules also interact with functional groups on the solutes, increasing solubilities and recoveries of polar compounds present in the matrix. However, it should be emphasized that, during an entrained-extraction run, organic solvent is carried over with the lipid sample and subsequently collected with the lipid extract (section 3.1.1.4). The inability to remove all traces of alcohol entrainer in the lipid extract becomes a source of error in subsequent analyses and thereby, is manifested in inflated data values. This error, though evident in both entrainer systems, is a function of entrainer concentration and more pronounced in the ethanol-entrained system. Difficulties in removing ethanol, with its lower volatility, from the lipid extracts has been a concern, especially in the interpretation of the results. Some entrainer may have been trapped with

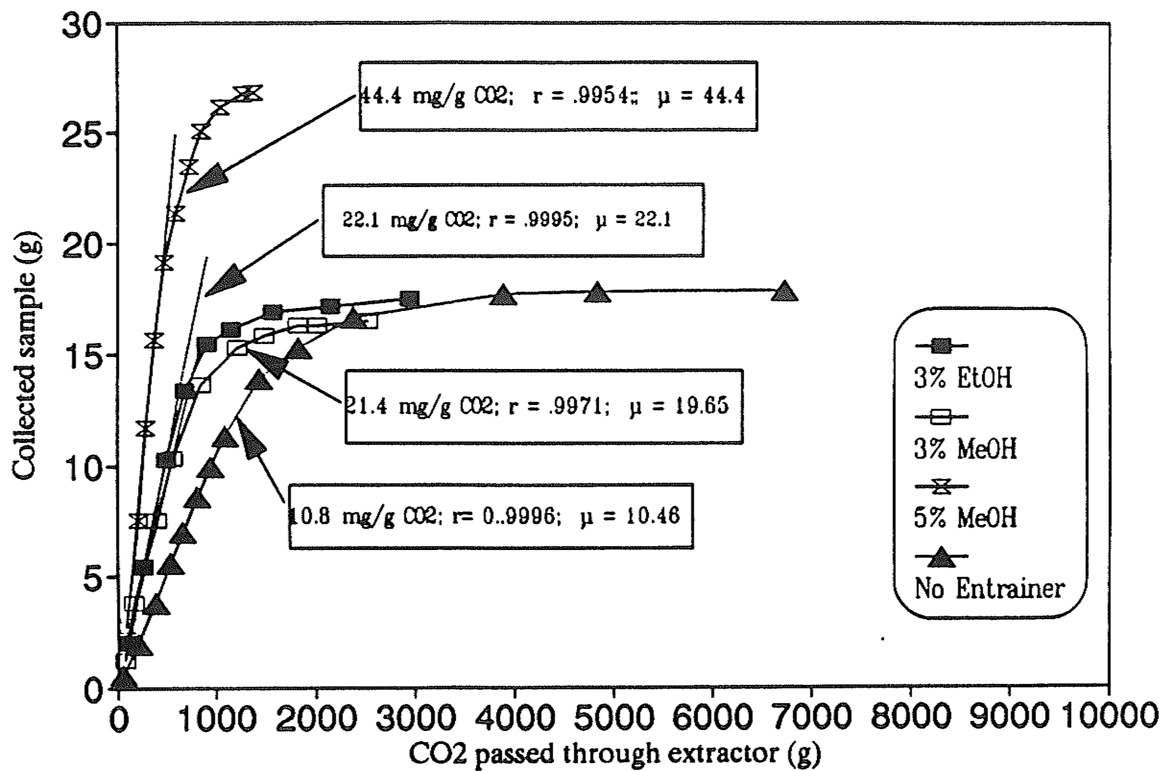


FIGURE 4.3: *Effect of the entrainer methanol and ethanol on egg yolk lipid solubility in supercritical CO_2 at constant pressure (36 MPa), temperature (40 °C) and CO_2 flowrate (2.0 g/minute). Average solubilites, indicated in the line descriptions, represent duplicate determinations.*

the extract leading to the higher apparent solubilities. As a result, the data must be looked on with care.

The solubilities of many substances in supercritical fluids have been found to be increased by the addition of small quantities of a component intermediate in volatility between the supercritical fluid and the compound to be extracted (Brunner, 1983; Brunner and Peter, 1982). Such compounds are known by their ability to 'entrain' the desired substance into the supercritical phase.

The extraction of polar compounds may be especially improved by the addition of substances able to form hydrogen, Lewis acid-base or other intermolecular bonds with the solute. In this study, the enhanced solubility of lipid could be partly attributed to the extraction of phospholipids in the polar extractant. However, with the phospholipid content comprising only 16% of the yolk solids, this effect would be marginal. The possible contribution of the individual egg lipid components on enhanced solubility in entrained systems will be discussed in section 4.4.3. Other researchers (Shimshick, 1983; Hardardottir and Kinsella, 1988) describe the influence of added entrainer on the increased size of non-polar fractions. Our study would appear to support such an effect.

The effects of entrainer type on the solubility of a compound and its selectivity are primarily dependent on the polarity of the entrainer (Hyatt, 1984; Dobbs *et al.*, 1986; McNally and Wheeler, 1988). In this study, varying the entrainer type, i.e. ethanol and methanol, resulted in insignficant differences in overall solubility. The close polarities of these two alcohols may account for the equivalent solubility values (dielectric constants: $\varepsilon_{EtOH} = 25.0$; $\varepsilon_{MeOH} = 36.6$).

In this study, the effects of entrainer concentration on the solubility of lipid components agrees with earlier work carried out by Dobbs and coworkers (1987) and researcher Van Alsten (1986) in which solubility enhancement by cosolvents, though

increasing solubilities up to an order of magnitude, was tied to concentration. Kamlet and researchers (1983) studied SC CO_2 systems using methanol as a cosolvent and described methanol's ability to act as either a Lewis acid or a Lewis base. However, Van Alsten (1986) and Schmitt (1984) present data which show that acid-base interactions are a secondary cosolvent effect superimposed on a primary effect determined by cosolvent concentration.

Ethanol was examined in addition to methanol because of its slightly lower polarity and lower toxicity for feed systems. The solubility of egg yolk lipid in methanol is not significantly different than in ethanol at 36 MPa and 40°C indicating that solubilities of solute in the liquid modifier can not necessarily predict overall extraction efficiency. An investigation of solute solubility in SC-CO_2 was conducted by McNally and Wheeler (1988) where modifier type and concentration were examined for their effect on the extraction efficiency of diron, linuron and 3,4-dichloroaniline from Sassafras oil. Acetronitrile was examined in addition to methanol and ethanol because of its slightly higher polarity. Increased extraction efficiency was exhibited for ethanol, with its the slightly lower polarity. The solubilities of diuron in methanol were not significantly different than in acetonitrile nor in ethanol. The authors current hypothesis is that the polarity of the solvent mixture must be optimized to match the polarity of the solute. The above examples illustrate that a polar co-solvent may increase markedly the solubility of a polar solute.

The impact of entrainer type and concentration will be discussed further in the examination of the solubility and extraction of individual egg yolk lipid components.

4.3 LIPID RECOVERY

4.3.1 Effect of Temperature, Pressure and Entrainer

The roles of temperature, pressure and entrainer type and concentration on the overall percentage of egg lipids that could be recovered from freeze dried egg yolk were examined with pressures ranging from 15 and 36 MPa and at temperatures between 40°C and 75°C. Table 4.1 is a tabulation of the results.

As mentioned earlier, egg lipids consisted of 65.5% triglycerides, 28.3% phospholipid and 5.2% cholesterol (Privett *et al.*, 1962). Assuming the lipid content of fresh yolk is 34% and the average total solids content of the fresh yolk is 53%, then freeze-dried egg yolk is 64.4% by-weight lipid. When CO_2 extractions were carried out to exhaustion at 40°C and 36 MPa, 72.8% of the total lipid was recovered. A plot of percent theoretical recovery versus CO_2 used, after standardizing for sample size, provides an indication of the rate of oil removal during a run (Figure 4.4) Since triglycerides and cholesterol are the main apolar lipids and make up about 70.7% of the total lipids with the balance being phospholipid, it appears that essentially all of the CO_2 extractable lipids are being removed. Even though recovery values for other pressures and temperatures were not determined at exhaustion, extractions were all carried to a point where oil removed per gram of CO_2 was less than 0.1g in 30 minutes. Based on a visual inspection of the curves, it is felt that if all systems were run to exhaustion, the recovery values obtained would have been the same as those at 40°C and 36 MPa. Hardardottir and Kinsella (1988) also found that different extraction temperatures and pressures did not affect the overall lipid recovery of oils extracted from freeze-dried fish muscle.

When 3% methanol was added as an entrainer and run to exhaustion, no overall increase in total extractable lipid was noted. When 3% ethanol was used as the entrainer however, the overall recovery increased to 84%. Although no phospholipid was found in lipid extracts collected in the early stages of a 3% ethanol run, significant

TABLE 4.1. SUMMARY OF LIPID RECOVERIES AS AFFECTED BY PRESSURE, TEMPERATURE AND THE USE OF ENTRAINERS DURING THE SUPERCRITICAL CO_2 EXTRACTION OF EGG YOLK

Temperature	Pressure	Entrainer type and concentration	Recovery				
°C	MPa	% wt. basis	Total specific CO_2 g CO_2 / g sample consumed	% of original sample mean ±S.D	% of total theoretical lipid	% original sample after passage of 50 g CO_2 / g sample	% of theoretical lipid after passage of 50 g CO_2 / g sample
40	36	-	112.6	39.8 ± 3.8 [a]	61.9	36.6 ± 2.3 [a]	56.9
			121.2	*46.9 ± 0.2 [b]	72.8	37.9 ± 0.3 [a]	58.9
40	20	-	189.2	34.7 ± 3.6 [a]	53.9	14.3 ± 0.6 [b]	22.2
40	15	-	570.4	33.3 [a]	51.8	1.4 [c]	2.2
55	36	-	64.4	35.8 ± 0.6 [a]	55.6	34.5 ± 0.2 [a]	53.6
75	36	-	113.8	31.8 ± 1.5 [a]	49.4	26.7 ± 0.4 [d]	41.5
40	36	3 % MeOH	72.6	*44.4 ± 2.9 [b]	69.0 [1]	43.4 ± 3.3 [e]	67.4
40	36	3 % EtOH	91.5	*54.2 ± 0.1 [c]	84.2 [1]	53.2 ± 0.8 [f]	82.7
40	36	5 % MeOH	107.2	*73.4 ± 2.3 [d]	114.1 [1]	64.7 ± 0.2 [g]	100.5

Means with different subscripts in the same column are significantly different ($P < 0.05$).

* run to exhaustion

[1] May be high due to contamination of sample with entrainer.

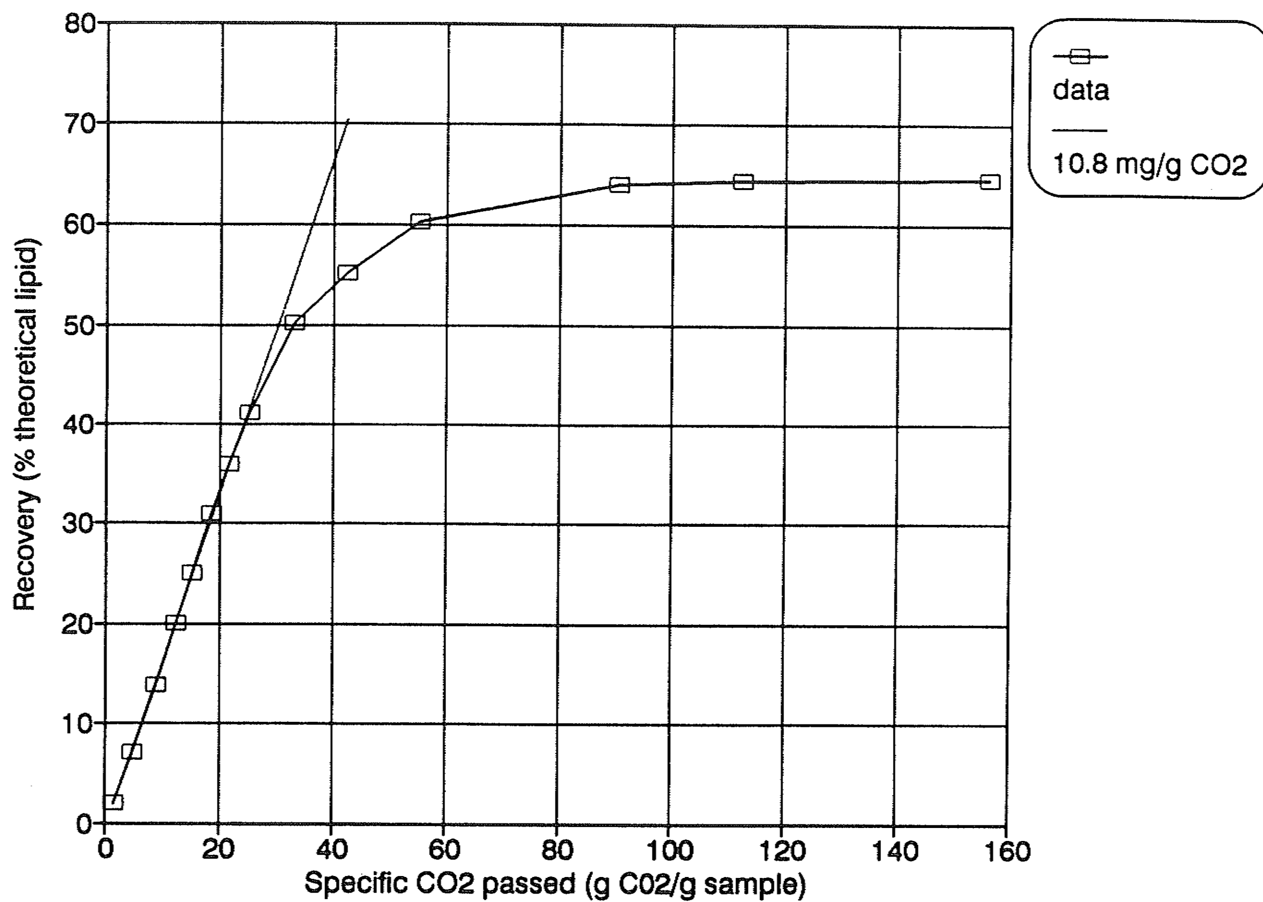


FIGURE 4.4: *Standardized extraction curve (36MPa, 40 ℃) for egg yolk lipids in which horizontal and vertical axes have been normalized by sample mass (~40g) The recovery is expressed as a percentage of theoretical lipid in egg yolk.*

quantities of phospholipid were measured in samples collected in the latter stages of a run and may account for the increase in lipid recovery collected near the end of the run. When the methanol concentration was increased to 5%, the total lipid recovered approached 100% of theoretical and contained larger amounts of phospholipid. Some entrainer may have been trapped with the extract leading to the apparent high recoveries.

Since extraction to exhaustion may not be practical in an industrial sense, the values of oil removed per gram of CO_2 for the first 50g of CO_2 that passed through the bed are also given in Table 4.1. At this interval, the values markedly differ reflecting differences in solubility. Since lipid removal is a function of lipid solubility and of bed geometry, the data must be looked on with care but it does provide some indication of overall extraction efficiencies as a function of time and CO_2 used. The total specific volume of CO_2 per gram sample consumed at the completion of the run has also been recorded (Table 4.1). It also reflects extraction efficiency. Low extraction volumes in conjunction with high recovery values at 50 g CO_2/g sample becomes the criterion for process efficiency. In this way, extraction conditions which maximize extraction rates and recoveries are determined without extracting to exhaustion. Based on these criteria, optimum temperature and pressure were 40°C and 36MPa and recovery was improved by the inclusion of entrainer, with greater recoveries obtained with higher entrainer concentration (5% methanol) and the more polar entrainer (ethanol).

4.4 CHEMICAL ANALYSIS OF EXTRACTS

The proximate composition of the freeze-dried egg material used in this study was 63.8% total lipid, 31.6% protein and 4.4% moisture. The composition of egg yolk lipid was 62.03% trigylcerides, 24.67% phosphatidylcholine and 4.99% cholesterol. These values are similar to those reported previously (65.5% triglycerides, 28.3% phospholipids, and 5.2% cholesterol; Privett *et al.*, 1962). SC CO_2 extraction of freeze-

dried egg yolk yielded an odorless, oily, viscous material. The first few fractions collected during an extraction run were pale yellow in appearance to the human eye. The yellow color intensified gradually during the progression of a run in subsequent fractions. The final fraction collected was perceived to be a deep yellow color resembling the color of the original yolk. The naturally occuring pigments in chicken egg yolk responsible for the yellow color are carotenoids, mainly including lipid-soluble lutein and zeaxanthin and alcohol-soluble xanthophylls (Baldwin, 1977). Several researchers (Favati *et al.*, 1988; Yamaguchi *et al.*, 1986) have documented the coextraction, recovery and isolation of carotenoids from natural products using SC CO_2 as the extractant (10 - 70 MPa ; 40 - 80°C).

4.4.1 Triglycerides

4.4.1.1 Effect of temperature, pressure, and entrainer on solubility

Examination of the results in Table 4.2 for egg triglycerides extracted at various pressures, temperatures and entrainer levels, in conjunction with the solubility values for egg lipids, indicate that the triglyceride solubility values correspond very closely to overall lipid solubility. Since triglycerides make up abut 62% of egg yolk lipids, this was to be expected unless significant separation / fractionation was occurring.

The neutral lipid fraction of egg yolk includes glycerides, sterol esters, carotenoid pigments, free fatty acids, cholesterol and vitamin alcohols. In this study, this fraction has been obtained using SC CO_2. The entrained SC CO_2 extracts included both the neutral and polar fractions. In this study, separation of neutral lipids into the various classes was not attempted with the analysis procedures employed. Nor was there an attempt to separate the polar lipids from the neutral lipids. For example, preparative TLC can be used to separate total neutral lipids to the solvent front and leave the polar lipids near the origin. Next, separation of the neutral lipid fraction into the classes of compounds mentioned above can be accomplished by chromatography on a column of silicic acid or by

TABLE 4.2: SOLUBILITY OF TRIGLYCERIDES AS AFFECTED BY EXTRACTION PRESSURE, TEMPERATURE AND USE OF ENTRAINER

Temperature °C	Pressure MPa	Entrainer conc. wt basis	Triglyceride solubility mg/g CO_2	Egg lipid solubility mg/g CO_2	TG solubility as a percentage of egg lipid solubility %
40	15	-	0.60 [a]	0.67 [a]	89.5
40	20	-	2.26 ± 0.11 [b]	2.47 ± 0.20 [b]	91.5
40	36	-	9.31± 0.74 [c]	10.46 ± 1.09 [c]	89.0
55	36	-	8.02 ± 0.21 [c]	8.99 ± 1.02 [c]	89.2
75	36	-	5.50 ± 0.74 [d]	6.16 ± 0.53 [d]	88.7
40	36	3 % MeOH	17.64 ± 0.38 [e]	19.7 ± 1.41 [e]	89.5
40	36	3 % EtOH	19.87 ± 2.5 [e]	22.1 ± 1.90 [e]	89.9
40	36	5 % MeOH	38.91 ± 0.43 [f]	44.4 ± 1.01 [f]	87.6

Means with different subscripts in the same column are significantly different ($P< 0.05$).

preparative TLC. In this study, triglycerides were first saponified and the resulting fatty acids converted to their methyl esters. Since there was no attempt to separate the neutral lipids into their various classes or to isolate the polar lipids, cleavage of fatty acids from all classes, including cholesterol esters and phospholipids was inevitable. Increased apparent triglyceride solubility with the use of the alcohol entrainers may be attributed to the contribution of fatty acids from other lipid classes including the polar lipids.

4.4.1.2 Fatty acid composition of CO_2 extracts

4.4.1.2.1 Fatty acid compostion of sequential CO_2 extracts

The fatty acid composition of SC CO_2 extracted egg lipid was determined as a function of extraction time. Samples were collected throughout the duration of the runs and analyzed independently for fatty acid composition. An example of the timing of sample collection is provided (Figure 4.5) In this extraction curve (20 MPa and 40°C), seventeen consecutive extract fractions were collected including early, intermediate and late fractions. In Figure 4.6, the fatty acid ester composition of each extract is shown. As can be seen, only small variations occur in the lipid composition during the extraction.

The various triglycerides that make up egg yolk lipid contain both saturated and unsaturated fatty acid moieties ranging from C16 to C22. Mixed triglycerides, formed from glycerol and molecules such as stearic acid, oleic acid, and linoleic acid, comprise most of the triglyceride fraction in egg yolk lipids. Simple triglycerides, for example tristearin, in which all fatty acid molecules are identical, are atypical and comprise only minute fractions of the egg yolk lipid. The triglycerides found in the egg yolk lipid exhibit similar solubility behaviors in SC CO_2. Due to the random distribution of the fatty acid moieties on the triglyceride molecules, the mixture of triglycerides' physical properites would not be expected to differ greatly and hence, they are extracted at the same rate and non-selectively; manipulation of the extraction parameters of temperature, pressure or the

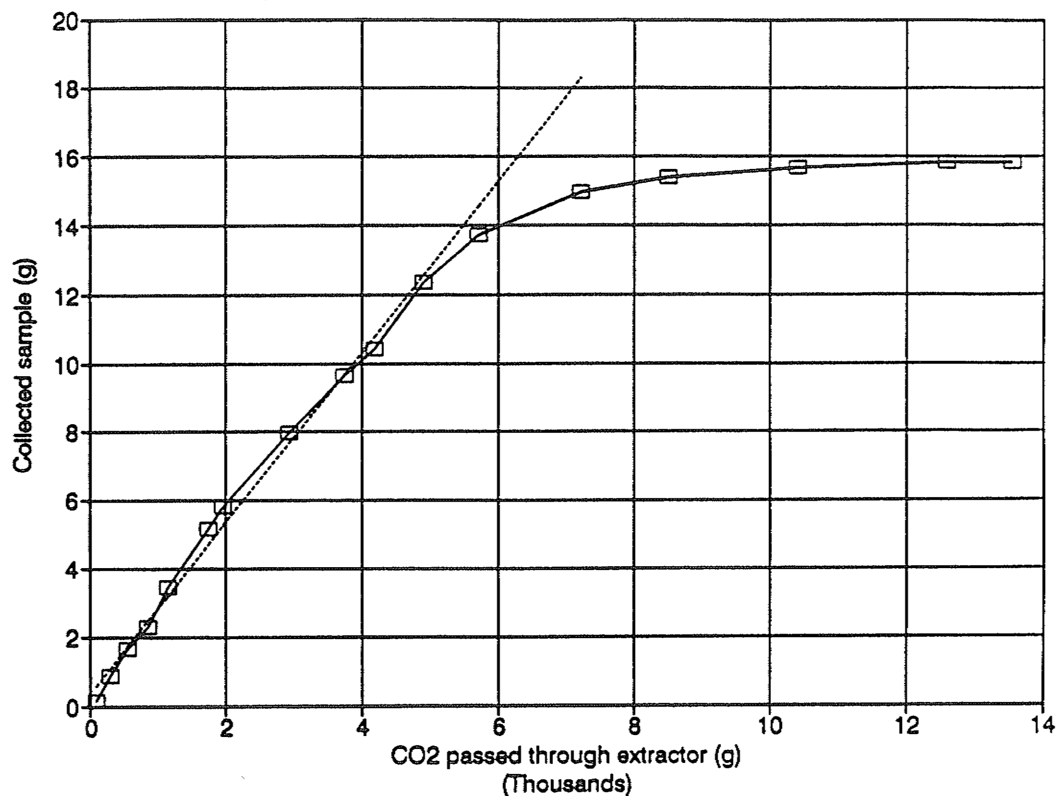


FIGURE 4.5: *Extraction curve for 39.7 g sample of freeze-dried egg yolk. The extraction was carried out at a pressure of 20 MPa and at a temperature of 40 ℃; the CO_2 flowrate was maintained at 2.0 g/minute. The data points on the curve represent discrete intervals over which lipid samples are collected for fatty acid anaysis. Seventeen consecutive extract fractions were collected.*

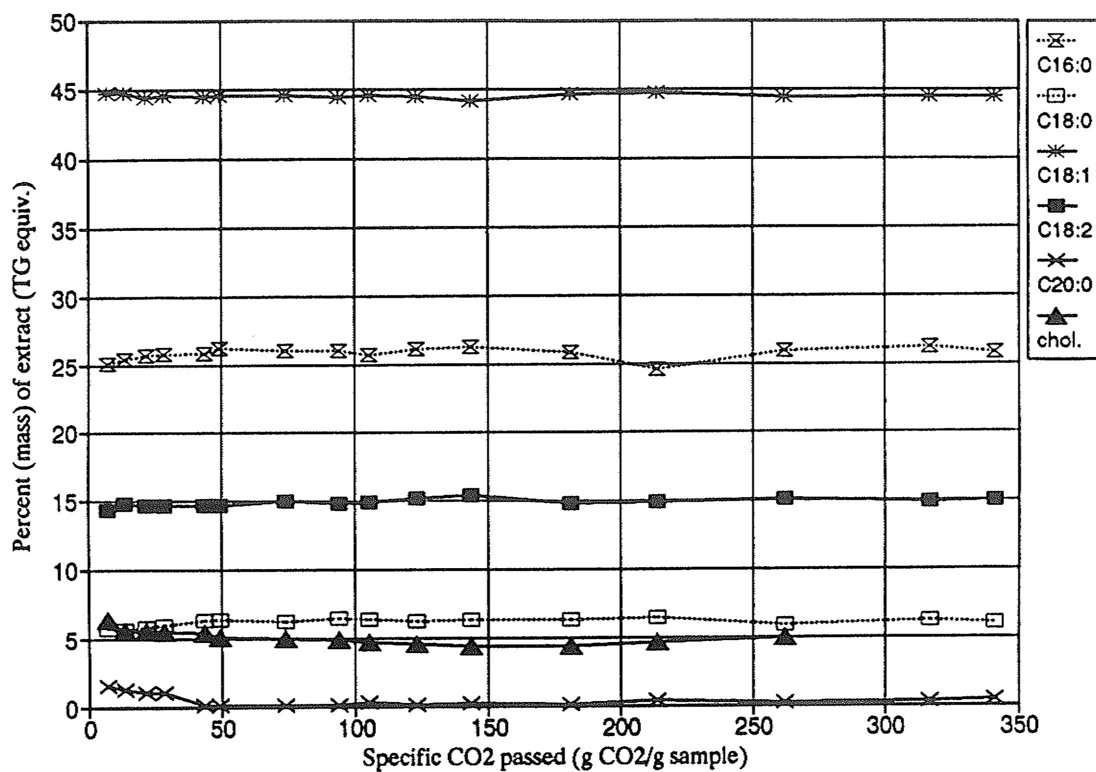


FIGURE 4.6: *Fatty acid composition (mass percent of extract) of sequential CO_2 extracts of freeze-dried egg yolk. Extract fractions correspond to those represented in Figure 4.5 except that data values at the point of origin have been omitted.*

inclusion of an entrainer will not enhance the extraction/fractionation of specific fatty acids Consequently, the composition of the extract remains constant throughout the run.

4.4.1.2.2 Effect of temperature, pressure and use of entrainer on fatty acid composition

The fatty acid compositions of the egg lipids extracted with SC CO_2 at the various extraction conditions studied are tabulated in Table 4.3. Individual fatty acid concentrations were unaffected by extraction parameters. These findings are similar to those reported for the CO_2 extraction of lipids from antarctic krill (Yamaguchi *et. al.*, 1986). It was reported that no appreciable differences were found in the fatty acid compositions of the oils extracted under various extraction pressures and temperatures. These results indicate that the fatty acids are distributed randomly throughout the triglycerides and that manipulation of the extraction parameters of temperature, pressure or the inclusion of an entrainer will not enhance the extraction/fractionation of specific fatty acids.

A mixture of compounds differing in physical properties, such as milk fat triglycerides, have been fractionated by varying the solvent power of supercritical carbon dioxide. Differences in molecular weight, melting temperature (molecular weight and entropy of fusion), volatility and intermolecular interaction energy of constituent triglycerides found in milk fat, have provided the physical property basis for separation of milk fat triglycerides using SC CO_2. Arul and coworkers (1987) using SC CO_2, fractionated milk fat into 8 fractions at temperatures of 50°C and 70°C over a pressure range of 100 - 350 bar. The peak melting temperature progressively increased (9.7 to 38.3°C) from fraction one to eight. The concentration of short chain (C24 - C34) triglycerides decreased from the first fractions to the final ones, while that of long chain (C42 - C54) increased gradually. The authors summarize that SC CO_2 fractionation offers

TABLE 4.3 EFFECT OF TEMPERATURE, PRESSURE, AND USE OF ENTRAINER ON FATTY ACID COMPOSITION

Temp. °C	Pressure MPa	Entrainer concentration. % wt basis	Fatty acid composition Percent of recovered sample [1] %				
			C16:0	C18:0	C18:1	C18:2	C20:0
40	36	-	23.9	5.5	45.1	15.0	0.6
40	20	-	24.4	6.1	44.7	15.3	0.7
40	15	-	-	-	-	-	-
55	36	-	24.6	5.2	44.2	15.9	0.2
75	36	-	24.0	6.5	46.0	16.8	1.0
40	36	3 % MeOH	26.3	6.7	45.1	15.3	0.5
40	36	3 % EtOH	23.9	5.8	44.8	16.7	0.3
40	36	5 % MeOH	25.1	5.8	44.6	15.7	0.6
F.A. composition of the lipid fraction of freeze-dried egg yolk (%)			24.2	7.1	46.1	15.4	0.3

[1] Represent mean values obtained during the course of a run for replicate trials.

an excellent means of obtaining milk fat fractions with distinctive differences in chemical and physical properties which could satisfy the requirements of many food applications including refridgerator-spreadable butter. Shishikura *et al.*, (1986) successfully fractionated triglycerides found in butter oil according to their carbon numbers; cholesterol was not isolated from the triglycerides by SC CO_2 extraction. It was noted that the polarity of cholesterol is higher than that of triglycerides, while the molecular weight of cholesterol is almost equivalent to that of triglycerides with carbon numbers 24. As a result of the combination of these two factors, cholesterol was extracted in a manner similar to the major triglycerides of butter oil with carbon numbers 38 and 40.

Solubilities of simple triglycerides in SC CO_2 have also been the studied (Cheok, 1984). Unsaturated triglycerides were more soluble than their saturated counterparts. For the C18 triglycerides studied, the presence of one double bond on each of the fatty acid chains caused a significant increase in the solubility of the triglyceride in CO_2. The addition of a second double bond to each of the fatty acid chains of the triglyceride showed no further increase in solubility. For the saturated triglycerides, a linear relationship existed between molecular weights of a saturated triglycerides and the logarithm of their solubilites in CO_2.

4.4.2 Cholesterol

4.4.2.1 Effect of temperature, pressure, and entrainer on solubility and recovery

The solubilities and recoveries of cholesterol from freeze-dried egg yolk obtained at different extraction conditions are reported in Table 4.4. An increase in pressure from 15 MPa to 36 MPa, keeping all other conditions the same, resulted in a substantial increase in cholesterol solubility due to decreased sterol solubility at the lower pressures. At a fixed temperature of 40°C, the solubility at 15 MPa was less than 0.001 mg/g CO_2, while that at 36 MPa reached 0.420 mg/g CO_2. This pressure effect on lipid

TABLE 4.4 SOLUBILITY AND RECOVERY OF CHOLESTEROL AS AFFECTED BY EXTRACTION PRESSURE, TEMPERATURE AND USE OF ENTRAINER

Temp. °C	Pressure MPa	Entrainer conc. % wt basis	Solubility mg/g CO_2	Percent theoretical recoverved after 50gCO_2/g sample %[1]	Cholesterol solubility as a percent of egg lipid solubility
40	36	-	0.420 ± 0.011^a	51.9 ± 0.55^a	4.0
40	20	-	0.162 ± 0.004^b	26.6 ± 0.21^b	6.5
40	15	-	0.001 * [c]	-	-
55	36	-	0.491 ± 0.023^d	57.0 ± 0.78^c	5.5
75	36	-	0.390 ± 0.001^e	52.0 ± 0.75^a	6.3
40	36	3 % MeOH	0.815 ± 0.098^f	63.5 ± 0.72^d	2.9
40	36	3 % EtOH	0.648 ± 0.001^g	48.8 ± 0.22^a	4.2
40	36	5 % MeOH	1.327 ± 0.015^h	76.5 ± 0.14^e	3.0

[1] Recovery = [extracted cholesterol / total theoretical cholesterol] x 100

Means with different subscripts in the same column are significantly different ($P < 0.05$)

* The minimum amount of cholesterol determined by the above procedure was found to be 0.001mg.

solubility has been well-documented by other researchers using SC CO_2 for the removal of oils and sterols from various biological materials (Ikushima, 1986; Shishikura *et al.*, 1986; Friedrich *et al.*, 1982).

Cholesterol solubility appeared to increase only marginally with a temperature increase of 40°C to 55°C at a fixed pressure of 36 MPa. Subsequently, a further increase in the extraction temperature to 75 °C resulted in a decrease in its solubility. The expected increase in cholesterol solubility with increase in temperature would probably be observed at higher pressures; but at this pressure (36 MPa) the increased solubility effect due to temperature may have been overcome by the decrease in density and related decrease in solute holding power.

The solubility of cholesterol in SC CO_2 increased substantially with the addition of an alcohol entrainer at the fixed extraction conditions of 40°C and 36 MPa. Without an added entrainer, cholesterol solubility is 0.420 mg/g CO_2. Using SC CO_2 entrained with 3 wt % methanol resulted in a two-fold solubility increase compared to its unentrained counterpart. Increasing the methanol concentration to 5 wt % resulted in a further solubility increase (1.327 mg/g CO_2). Though the inclusion of ethanol as an entrainer increased cholesterol solubility, it was to a lesser degree. Solubility values may be slightly inflated due to contaimination of entrainer in the sample.

The cholesterol molecule bears a polar functional group, a hydroxyl group, on carbon 3 (Figure 2.2); the presence of the OH group increases the hydrophilicity of the sterol and consequently its solubility in polar solvents. This solubility in polar solvents is directly attributable to hydrogen bonding between the hydroxyl groups. As described earlier, carbon dioxide exhibits properties typical of hydrocarbon solvents (Hyatt, 1984). Hyatt defines CO_2 in the supercritical state as a "hydrocarbon" solvent with unusual properties (infinite compressibility, low surface tension and viscosity, low polarizability, and ease of solute recovery). While supercritical carbon dioxide has many desirable

properties, its polarizability is less than that of all of the hydrocarbons except methane (Dobbs *et al.*, 1986). Consequently, the addition of small amounts of polar solvents has been used to enhance the polarizability and therefore the strength of supercritical solvent, while maintaining the sensitivity of the solubility with respect to temperature and pressure. Larson and King (1986) demonstrated that the solubility of several steroids in supercritical CO_2 were greatly improved with the aid of polar co-solvents. For example, efrotomycin, a steroid of pharmaceutical interest, was found to increase by a factor of nearly five in SC CO_2 entrained with acetone. Extracting mevinolin, an anti-cholesterol drug, with SC CO_2 entrained with 5 wt % methanol increased its solubility one order of magnitude. Dobbs and co-workers (1986) studied the effects of nonpolar co-solvents on the solubility behavior of nonpolar solutes. They demonstrated that the solubilities of the solids were increased more by the presence of several mole concentrations of co-solvent than by changes in the pressure of several hundred bar. For example, an increase in the pressure from 150 to 350 bar raised the solubility of phenanthrene by a factor of 1.7 in pure CO_2; the solubility was increased by a factor of 3.6 for SC CO_2 doped with 3.5% undecane.

The addition of polar cosolvents can enhance the solubility of the solute through preferential intermolecular forces such as hydrogen bonding (Larson and King, 1986). However, as the complexity of the molecule increases, that is, molecular weight, number of polar functional groups, charged functional groups, the effect of the entrainer becomes more ambigious (Dobbs *et al.*, 1986) and more so in biological matrices. Hardardottir and Kinsella (1988) using an ethanol entrained SC solvent system to extract lipids from fish muscle attributed increased lipid solubility to the dissociation of phospholipid-protein complexes. In egg yolk, using a SC CO_2 extractant enriched with an organic solvent, e.g.methanol, may serve to disrupt the lipoprotein complexes to which cholesterol and its esters are intimately bound.

Table 4.4 shows the recovery for cholesterol obtained at different extraction pressures, temperatures and entrainer concentrations. When the temperature was increased from 40 to 55 to 75°C at a fixed pressure of 36 MPa, percent recovered cholesterol remained almost constant. Increasing the gas pressure to 36 MPa resulted in a significant increase in the amount of cholesterol extracted with respect to total gas volume passed through the extractor. At this level of compression, there was a two-fold increase in the amount of cholesterol removed from the egg material after the passage of 50 grams of carbon dioxide per gram of sample. That is, when the extractor was operated at 20 MPa, passing the same quantity of dense gas recovered only 27% of the theoretical cholesterol available (Figure 4.7). At 36 MPa, over 50% of the theoretical cholesterol available was recovered (Figure 4.8). Cholesterol recovery from the egg yolk samples dropped to negligible quantities when extracted at 15 MPa and 40 °C.

After passing 50 grams of pure CO_2 per gram of sample (40°C, 36 MPa), 51.9% of the total theoretical cholesterol in the egg sample was extracted; 56.9% of the egg lipid was also extracted at that point. The passage of the same volume of SC CO_2 entrained with 3 wt % methanol, recovered 63.5% of the total theoretical cholesterol; 67.4% of the egg lipid was also extracted at that point. Even higher yields of cholesterol were recorded when the methanol concentration was increased to 5 wt %. The higher concentration of methanol recovered over 75% of the total theoretical cholesterol with over 95% of the egg lipid recovered at that point. Using a SC CO_2 - ethanol solvent medium yielded 48.8% cholesterol with the passage of 50 grams CO_2 g per gram sample. At this point, 82.7% of the egg lipid was also extracted. With the operation of the extraction system at 40°C and 36 MPa, the entrained solvent systems increased cholesterol recovery with respect to its unentrained counterpart. This effect is not evident with the use of the ethanol entrained system.

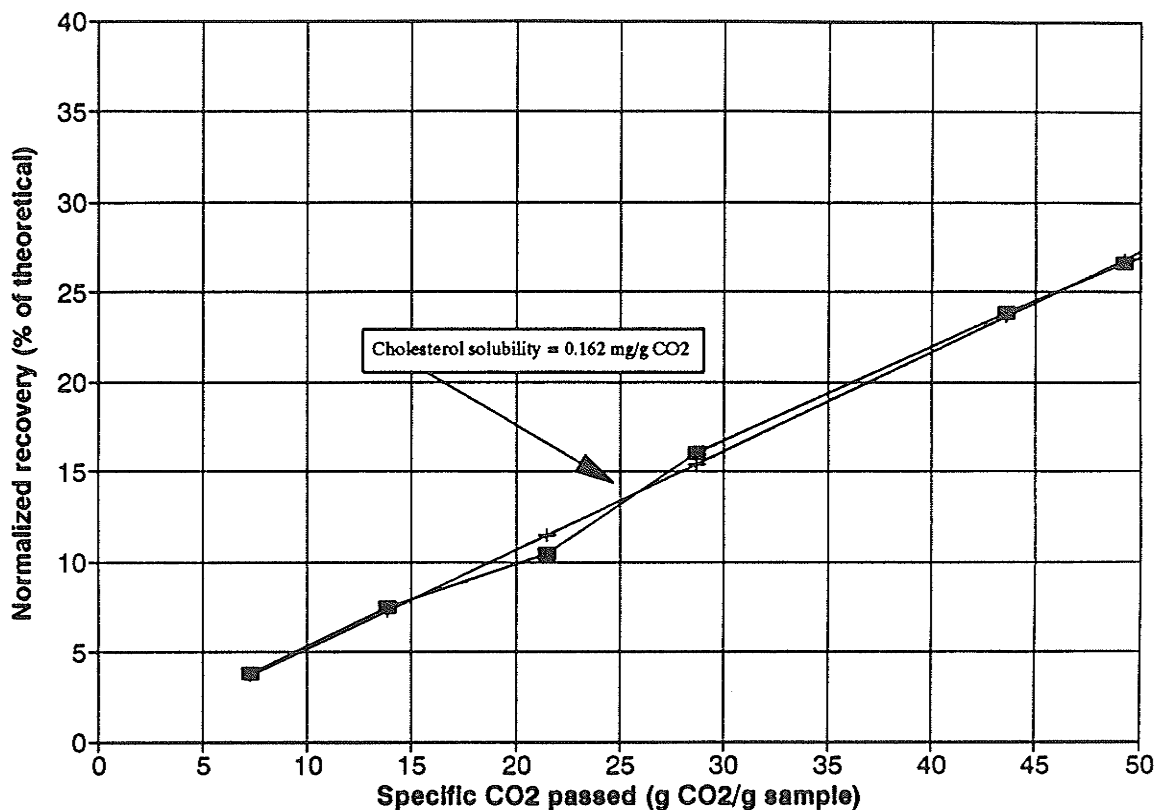


FIGURE 4.7: *Extraction curve (20MPa, 40 ℃) for cholesterol in which horizontal and vertical axes have been normalized by sample mass (~40g). Cholesterol recovery is plotted as a function of consumed CO_2 and is expressed as a percentage of theoretical cholesterol content of egg yolk.*

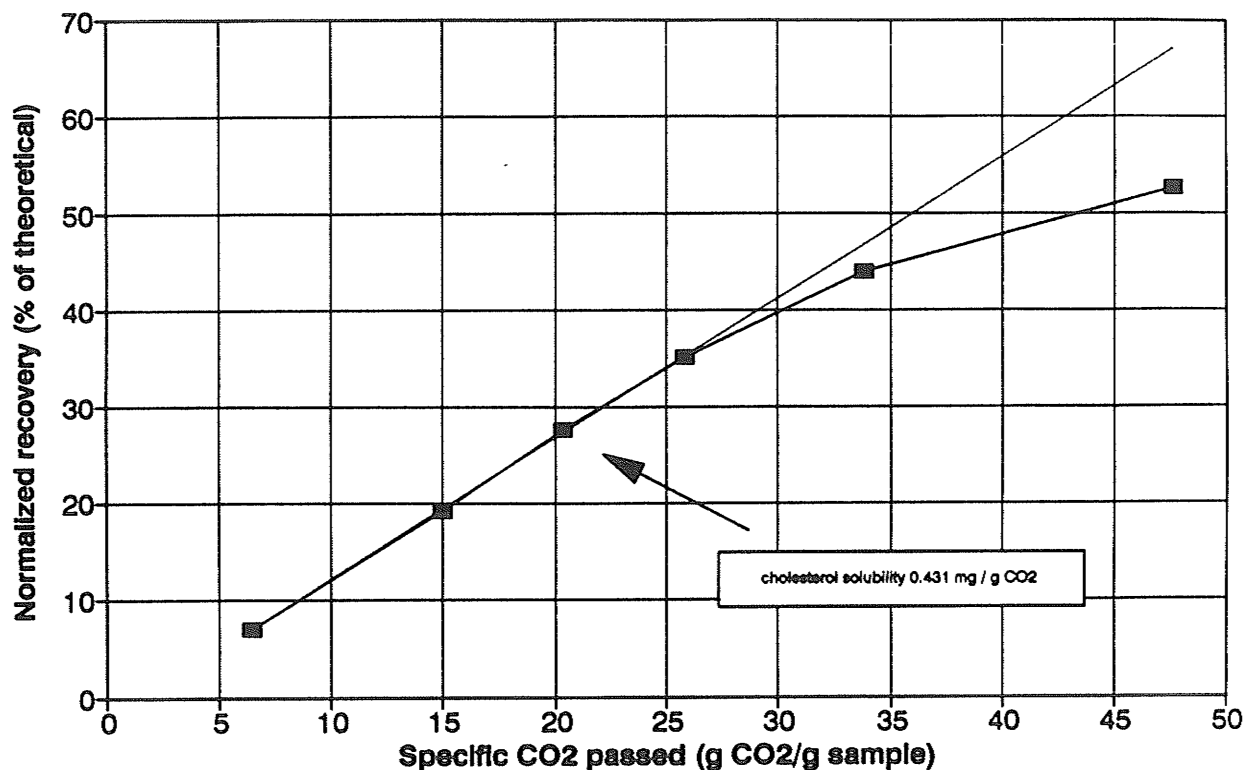


FIGURE 4.8: *Extraction curve (36 MPa, 40 °C) for cholesterol in which horizontal and vertical axes have been normalized by sample mass (~40g). Cholesterol recovery is plotted as a function of consumed CO_2 and is expressed as a percentage of the cholesterol content of egg yolk.*

4.4.2.2 Effect of extraction time

The relative concentration of triglycerides, as represented by fatty acid values, to cholesterol was constant throughout the run. This relationship was independent of the pressures and temperatures examined and entrainer treatments applied (Figure 4.6). The cholesterol content ranged between 2.9 and 6.5% of the recovered extract (Table 4.4). Cholesterol content as a percent of egg lipid extract appeared to be affected by both temperatures and pressure. The results indicate that with increasing temperature there was a corresponding increase in the relative concentration of cholesterol in the extract. Although the absolute cholesterol content only increased by 1 or 2 percent, the relative percent change is significant and could represent as much as a 50% increase in cholesterol concentration with a temperature increase from 40°C to 75°C. This study suggested a similar relationship with decreasing extraction pressure. It is noted that the manipulation of the extraction parameters of pressure or temperature *may* enhance the extraction/fractionation of cholesterol. Cholesterol is largely removed with the other egg lipids and this trend must be looked on with care. Further experimentation is recommeded before a final relationship can be stated. Earlier work by Levi and Sims (1988) support the selective isolation of cholesterol. To selectively remove cholesterol from extracted lipids several researchers have incorporated a physical method to complement SC CO_2 extraction. Shishikura and coworkers (1986) after finding that cholesterol was extracted with the major triglycerides (C38 and C40) in butter oil with SC CO_2, used a silica gel to further fractionate the extract and selectively remove cholesterol.

4.4.3 Phospholipid recovery

The phospholipid data recorded in Table 4.5 was obtained from the enzymatic analysis of the SC CO_2 treated egg yolk material. In addition to this work, Iatroscan TLC/FID analysis was also performed on the egg lipid extract. However, the

TABLE 4.5: LECITHIN CONTENT IN LIPIDS EXTRACTED FROM FREEZE-DRIED EGG YOLK WITH PURE SC-CO_2 AND WITH ALCOHOL ENTRAINED SC-CO_2

Pressure (MPa)	Temperature (°C)	Entrainer concentration (% wt basis)	Lecithin content in lipid extract (% dry weight basis)
40	36	-	0.2
40	36	3 % MeOH	6.8
40	36	3 % EtOH	8.8
40	36	5 % MeOH	17.0
Freeze-dried egg yolk			15.8

values obtained with the Iatroscan were not reproducible as values for standards were inaccurate and unreliable. It is recommended that those researchers considering this methodology for egg lipid studies review previous Iatroscan literature (Christie, 1982).

Phospholipids represent approximately 30% of the total lipids found in egg yolk solids of which lecithin (PC) makes up 79% (Figure 2.2). The lecithin content of 15.8% of the egg yolk solids obtained in the present study compares to the expected lecithin content of 16.7% in egg yolk. No significant amounts of these polar lipids were found in the pure SC CO_2 extracts for samples collected at different extraction temperatures and pressures and at either early or late stages in the extraction. Supercritical carbon dioxide extraction of freeze-dried egg material yielded oils that were composed solely of nonpolar lipids, largely triglycerides with lecithin concentrations < 0.2 %.

SC CO_2 preferably extracted the triglycerides, whereas the alcohol entrained SC CO_2 extracted both triglycerides and phospholipids. In fractions that were collected at the onset of an entrained extraction run, lecithin concentrations of $< 0.2\%$ were found. Appreciable concentrations of lecithin were found in the samples collected near the end of an extraction run (i.e. after the passage of approximately 60 g CO_2/g sample) when an entrainer was present (Table 4.5). The concentrations ranged from 6.8 to 8.8% when 3% entrainer was used to 17% when 5% methanol was used. Evidence that fractionation is occurring is shown by the increasing concentrations of lecithin detected in the later fractions. These results should be considered very preliminary since problems related to the analysis due to contamination of the samples with residual entrainer were experienced. The results do show that when the lipid pool was reduced and the SC CO_2 was no longer saturated with lipid, the CO_2 with entrainer will begin to pick up phospholipid in significant quanitities.

As described earlier, phospholipids are composed of one unit of glycerol, two units of fatty acids, and a phosphate group esterified to an organic acid or alcohol.

Because the phosphate group has a marked tendency to lose a hydrogen ion, one of the oxygens becomes negatively charged. Similarly, the amino group tends to attract a hydrogen ion and thus to become positively charged. In short, the end of the phospholipid molecule with the phosphate and nitrogenous group is strongly polar and hence soluble in polar extractants, whereas, the other end, composed of the two long hydrocarbon tails of the fatty acids is nonpolar and insoluble and makes phospholipids especially well suited to a SC-CO_2 solvent system modified with a polar entrainer. The sparing solubility of phospholipids in SC-CO_2 has been well documented (Friedrich *et al.*, 1982; Friedrich and Pryde, 1984; Froning *et al.*, 1990; Yamaguchi *et al.*, 1986; Hardardottir and Kinsella, 1988; List *et al.*, 1984a; Fattori *et al.*, 1987). Sunol *et al.* (1985) have demonstrated that polar compounds such as esters, phenolics, amines, nitrated aromatics can be rendered more soluble in a polar-enriched SC-CO_2 system.

4.5 CHEMICAL ANALYSIS OF RESIDUE

A crude characterization of the residual proteinaceous matter left after extraction with the designated treatments is recorded in Table 4.6. There was an appreciable drop in the fat content of the residual raffinate over that found in the original sample for all treatments. The removal of these lipid components conversely affected the protein level of the residual product, raising the crude protein from the original 31.06 to 66.47 after extraction with 5 wt % methanol.

As mentioned previously, the egg yolk has a very high quality protein while the fatty acid composition is highly desireable, for example, for infant formula (Tokarska and Clandinin, 1986). The effect of supercritical carbon dioxide on foods of high protein content has been the subject of recent studies (Weder, 1980; Weder, 1984; Eldridge *et al.*, 1986; Christianson *et al.*, 1984; Stahl *et al.*, 1984). Proteins, amino acids and amines are known to react with carbon dioxide under physiological and/or very specialized

TABLE 4.6: PROXIMATE COMPOSITION OF UNTREATED AND RESIDUAL EGG YOLK (DRY BASIS) AT DIFFERENT EXTRACTION CONDITIONS

		Extraction Conditions (40°C; 36 MPa)			
	Untreated freeze dried egg yolk	No entrainer	3 wt% MeOH	3 wt% EtOH	5 wt% MeOH
Crude fat (%)	63.81	40.73	38.75	32.36	22.13
Crude protein (N x 6.25)(%)	31.60	52.70	54.05	59.04	66.47
Moisture (%)	4.42	4.51	5.29	4.96	5.64

experimental conditions (Weder, 1980). Weder (1980), using ribonuclease as a model protein system, found only minor alterations such as unfolding of the protein molecule and some oligomerization and fragmentation when extracted under supercritical conditions. Lysozyme, treated with humid supercritical carbon dioxide (300 bar and 80°C), demonstrated similar results (Weder, 1984). The author summarizes that similar protein alterations were caused by heating proteins in the presence of water; the alterations would not negatively influence nutritional quality under the reaction conditions used in SC CO_2 (Weder, 1984). It should be noted that Weder addresses the nutritional quality of the protein and has omitted discussion on the functional quality of the protein. The functional quality of proteins can be affected by unfolding. Similarly, treatment of oilseeds with supercritical carbon dioxide had no deleterious effects on the protein quality of the oilseed meal (Stahl *et al.*, 1984). Full-fat soybean flakes were extracted with supercritical carbon dioxide at pressures ranging from 10,600-12,400 psi, at temperatures of 80 - 100°C and at moisture levels of 5 - 13 (Eldridge *et al.*, 1986). The remaining protein meal possessed high protein solubility. Contrary to these studies, other researchers have shown denaturing effects of SC CO_2 on protein quality (Christianson *et al.*, 1984), including the reduction of enzymatic activity (Friedrich and Pryde, 1984).

Though the effects of SC CO_2 on the residual proteinaceous material has not been determined in the present study, it warrants further consideration.

5. *Conclusions and Recommendations*

5.1 CONCLUSIONS

This study has demonstrated that supercritical carbon dioxide, under the appropriate conditions, can be an effective solvent for extracting lipid components from egg yolk. The findings of this study can be summarized as follows:

1. The solubility of egg yolk lipid in supercritical carbon dioxide is a direct function of the CO_2 density and the extraction temperature. Results confirm previous lipid studies that solubility increases with increasing pressure at constant temperature. The solubility of egg yolk lipid remained constant with increasing temperature from 40°C to 55°C at constant density. The solubility of egg yolk lipid decreases when extracted at 75°C. Over the range of pressures and temperatures studied, the maximum solubility of egg yolk lipid in CO_2 was observed at 36 MPa and 40°C.

2. The overall solubility of egg lipids in SC CO_2 increases with the addition of a polar entrainer. Increasing the percent entrainer concentration from 3% to 5% methanol increases the overall solubility of the egg lipid. At 3 wt percent concentrations of either methanol or ethanol, the solubilities of egg lipid in entrained CO_2 are not significantly different.

3. The overall percentage of egg lipid recoverable from freeze-dried egg yolk is independent of extraction pressure and temperature over the range of pressures and temperatures tested in this study. Greater percent recoveries of total lipid when run to exhaustion were observed when higher entrainer concentrations were used or when using the co-solvent ethanol with its higher polarity.

4. Triglyceride as a percent of egg lipid recovered is independent of pressure and temperature and the addition of entrainers. The relative concentration of cholesterol as a percent of egg yolk lipid was found to be affected by the extraction parameters. An increase in temperature increases the relative concentration of cholesterol in the extract. Likewise, a decrease in pressure increases the relative concentration of cholesterol in the extract. The manipulation of the extraction parameters of temperature and pressure may enhance the fractionation of cholesterol.

5. The apparent solubility of cholesterol is dependent on pressure and the addition of entrainer. An increase in CO_2 density increases the apparent solubility of cholesterol. The effect of a change in the temperature at constant pressure on the apparent solubility of cholesterol was insignificant over the temperatures studied. The apparent solubility of cholesterol was increased more by the presence of 3-5 % concentrations of entrainer than by changes in the pressure of several MPa.

6. The apparent solubility of triglycerides were similar to the trends of overall egg lipid solubility in supercritical carbon dioxide. The addition of 3% methanol or ethanol as an entrainer doubled the triglyceride solubility in SC CO_2. No significant differences in triglyceride solubility were observed for SC CO_2 entrained with 3% methanol compared to to 3% ethanol. The addition of 5% methanol increased the solubility significantly over the extractions at the 3% entrainer concentrations.

7. The fatty acid composition of the lipid extract is independent of temperature, pressure and the addition of entrainer. The fatty acid composition of lipid samples collected during the course of a run does not vary during the extraction.

8. When no entrainer was present, the CO_2 extracted lipid was essentially free from phospholipid content ($< 0.2\%$) at both early and late stages of an extraction trial. No appreciable quantities of phospholipids were detected in the extracts obtained at the onset of an entrained extraction run. However, significant phospholipid levels were found in the entrainer-enhanced SC-CO_2 lipid extracts collected in the latter part of the run.

5.2 RECOMMENDATIONS

The following recommendations are suggested for future research.

1. Lipid analysis procedures must be specialized for the nature of lipids recovered by SC-CO_2. To accurately determine the composition of the extracted lipids, partitioning of the extracted lipids into neutral and polar classes by chromatography on a silica gel column is recommended. Other chromatographic methods which could be further investigated are Iatroscan TLC/FID and HPLC analyses. The properties of these techniques should find wide applications for the rapid identification, purification and quantitiation of lipids in biological samples.

2. Phospholipid analysis of the CO_2 lipid extracts obtained was frustrated by instrumental / analytical difficulties. Extraction trials should further investigate the applications of supercritical fluid extraction technology to obtain purified phospholipids from egg yolk. Studying the solubility behavior of standard phospholipid mixtures in SC-CO_2 with the addition of different entrainers under different temperature and pressure conditions would serve to complement the results obtained from biological matrices.

3. SC-CO_2 sample collection systems which are to be utilized in research employing the use of an entrainer must be specialized rather than general purpose collection systems. They must be able to handle the recovery of large volumes of organic solvent. Likewise, optimizing the recovery of lipids from entrained mixtures can not be over emphasized. Complete removal of the organic solvents from the extracted lipid material would probably improve the accuracy and reliability of the results.

4. Successful fractionation and development of a highly purified phospholipid fraction from egg yolk may be obtained by the re-extraction of the raffinate with an

entrained SC-CO_2 solvent system. Extraction trials should be conducted under various conditions to optimize maximum recovery.

References

American Heart Association: Dietary guidelines for healthy American adults: A statement for physicians and health professionals by the Nutrition Committee. 1988. American Heart Association. *Circulation* **77**, 721A-723A.

Angus, F., Armstrong, B., deReuck, K.M., Shapela, G.A. and Rolinson, J.S. 1976. International Uninion of Pure and Applied Chemistry. *International Thermodynamic Tables of the Fluid State-CO_2*. Pergamon Press, Oxford, England.

Anonymous. 1990. The liposome solution. *The Scientist,* **January 8**.

Arul, J., Boudreau, A., Makhlouf, J., Tardif, R. and Sahasrabudhe, M.R. 1987. Fractionation of anhydrous milk fat by supercritical carbon dioxide. *J. Food Sci.* **52**, 1231-1236.

Association of Official Analytical Chemists. 1984. Official Methods of Analysis. 14th ed. Assoc. Offic. Anal. Chem., Arlington, VA.

Baldwin, R.E. 1986 Functional properties in foods. In *Egg Science and Technology, 3rd edition.* W.J. Stadelman and O.J. Cotterill (eds). AVI Publishing Company, Westport, Connecticut, 247-277.

Bangham, A.D. 1989. The first description of liposomes. Current contents, March 27, **no. 13**, 14.

Bangham, A.D., Standish, M.M. and Watkins, J.C. 1965. Diffussion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **13**, 238-252.

Barenholz, Y. and Yechiel, E. 1989. Phosphatidylcholine liposomes for medical treatment - especially to restore heart lipid compositions and increase respiratory stress resistance. *U.S. Patent 4,812,314.*

Bernardi, G., and Cook, W.H. 1960a. An electrophoretic and ultracentrifugal study on the proteins of the high density fraction of egg yolk. *Biochim. Biophys. Acta* **44**, 86-96.

Bernardi, G., and Cook, W.H. 1960b. Separation and characterization of the two high-density lipoproteins of egg yolk, α– and β–lipovitellin. *Biochim. Biophys. Acta* **44**, 96-105.

Bethuel, L., Carles, M., Neige, R. 1989. Extraction of essential oils, flavourings, perfumes and alkaloids from vegetables and flowers using supercritical extraction carbon dioxide to dissolve out required constituents. *U.S. Patent 4,824,570.*

Beyer, J.D., Milani, F.X., Dutelle, M.J. and Bradley, R.L. 1989. Gas chromatographic determination of cholesterol in egg products. *J. Assoc. Off. Anal. Chem.* **72**, 746-748.

Blackwelder, J.A. and Pike, O.A. 1990. Oxidative stability of cholesterol-free egg lipid fractions. *J. Food Sci.* **55**, 92-94.

Brogle, H. 1982. Carbon dioxide as a solvent - its properties and applications. Carbon Dioxide in Solvent Extraction: Proceedings of SCI Food Group Food Engineering Panel Meeting. London, SCI, London.

Brooks, J., and Taylor, D.J. 1955. Eggs and egg products. *G.B., Dep. Sci. Ind. Res.*, Food Invest. Board, Spec. Rep. **60**.

Brunner, G. 1983. Selectivity of supercritical compounds and entrainers with respect to model substances. *Fluid Phase Equilib.* **10**, 289.

Brunner, G. and Peter, S., 1982. On the solubility of glycerides and fatty acids in compressed gases in the presence of an entrainer. *Sep. Sci. Technol.* **17**, 199.

Bulley, N.R. and Fattori, M.J., Meisen, A. and Moyls, L. 1984. Supercritical fluid extraction of vegetable oil seeds. *J. Amer. Oil Chemists' Soc.* **61**, 1362-1365.

Burley, R.W. 1975. Recent advances in the chemistry of egg yolk. *CSIRO Food Res. Q.* **35**, 1-5.

Burley, R.W. and Cook, W.H. 1961. Isolation and compostion of avian egg yolk granules and their constituent α– and β–lipovitellins. *Can. J. Biochem. Physiol.* **39**, 1295-13307.

Burley R.W. and Kushner, D.J. 1963. The action of *Clostridium perfringens* phosphatidase on the lipovitellins and other yolk constituents. *Can. J. Biochem. Physiol.* **41**, 409-16.

Burley, R.W., Sleigh, R.W., and Fretheim. 1985. Protein-lipid interactions in food: with special reference to egg yolk. *CSIRO Food Res.* **45**, 64-69.

Carey, F.A. 1987. *Organic Chemistry*. McGraw-Hill Book Company, New York, NY.

Cheok, N.T. 1984. The effect of molecular structure and operating conditions on the solubility of triglycerides in supercritical carbon dioxide. M.S. thesis, Univeristy of British Columbia, Vancouver, B.C.

Chrastil, J. 1982. Solubility of solids and liquids in supercritical gases. *J. Phys. Chem.* **86**, 3016-3021.

Christianson, D.D., Friedrich, J.P., List, G.R., Warner, K., Bagley, E.B., Stringfellow, A.C. and Inglett, G.E. 1984. Supercritical fluid extraction of dry-milled corn germ with carbon dioxide. *J. Food Sci.* **49**, 229-232.

Christie, W.W. 1982. The analysis of simple lipid classes. In *Lipid Analysis. Isolation, Separation, Identification and Structural Analysis of Lipids. 2nd Edition.* Pergamon Press, Toronto, 93-106.

Chung, R.A., Rogler, J.C. and Stadelman, W.J. 1965. The effect of dietary cholesterol and different dietary fats on cholesterol content and lipid composition of egg yolk and various body tissues. *Br. Poultry Sci.* **6**, 221-228.

Connor, W.E., and Connor, S.L. 1989. Dietary treatment of familial hypercholesterolemia. *Arteriosclerosis Supplement I* **9**, I-91-I-105.

Consumer and Food Economics Institute. Composition of Foods, Dairy and Egg products, Raw-Processed-Prepared. USDA Agric. Handbook 1976, 8-1, 1-167.

Cook, F. and Briggs, G.M. 1986. Nutritive value of eggs. In *Egg Science and Technology, 3rd edition.* W.J. Stadelman and O.J. Cotterill (eds). AVI Publishing Company, Westport, Connecticut, 92-108.

Cornell University News Service. 1989. Cornell unveils commercial process for low-cholesterol milk. *Food Engineering* **61**, 172-174.

Crapo, P.A. 1988. Use of alternative sweetners in diabetic diet. *Diabetes Care* **11**, 174-182.

Deftos, L.J. and Hostetler, K.Y., 1989. Calcitonin liposome compositions for lowering serum calcium levels by parenteral administration. *U.S. patent 4,837,380.*

Dobbs, J.M. Wong, J.M. and Johnston, K.P. 1986. Nonpolar co-solvents for solubility enhancement in supercritical fluid carbon dioxide. *J. Chem. Eng. Data* **31**, 303-308.

Dobbs, J.M., Wong, J.M., Lahiere, R.J. and Johnston, K.P. 1987. *Ind. Eng. Chem. Res.* **26**, 56-65.

Duncan, D.B. 1955. Multiple range and multiple F tests. *Biometrics* **11**, 1.

Eisai, K.K. 1989. Reproductive agents for nerve fibres - contain natural or synthetic phosphatidylinositol, phosphatidylcholine, phosphatidylserine and/or sphingomyelin - for therapy of brain diseases. *JP Patent 1,135,720.*

Eldridge, A.C., Friedrich, J.P., Warner, K., and Kwolek, W.F. 1986. Preparation and evaluation of supercritical carbon dioxide defatted soybean flakes. *J. Food Sci.* **51**, 584-587.

Fattori, M. J. 1986. Supercritical fluid extraction of canola seed. Ph.D thesis, University of British Columbia, Vancouver, B.C.

Fattori, M., Bulley, N.R. and Meisen, A. 1987. Fatty acid and phosporous contents of canola seed extracts obtained with supercritical carbon dioxide. *J. Agric. Food Chem.* **35**, 739-743.

Favati, F.,. King, J.W., Friedrich, J.P. and Eskins, K. 1988. Supercritical carbon dioxide extraction of carotene and lutein from leaf protein concentrates. *J. Food Sci.* **53**, 1532-1536.

Fevold, H.L. 1951. *Adv. Protein Chem.* **6**, 187-252.

Fletcher, D.L., Britton, W.M. and Cason, J.A. 1984. A comparison of various procedures for determining total yolk lipid content. *Poultry Sci.* **63**, 1759-1763.

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1956. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509.

Francis, A.W. 1954. Ternary systems of liquid carbon dioxide. *J. Phys. Chem.* **58**, 1099-1114.

Friedrich, J.P. and Pryde, E.H. 1984. Supercritical carbon dioxide extraction of lipid-bearing materials and characterization of the products. *J. Amer. Oil Chemists' Soc.* **61**, 223-228.

Friedrich, J.P., List, G.R. and Heakin, A.J. 1982. Petroleum-free extraction of oil from soybeans with supercritical carbon dioxide. *J. Amer. Oil Chemists' Soc.* **59**, 288-292.

Froning, G.W., Wehling, R.L., Cuppett, S.L., Pierce, M.M., Niemann, L. and Seikman, D.K. 1990. Extraction of cholesterol and other lipids from dried egg yolk using supercritical carbon dioxide. *J. Food Sci.* **55**, 95-98.

Gere, D.R. 1983. Supercritical fluid chromatography. *Science* **222**, 253-259.

Goodrum, J.W. and Kilgo, M.B. 1989. Rapeseed oil recovery by carbon dioxide solvent: Recovery kinetics and extraction model. *Transactions of the ASAE* **32**, 727-731.

Gotto, A.M. 1989. Diet and cholesterol guidelines and coronary heart disease. *Journal of the American College of Cardiology* **13**, 503-507.

Grierson, H.L., Irvine, W.A., and Goldsmith, J.C. 1988. Effect of egg lecithin on immune function and clinical status in patients with AIDS-related complex. *Clin. Res.* **36**, 882A.

Hardardottir, I. and Kinsella, J.E. 1988. Extraction of lipid and cholesterol from fish muscle with supercritical fluids. *J. Food Sci.* **53**, 1656-1658, 1661.

Hargis, P.S. 1988. Modifying egg yolk cholesterol in the domestic fowl: A review. *Worlds Poultry Science Journal* **44**, 17.

Hatta, H., Sim, J.S. and Nakai, S. 1988. Separation of phospholipids from egg yolk and recovery of water-soluble proteins. *J. Food Sci.* **53**, 425.

Havel, R.J. 1988. Lowering cholesterol: Rationale, mechanisms, means. *J. Clin. Invest.* **81**, 1653-1660.

Hepburn, F.N., Exler, J., and Weihrauch, J.L. 1986. Provisional tables on the content of omega-3 fatty acids and other fat components of selected foods. *J. Am. Diet. Assoc.* **86**, 788-793.

Hirsch, J. and Ahrens, E.H. 1958. The separation of complex lipid mixtures by the use of silicic acid chromatography. *J. Biol. Chem.* **233**, 311.

Hostetler, K.Y. and Deftos, L.J., 1987. Regulation of blood calcium levels in mammals by oral administration of parathyroid hormone or calcitonin encapsulated in liposomes with cationic amphiphilic agent. *U.S. patent 4,692,433.*

Hubert, P. and Vitzthum, O.F. 1978. Extraction of hops, spices, and tobacco with supercritical gases. *Agnew. Chem. Int. Ed. Engl.* **17**, 710.

Hyatt, J.A. 1984. Liquid and supercritical carbon dioxide as organic solvent. *J. Org. Chem.* **49**, 5097-5101.

Ikushima, Y., Saito, N., Hatakeda, K., Ito, S., Asano, T. and Goto, T. 1986. A supercritical carbon dioxide extraction from mackerel (*Scomber Japonicus*) powder: Experiment and modeling. *Bull. Chem. Soc. Japan* **59**, 3709-3713.

Kamlet, M.J., Abboud, J.M., Abraham, M.H. and Taft, R.W. 1983. *J. Org. Chem.* **48**, 2877. Cited in *Supercritical Fluid Extraction and Chromatography: Techniques and Applications, ACS Symposium Series 366.* B.A. Charpentier and M.R. Sevenants (eds.). 1988. American Chemical Society, Washington, D.C.

Kaufmann, V.W., Biernoth, G., Frede, E., Merk, W., Precht, D., and Timmen, H. 1982. Fraktionierung fon butterfett durch extraktion mit Uberkritischem CO_2. *Milchwissenschaft* **37**, 92.

Krishnan, K.R.R., Heyman, A., Ritchie, J.C., Utley, C.M., Dawson, D.V. and Rogers, H. 1988. Depression in early-onset Alzheimer's disease: Clinical and neuroendocrine correlates. *Biol. Psychiatry* **24**, 937-940.

Krukonis, V.J. and Kurnik, R.T. 1985. Solubility of the solid aromatic isomers in carbon dioxide. *J. Chem. Eng. Data* **30**, 247.

Kuksis, A. 1985. Animal lecithins. In *Lecithin.* B.F. Bernard and G.R. List (eds.). American Oil Chemists' Society, USA.

Larsen, Y.E. and Froning, G.W. 1981. Extraction and processing of various components from egg yolk. *Poultry Sci.* **60**, 160-

Larson, K.A. and King, M.L. 1986. Evaluation of supercritical fluid extraction in the pharmaceutical industry. *Biotechnology Progress* **2**, 73-81.

Lea, C.H. 1962. In *Recent advances in Food Science, Volume 1* , J.B Hawthorn and R. Muil Leitch (eds.). Butterworths, London.

Leatherwood, P.D. 1986. Neurotransmitter precursors: from animal experiments to human applications. *Nutr. Rev.* **44**, 193-204.

Lee, A.A.K., Bulley, N.R., Fattori, M.J., and Meisen, A. 1986. Modelling of supercritical carbon dioxide extraction of canola oilseeds in fixed beds. *J. Amer. Oil Chemists' Soc.* **63**, 921-925.

Legros, F. and Ruysschaer, J.M. 1987. Epidural anaesthetic compositions containing local anaesthetic and / or analgestic encapsulated in liposome. *EP Patent 0233100.*

Lehninger, A.L. 1982. *Principles of Biochemistry.* Worth Publishers, Inc., New York, N.Y.

Leiner, S. 1986. In *Dense gases for Extraction and Refining.* E. Stahl, K.W.Quirin, and D. Gerald (eds.). Springer-Verlag, New York, NY.

Levi, S. and Sim, J.S. 1988. Selective removal of cholesterol from egg yolk products by supercritical carbon dioxide fluid extraction. *Can. Inst. Food Sci. Technol. J.* **21**, 369.

List, G.R., Friedrich, J.P. and Christianson, D.D. 1984a. Properties and processing of corn oils obtained by extraction with supercritical carbon dioxide. *J. Amer. Oil Chemists' Soc.* **61**, 1849-1851.

List, G.R., Friedrich, J.P. and Pominski, J. 1984b. Characterization and processing of cottonseed oil obtained by extraction with supercritical carbon dioxide. *J. Amer. Oil Chem. Soc.* **61**, 1847-1849.

Lopezberes, G., Mehta, R., Hopfer, R.L., and Juliano, R.L. 1989. Liposome-encapsulated mepartricin composition for treating disseminated fungal infections. *WO Patent 8,903,677.*

Mayer, L., Balley, M.B., Cullis, P.R., Ginsberg, R.S. 1988. High drug-lipid compositions of liposome antineoplastic agents containing release inhibiting buffer so the liposome has transmembrane pH gradient. *WO Patent 8,806,442.*

Mayes, P.A. 1988a. Cholesterol Synthesis, Transport, and Excretion. In *Harper's Biochemistry*, 21st edition. R.K. Murray, D.K. Granner, P.A. Mayes, and V.W. Rodwell (eds.). Appleton and Lange, Norwalk, CT., 241-252.

Mayes, P.A. 1988b. Lipids of physiologic significance. In *Harper's Biochemistry*, 21st edition. R.K. Murray, D.K. Granner, P.A. Mayes, and V.W. Rodwell (eds.). Appleton and Lange, Norwalk, CT., 130-141.

McHugh, M.A., Seckner, A.J. and Yogan, T.J. 1984. High pressure phase behavior of octacosame and carbon dioxide. *Ind. Eng. Chem. Fundam.* **23**, 493.

McNally, M.E. P. and Wheeler, J.R. 1988. Increasing extraction efficiency in supercritical fluid extraction from complex matrices. Predicting extraction efficiency of diuron and linuron in supercritical fluid extraction using supercritical fluid chromatographic retention. *J. Chromatogr.* **447**, 53-63.

Mehta, J.L., Lopez, L.M., Lawson, D., Wargovich, T.J., Williams, L.L. 1988. Dietary supplementation with omega-3 polyunsaturated fatty acids in patients with stable coronary heart disease. *Amer. J. Med.* **84**, 45-51.

Metcalfe, L.D., Schmitz, A.A. and Pelka, J.R. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal. Chem.* **38**, 514-515.

Mosby's Medical and Nursing Dictionary, 2nd Edition. 1986. The C.V. Mosby Company, Toronto.

Mountney, G.J. 1976. *Poultry Products Technology*, 2nd ed. AVI Publishing Company, Inc., Westport, Connecticut.

Multiple Risk Factor Intervention Trial Research Group. 1982. Multiple risk factor intervention trial: risk factor changes and mortality results. *JAMA.* **248**, 1465-77.

Naito, H.K. 1988. The cholesterol challenge: From laboratory to clinician. *Clin. Chem. 34,* 444-449.

Newitt, D.E. Pai, M.V., Kuloor, N.R. and Huggill, A.W. 1956. In *Thermodynamic Functions of Gases, Volume 1.* F. Din (ed.). Butterworths Scientific Publications, London, England.

Nilsson, W.B., Gauglitz Jr., E.J., Hudson, J.K., Stout, V.F. and Spinelli, J. 1988. Fractionation of menhaden oil ethyl esters using supercritical fluid carbon dioxide. *J. Amer. Oil Chemists' Soc.* **65**, 109-117.

Nippon Kayaku, K.K. 1989. Preventative and therapeutic agents for ischaemic diseases - liposome containing superoxidedismutase. *JP Patent 1,175,944.*

Nippon Lederle, K.K. 1989. Liposome enclosing antibiotics for listeriosis therapy - comprises liposome containing antibiotic coated with polysaccharide derived from natural sources. *JP Patent 1,197,431.*

Nippon Shinyaku, K.K. 1986. Liposome containing steryl glucoside (monopalmitate) suitable for intravenous injection. *U.S. Patent 4,614,796.*

Parkinson, T.L. 1966. The chemical composition of eggs. *J. Sci. Food Agric.* **17**, 101-111.

Peter, S. and Brunner, G. 1978. The separation of nonvolatile subtances by means of compressed gases in countercurrent processes. *Angew. Chem. Int. Ed. Engl.* **17**, 746-750.

Pike, O.A. and Peng, I.C. 1985. Stability of shell egg and liquid yolk to lipid oxidation. *Poultry Sci.* **64**, 1470-1475.

Polson, A., Von-Wechmar, M.B. and Fazakerley, G. 1980. Antibodies to proteins from yolk of immunized hens with a variety of plant viruses. *Immunol. Commun.* **9**, 495-514.

Privett, O.S., Bland, M.L. and Schmidt, J.A. 1962. Studies on the composition of egg lipid. *J. Food Sci.* **27**, 463-468.

Proctor, V.A. and Cunningham, F.E. 1988. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. *Crit. Rev. Food Sci. Nutr.* **26**, 359.

Prosise, W.E. 1985. Food use of soybean lecithin. In *Lecithin.* B.F. Bernard and G.R. List (eds.). American Oil Chemists' Society, USA., 163-182.

Randall, L. 1982. The present status of dense (supercritical) gas extraction and dense gas chromatography: impetus for DGC/MS development. *Sep. Sci. Tech.* **17**, 1-118.

Redziniak, G. and Meybeck, A. 1985. Hydrated, lipid lamellar phases containing hydrophobic substance prepared by dissolving lipidic amphiphile. *U.S. Patent 4,508,703.*

Rhodes, D.N. and Lea, C,H. 1957. Phospholipids. On the composition of hen's egg phospholipids. *Biochem. J.* **54**, 526-533.

Rizvi, S.S.H., Daniels, J.A., Benado, A.L. and Zollweg, J.A. 1986. Supercritical fluid extraction: Operating principles and food applications. *Food Technol.* **40**, 57-64.

Saari, A., Powrie, W.D., and Fennema, O. 1964. Isolation and characterization of low-density lipoproteins in native egg yolk plasma. *J. Food Sci.* **29**, 307-315.

Saito, A., Martin, W.G. and Cook, W.H. 1965. Changes in the major macromolecular fractions of egg yolk during embryogenesis. *Can. J. Biochem.* **43**, 1755.

SAS. 1982. *SAS User's Guide: Statistics.* SAS Institute Inc., Cary, NC.

Schmidt, J.C. and Orthoefer, F.T. 1985. Nonfood uses of lecithin. In *Lecithin.* B.F. Bernard and G.R. List (eds.). American Oil Chemists' Society, USA., 183-202.

Schmitt, W.J. 1984. Cited in *Supercritical Fluid Extraction and Chromatography: Techniques and Applications, ACS Symposium Series 366.* B.A. Charpentier and M.R. Sevenants (eds.). 1988. American Chemical Society, Washington, D.C.

Scholfield, R.C. 1985. Occurence, structure, compostion and nomenclature. In *Lecithin.* B.F. Bernard and G.R. List (eds.). American Oil Chemists' Society, USA., 1-20.

Schuler, G., Schlierf, G., Wirth, A., Mautner, H.P, Scheurlen, H., Thumm, M., Roth, H., Schwarz, F., Kohlmeier, M., Mehmel, H.C. and Kubler, W. 1988. Low-fat diet and regular, supervised physical exercise in patients with symptomatic coronary artery disease: reduction of stress-induced myocardial ischemia. *Circulation* **77**, 172-181.

Sempos, C., Fulwood, R., Haines, C., Carroll, M., Anda, R., Williamson, D.F., Remington, P. and Cleeman, J. 1989. The prevalence of high blood cholesterol levels amoung adults in the United States. *J. Amer. Med. A* ssoc. **262**, 45-52.

Shimamoto, T., Komachi, Y., Inada, H., Doi, M., Iso, H., Sato, S., Kitamura, A., Iida, M., Konishi, M., Nakanishi, N., Terao, A., Naito, Y. and Kojima, S. 1989. Trends for coronary heart disease and stroke and their risk factors in Japan. *Circulation* **79**, 503-315.

Shimshick, E.J. 1983. Extraction with supercritical CO_2. *Chemtech* **June**, 136-138.

Shishikura, A., Fujimoto, K., Kaneda, T., Arai, K. and Saito, S. 1986. Modification of butter oil by extraction with supercritical carbon dioxide. *Agric. Biol. Chem.* **50**, 1209-1215.

Sim, J.S. 1987. Eggs and cholesterol. *Agric. Bull. Alberta Univ. Fac. Ext.*, University of Alberta, Edmonton, AB. (special edition), 32-35.

Snyder, J.M., Friedrich, J.P. and Christianson, D.D. 1984. Effect of moisture and particle size on the extractability of oils from seeds with supercritical carbon dioxide. *J. Amer. Oil Chemists' Soc.* **61**, 1851-1856.

Stahl, E., Quirin, K.W. and Blagrove, R.J. 1984. Extraction of seed oils with supercritical carbon dioxide: Effect on residual proteins. *J. Agric. Food Chem.* **32**, 938-940.

Stahl, E., Schutz, E. and Mangold, H.K. 1980. Extraction of seed oils with liquid and supercritical carbon dioxide. *J. Agric. Food Chem.* **28**, 1153-1157.

Stone, N.J. and Van Horn, L.V. 1988. Controlling cholesterol levels though diet. *Postgraduate Medicine,* **83**, 22-237.

Sunol, A.K., Hagh, B. and Chen, S. 1985. Entrainer selection in supercritical extraction. In *Supercritical Fluid Technology*. J.M.L. Penninger, M. Radosz and M.A. McHugh (eds). Elsevier Science Publishers, Amsterdam, 451-464.

Sweeley, C.C. 1969. Chromatography on columns of silicic acid. *Meth. Enzymology* **14**, 45.

Swientek, R.J. 1987. Supercritical fluid extraction separates components in foods. *Food Processing* **July**, 32.

Taniguchi, M., Tsuji, T., Shibata, M. and Kobayashi, T. 1985. Extraction of oils from wheat germ with supercritical carbon dioxide. *Agric. Biol. Chem.* **49**, 2367-2372.

The Expert Panel. 1988. Report of the National Cholesterol Education Program Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults. *Arch. Intern. Med.* **148**, 36-69.

Tokarska, B. and Clandinin, M.T. 1985. Extraction of egg yolk oil of reduced cholesterol content. *Can. Inst. Food Sci.Technol.J.* **18**, 256-258.

Tremblay, P.A., Suddith, R.L., and Kearns, J.J. 1988. Lipid composition for preparation of liposomes for drug delivery - containing mostly phosphatidylcholine and small amount of sphingomyelin. *WO Patent 8,803,797.*

Turk, D.E. and Barnett, B.D. 1971. Cholesterol content of market eggs. *Poultry Sci.* **50**, 1303.

Van Alsten, J.G. 1986. Cited in *Supercritical Fluid Extraction and Chromatography: Techniques and Applications, ACS Symposium Series 366*. B.A. Charpentier and M.R. Sevenants (eds.). 1988. American Chemical Society, Washington, D.C.

Van Leer, R.A. and Paulaitis, M.E. 1980. Solubilities of phenol and chlorinated phenols in supercritical carbon dioxide. *J. Chem. Eng. Data* **25**, 257.

Vance, D.E. 1985. Phospholipid metabolism in eucaryotes. In *Biochemistry of Lipids and Membranes.* D.E. Vance and J.E. Vance (eds.). The Benjamin-Cummings Publishing Company, Inc., Menlo Park, CA., 242-255.

Vida, S., Gauthier, L. and Gauthier, S. 1989. Canadian collaborative study of tetrahydroaminoacridine (THA) and lecithin treatment of Alzheimer's disease: effect on mood. *Can. J. Psychiatry* **34**, 165-170.

Vukalovich, M.P. and Altunin, V.V. 1968. *Thermophysical Properties of Carbon Dioxide.* London, Wellingborough, Collets.

Warren, M.W., Brown, H.G. and Davis, D.R. 1988. Solvent extraction of lipid components from egg yolk solids. *J. Amer. Oil Chemists' S oc.* **65**, 1136-1139.

Washburn, K.W. and Nix, N.F. 1974. Genetic basis of yolk cholesterol content. *Poultry Sci.* **53**, 109.

Weder, J.K.P. 1980. Effect of supercritical carbon dioxide on proteins. *Z. Lebensm. Unters. Forsch.* **171**, 95-100.

Weder, J.K.P. 1984. Studies on proteins and amino acids exposed to supercritical carbon dioxide extraction conditions. *Food Chem.* **15**, 175-190.

Williams, D.F. 1981. Extraction with supercritical gases. *Chem. Eng. Sci.* **36**, 1769-1788.

Willson, R.C. 1985. Supercritcial fluid extraction. In *Comprehensive Biotechnology. The Principles, Applications, Regulations of Biotechnology in Industry, Agriculture and Medicine.* C.L. Cooney and A.E. Humphrey (eds.). Pergamon Press, New York, NY., 567-574.

Wong, J.M. and Johnston, K.P. 1986. Solubilization of biomolecules in carbon dioxide based supercritical fluids. *Biotechnology Progress* **2**, 30.

Wood, P.D., Stefanick, M.L., Dreon, D.M., Frey-Hewitt, B., Garay, S.C., Williams, P.T., Superko, H.R., Fortman, S.P., Albers, J.J., Vranizan, K.M., Ellsworth, N.M., Terry, R.B. and Haskell, W.L. 1988. Changes in plasma lipids and lipoproteins in overweight men during weight loss through dieting as compared with exercise. *New England J. Med.* **319**, 1173-1179.

Yagi, K., Kojima, N., and Takano, M. 1987. Adriamycin entrapping liposome preparation for improved antitumor use comprises phosphatidylcholine, cholesterol, and sulphatide in specific molar ratio for increased efficacy and miminal cardiac toxicity. *EP Patent 0226370.*

Yamaguchi, K., Murakami, M., Nakano, H., Konosu, S., Kokura, T., Yamamoto, H., Kosaka, M., Hata, K. 1986. Supercritical carbon dioxide extraction of oils from antarctic krill. *J. Agric. Food Chem.* **34**, 904-907.

Zeisel, S.H. 1985. Lecithin in Health and disease. In *Lecithin.* B.F. Bernard and G.R. List (eds.). American Oil Chemists' Society, USA., 323-345.

APPENDIX A

UV- METHOD FOR THE DETERMINATION OF L-α-LECITHIN IN FOODSTUFFS AND OTHER MATERIALS

(BOEHRINGER-MANNHEIM, MONTREAL, CANADA)

Not for use in *in vitro* diagnostic procedures for clinical diagnosis

biochemical analysis
food analysis

L-α-Lecithin

UV-method

for the determination of L-α-lecithin in foodstuffs and other materials

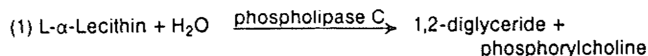
Determination of choline see under Pt. 3

Cat. No. 529 362
Test-Combination for ca. 10 determinations

0
3
5

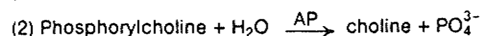
Principle (Ref. 1-3)

L-α-Lecithin is hydrolyzed by the enzyme phospholipase C to a diglyceride and phosphorylcholine at pH 8.0 (1).



(1) L-α-Lecithin + H_2O $\xrightarrow{\text{phospholipase C}}$ 1,2-diglyceride + phosphorylcholine

The phosphorylcholine formed is hydrolyzed by alkaline phosphatase (AP) to choline and inorganic phosphate (2).



(2) Phosphorylcholine + H_2O $\xrightarrow{AP}$ choline + PO_4^{3-}

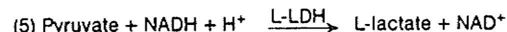
After inactivation of the alkaline phosphatase by heating the assay solution in a boiling water-bath, the formed choline is phosphorylated in the presence of adenosine-5'-triphosphate (ATP) by the enzyme choline kinase to phosphorylcholine (3).

(3) Choline + ATP $\xrightarrow{\text{choline kinase}}$ phosphorylcholine + ADP

The adenosine-5'-diphosphate formed in the above reaction is reconverted by pyruvate kinase (PK) with phosphoenolpyruvate (PEP) into ATP with the formation of pyruvate (4).

(4) ADP + PEP $\xrightarrow{PK}$ ATP + pyruvate

In the presence of the enzyme lactate dehydrogenase (L-LDH) pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the oxidation of NADH to NAD (5).



(5) Pyruvate + NADH + H^+ $\xrightarrow{L\text{-}LDH}$ L-lactate + NAD^+

The amount of NADH oxidized in the above reaction is stoichiometric with the amount of L-α-lecithin. NADH is determined by means of its absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 10 ml solution, consisting of: boric acid/borax buffer, pH 8.0; stabilizers.
2. Bottle 2 with approx. 0.5 ml enzyme suspension, consisting of: phospholipase C, 60 U; alkaline phosphatase, 70 U.
3. Bottle 3 with approx. 90 mg coenzyme/D-glucose mixture, consisting of: NADH, 5 mg; ATP, 20 mg; PEP, 10 mg; D-glucose and stabilizers.
4. Bottle 4 with approx. 0.2 ml enzyme suspension, consisting of: pyruvate kinase, 130 U; lactate dehydrogenase, 130 U.
5. Bottle 5 with approx. 50 mg lyophilisate choline kinase, 1.5 U.

Preparation of solutions

1. Use solution of bottle 1 undiluted.
2. Use suspension of bottle 2 undiluted.
3. Dissolve contents of bottle 3 with 1.5 ml redist. water.
4. Use suspension of bottle 4 undiluted.
5. Dissolve contents of bottle 5 with 0.6 ml solution (1).

Stability of solutions

Solution 1 is stable for 1 year at +4°C.
Bring solution 1 to 37°C before use.
Suspension 2 is stable for 1 year at +4°C.
Solution 3 is stable for 1 week at +4°C.
Suspension 4 is stable for 1 year at +4°C.
Solution 5 is stable for 1 week at +4°C, for 2 weeks at -20°C and for 24 h at room temperature.

Procedure

Wavelength[1]:	340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]:	1 cm light path
Temperature for incubation:	37°C, or 20–25°C
Temperature for measurement:	20–25°C
Final volume:	2.22 ml

Read against air (without a cuvette in the light path), against water or against reagent blank[3].

Sample solution:	10–200 µg L-α-lecithin/cuvette[4] (in 0.1–1.0 ml sample volume)

Pipette into 10 ml glass centrifuge tubes	blank	sample
solution 1 suspension 2 sample solution* redist. water	1.00 ml 0.05 ml - 1.00 ml	1.00 ml 0.05 ml 0.10 ml 0.90 ml
mix, stopper centrifuge tubes[5] and incubate for approx. 20 min at 37°C (or for approx. 50 min at 20–25°C). Keep in a boiling water-bath (95–100 °C) for at least 5 min, allow to cool to 20–25°C and add		
solution 3 suspension 4	0.10 ml 0.02 ml	0.10 ml 0.02 ml
mix thoroughly**, incubate for 10 min, and transfer quantitatively into cuvettes. Read absorbances of reagent blank and sample (A_1). Addition of		
solution 5	0.05 ml	0.05 ml
mix** and incubate for 30 min at 20–25°C. Read absorbances (A_2) of sample and blank immediately one after another. Determine the absorbance differences (A_1–A_2). $\Delta A = (A_1-A_2)_{sample} - (A_1-A_2)_{blank}$		

* Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm® (registered trademark of the American Can Company, Greenwich, Ct., USA).

The absorbance differences measured should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results (see "Instructions for performance of assay").

If the absorbance difference of the sample (ΔA_{sample}) is higher than 0.770 (measured at 340 nm or Hg 334 nm, respectively) or 0.420 (measured at 365 nm), the concentration of L-α-lecithin in the sample solution is too high. The sample is to be diluted according to the dilution table in that case.

Calculation

According to the general equation for calculating the concentrations:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l], where}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ε = absorption coefficient of NADH at:
340 nm = 6.3 [$l \times mmol^{-1} \times cm^{-1}$]
Hg 365 nm = 3.4 [$l \times mmol^{-1} \times cm^{-1}$]
Hg 334 nm = 6.18 [$l \times mmol^{-1} \times cm^{-1}$]

It follows for L-α-lecithin[6]:

$$c = \frac{2.22 \times 733}{\varepsilon \times 1 \times 0.1 \times 1000} \times \Delta A = \frac{16.27}{\varepsilon} \times \Delta A \text{ [g L-α-lecithin/l sample solution]}$$

If the sample has been diluted during preparation the result must be multiplied by the dilution factor F.

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; when spectralline photometers equipped with a mercury vapour lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 For example, when using a double-beam photometer.
4 See instructions for performance of the assay.
5 For example, stopper with glass marbles.
6 Molecular weight for L-α-lecithin is taken with 733 = dipalmitoyl lecithin.

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{content}_{L\text{-}\alpha\text{-lecithin}} = \frac{c_{L\text{-}\alpha\text{-lecithin}} \text{ [g/l sample solution]}}{c_{sample} \text{ [g/l sample solution]}} \times 100 \text{ [g/100 g]}$$

Instructions for performance of assay

The L-α-lecithin content present in the cuvette should range between 10 µg and 200 µg. The sample solution must therefore be diluted sufficiently to yield an L-α-lecithin concentration between 0.1 and 2.0 g/l.

Dilution table

estimated amount of L-α-lecithin per liter	dilution with water	dilution factor F
< 2.0 g	–	1
> 2.0 g	1 + 9	10

If the absorbance difference measured (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared anew (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 1.0 ml. The volume of water added must then be reduced so as to obtain the same final volume for the sample and blank in the cuvettes. The new sample volume v must be taken into account in the calculation.

1. Instructions for sample preparation

1.1. Direct measurement

For egg products (e.g. liquid egg, liquid egg yolk, frozen egg, dry egg) and egg liqueur it is possible to use a sample solution with tertiary butanol as solvent for the direct measurement of L-α-lecithin. Otherwise a suspension with water has to be prepared by ultrasonics (e.g. with Sonifier Cell Disrupter, Branson Sonic Power Co.).

Examples:

Determination of L-α-lecithin in egg liqueur

Weigh approx. 2 g egg liqueur accurately into a 100 ml volumetric flask and dissolve with approx. 10 ml tertiary butanol. Fill up with water to 100 ml (add water in 10 ml portions, each and shake after each addition). Use 0.2 ml for the assay.

Determination of L-α-lecithin in L-α-lecithin substance and in L-α-lecithin raw materials

Weigh approx. 100 mg substance accurately into an Erlenmeyer flask, add approx. 60 ml water (heated to 37°C, if necessary) and dissolve by ultrasonics for 5 min. Allow to cool the heated solution for approx. 10 min and treat with ultrasonics a second time for 5 min. Transfer the solution into a 100 ml volumetric flask, fill up to the mark with water and mix. Use 0.1 ml of the solution for the assay.

1.2. After alkaline hydrolysis

The hydrolysis is carried out with methanolic potassium hydroxide solution by boiling under a reflux condenser. Hereby the L-α-lecithin in the sample is dissolved and simultaneously saponified. After this sample preparation the total choline is measured and can be calculated as L-α-lecithin.

Examples:

Determination of L-α-lecithin in mayonnaise, fat and ice-cream

Weigh approx. 3 g sample accurately into a round-bottomed flask, add approx. 20 ml methanolic potassium hydroxide solution (0.5 mol/l) and boil for 15 min under a reflux condenser. Allow to cool to room temperature, add approx. 30 ml water and adjust to pH 6.0–6.5 by dropwise addition of hydrochloric acid (8 mol/l). Transfer the solution into a 100 ml volumetric flask and fill up to the mark with water and mix. Place the volumetric flask for 10–20 min into a refrigerator for separation of the fatty acids. Filter solution and discard the first few ml. Use up to 0.5 ml of the clear, possibly slightly opalescent solution for the assay.

Determination of L-α-lecithin in pastries, noodles and chocolate products

Weigh approx. 5 g of a sample, minced and homogenized in a mixer, accurately into a round-bottomed flask and add approx. 20 ml methanolic potassium hydroxide solution (5 mol/l). Continue sample preparation as described under "Determination of L-α-lecithin in mayonnaise". Use the clear or yellow colored solution for the assay.

Determination of L-α-lecithin in yolk and dried yolk

Weigh approx. 1 g yolk or 0.5 g dried yolk, respectively, accurately into a round-bottomed flask and add approx. 20 ml water; stir gently until the sample has been homogenized. Add approx. 20 ml methanolic potassium hydroxide solution (0.5 mol/l). Continue sample preparation as described under "Determination of L-α-lecithin in mayonnaise". Use the slightly turbid solution for the assay.

1.3. After extraction with organic solvents

The extraction is carried out with a mixture of ethanol/benzene.

Examples:

Determination of L-α-lecithin in mayonnaise, fat and ice-cream

Accurately weigh out approx. 5 g sample and shake with 30 ml ethanol/benzene mixture (1 + 1, v/v) and 50 ml water in a separation funnel for approx. 5 min. Separate the organic phase. Extract the aqueous phase two times with approx. 20 ml ethanol/benzene mixture. Evaporate the combined organic phases in a rotation evaporator to nearly dryness*. Dissolve the residue in 5 ml tertiary butanol, transfer into a 50 ml volumetric flask, fill up to the mark with water (add water in portions and shake after each addition). Filter the solution and use 1 ml of the nearly clear solution for the assay.

Determination of L-α-lecithin in pastries

Accurately weigh approx. 2 g of a sample of pastries, minced and homogenized in a mixer, into a 100 ml separation funnel, and shake with 30 ml ethanol/benzene mixture (1 + 1, v/v) and 50 ml water for approx. 5 min. After separation of the organic phase extract the aqueous phase two times with 10 ml ethanol/benzene mixture each. Filter the combined organic phases and evaporate in a rotation evaporator to nearly dryness.* Dissolve the residue in approx. 10 ml tertiary butanol, transfer quantitatively into a 50 ml volumetric flask, fill up to the mark with water (add water in portions and shake after each addition). Filter the solution. Use 0.1–1 ml – depending on the L-α-lecithin content (see dilution table) – for the assay.

When extracting with ethanol/benzene in some cases it is difficult to separate the phases which can often be avoided by addition of ethanol or sodium chloride. **Because of danger to health this kind of extraction should be avoided.**

2. Specificity

Phospholipase C hydrolyzes phosphatides of the L-α-lecithin type, but does not attack L-β-lecithins, lysophosphatides, glycerylphosphorylcholine and sphingomyelin. Under the mentioned assay conditions the choline formed is completely converted, the possibly present base dimethylaminoethanol is converted only to about 50%. Other bases do not react with choline kinase. The main part of the L-α-lecithin is hydrolyzed by the treatment with potassium hydroxide solution, and choline is formed. The remaining part of the L-α-lecithin is cleaved by phospholipase C and converted to choline by alkaline phosphatase. In the assay procedure the sum of free choline and in L-α-lecithin bound choline is determined and calculated as L-α-lecithin.

3. Sources of error

The enzyme choline kinase contains a small hexokinase side activity. Therefore, a slow creep reaction occurs in the presence of D-glucose in the sample. However, this error is compensated in the assay procedure by addition of D-glucose, thus, the same creep reaction will occur in the reagent blank and sample. Further calculation is not necessary.

If turbidity occurs during the procedure (determination of L-α-lecithin) it is recommended to proceed as follows:

Reagents

1. Glycine
2. Magnesium sulfate, $MgSO_4 \cdot 7H_2O$, A.R.
3. Sodium hydroxide, 5 mol/l
4. Test-Combination L-α-Lecithin, bottles 2–5

---

* Heat rotation evaporator till 45°C at maximum.

Preparation of solutions

I. Buffer
(Glycine, 0.2 mol/l; Mg^{2+}, 10 mmol/l; pH 8.0):
Dissolve 1.50 g glycine and 0.25 g $MgSO_4 \cdot 7H_2O$ with approx. 80 ml redist. water, adjust to pH 8.0 with sodium hydroxide (5 mol/l) and fill up to 100 ml with redist. water.
The buffer is stable for 3 months at +4°C.

II. Further reagents prepare as decribed for the determination of L-α-lecithin.

Procedure

a) Hydrolysis of L-α-lecithin

Pipette into 10 ml centrifuge tubes	blank	sample
buffer (I) suspension 2 sample solution redist. water	1.00 ml 0.05 ml – 2.00 ml	1.00 ml 0.05 ml 0.10 ml 1.90 ml
mix, stopper centrifuge tubes[5] and incubate for approx. 20 min at 37°C (or for approx. 50 min at 20–25°C). Place the tubes into a boiling water-bath (95–100°C) for at least 5 min, allow to cool to 20–25°C and centrifuge.		

b) Determination of the hydrolyzed L-α-lecithin

Pipette into cuvettes	blank	sample
supernatant of the blank supernatant of the sample solution 3 suspension 4	2.00 ml – 0.10 ml 0.02 ml	– 2.00 ml 0.10 ml 0.02 ml
mix and read absorbances after approx. 10 min (A_1). Addition of		
solution 5	0.05 ml	0.05 ml
mix and incubate for 20 min at 20–25°C. Read absorbances (A_2) of sample and reagent blank immediately one after another. Determine absorbance differences (A_1–A_2). $\Delta A = (A_1-A_2)_{sample} - (A_1-A_2)_{blank}$		

Calculation

$$c = \frac{3.05 \times 2.17 \times 733}{\varepsilon \times 1 \times 0.1 \times 2.0 \times 1000} \times \Delta A = \frac{24.26}{\varepsilon} \times \Delta A \text{ [g L-α-lecithin/l sample solution]}$$

4. Determination of choline

Reagents

1. Glycine
2. Magnesium sulfate, $MgSO_4 \cdot 7H_2O$, A.R.
3. Sodium hydroxide, 5 mol/l
4. Test-Combination L-α-Lecithin, bottles 3–5

Preparation of solutions

I. Buffer
(Glycine, 0.2 mol/l; Mg^{2+}, 10 mmol/l; pH 8.0):
Dissolve 1.50 g glycine and 0.25 g $MgSO_4 \cdot 7H_2O$ with approx. 80 ml redist. water, adjust to pH 8.0 with sodium hydroxide (5 mol/l) and fill up to 100 ml with redist. water.
The buffer is stable for 3 months at +4°C.

II. Further reagents prepare as described for the determination of L-α-lecithin.

Procedure

Pipette into cuvettes	blank	sample
buffer (I) sample solution redist. water solution 3 suspension 4	1.00 ml – 1.00 ml 0.10 ml 0.02 ml	1.00 ml 0.10 ml 0.90 ml 0.10 ml 0.02 ml
mix and incubate for 10 min. Read absorbances (A_1) of the solutions. Addition of		
solution 5	0.05 ml	0.05 ml
mix and incubate for 30 min at 20–25°C. Read absorbances (A_2) of the sample and the blank immediately one after another. Calculate the result according to the general formula (molecular weight of choline hydroxide, $C_5H_{15}NO_2$ = 121.2).		

Calculation

$$c = \frac{2.17 \times 121.2}{\varepsilon \times 1 \times 0.1 \times 1000} \times \Delta A \text{ [g choline hydroxide/l sample solution]}$$

5. Further applications

The method may also be used in the examination of pharmaceuticals and in research when analyzing biological samples (see Ref. 2). For details of sampling, treatment and stability of the sample see Ref. 3.

References

1 Beutler, H.-O. & Henniger, G. (1981) Enzymatische Bestimmung von Lecithin, Swiss Food 3, 27–29
2 Diedrich, K., Hepp, S., Welker, H., Krebs, D., Beutler, H.-O. & Michal, G. (1979) Die enzymatische Lecithin-Bestimmung im Fruchtwasser zur Beurteilung der fötalen Lungenreife, Geburtshilfe und Frauenheilkunde 39, 849–856.
3 Schiefer, S. & Beutler, H.-O. (1985) in Methods of Enzymatic Analysis (Bergmeyer, H. U, ed.) 3rd ed., vol. VIII, pp. 87–104, Verlag Chemie, Weinheim Deerfield Beach/ Florida, Basel.
4 Möllering, H. & Bergmeyer, H. U. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., Hrsg.) 3. Aufl., Bd. 2, S. 1861–1862, Verlag Chemie, Weinheim and (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd ed., vol 4, pp. 1813–1815, Verlag Chemie, Weinheim, Academic Press, Inc. New York and London.

©1987

BOEHRINGER MANNHEIM GMBH
Biochemica

787.1223.2.537 209 1

Preparation of solutions

I. Buffer
(Glycine, 0.2 mol/l; Mg^{2+}, 10 mmol/l; pH 8.0):
Dissolve 1.50 g glycine and 0.25 g $MgSO_4 \cdot 7H_2O$ with approx. 80 ml redist. water, adjust to pH 8.0 with sodium hydroxide (5 mol/l) and fill up to 100 ml with redist. water.
The buffer is stable for 3 months at +4°C.

II. Further reagents prepare as decribed for the determination of L-α-lecithin.

Procedure

a) Hydrolysis of L-α-lecithin

Pipette into 10 ml centrifuge tubes	blank	sample
buffer (I) suspension 2 sample solution redist. water	1.00 ml 0.05 ml - 2.00 ml	1.00 ml 0.05 ml 0.10 ml 1.90 ml
mix, stopper centrifuge tubes[5] and incubate for approx. 20 min at 37°C (or for approx. 50 min at 20-25°C). Place the tubes into a boiling water-bath (95-100°C) for at least 5 min, allow to cool to 20-25°C and centrifuge.		

b) Determination of the hydrolyzed L-α-lecithin

Pipette into cuvettes	blank	sample
supernatant of the blank supernatant of the sample solution 3 suspension 4	2.00 ml - 0.10 ml 0.02 ml	- 2.00 ml 0.10 ml 0.02 ml
mix and read absorbances after approx. 10 min (A_1). Addition of		
solution 5	0.05 ml	0.05 ml
mix and incubate for 20 min at 20-25°C. Read absorbances (A_2) of sample and reagent blank immediately one after another. Determine absorbance differences (A_1-A_2). $\Delta A = (A_1-A_2)_{sample} - (A_1-A_2)_{blank}$		

Calculation

$$c = \frac{3.05 \times 2.17 \times 733}{\varepsilon \times 1 \times 0.1 \times 2.0 \times 1000} \times \Delta A = \frac{24.26}{\varepsilon} \times \Delta A \text{ [g L-}\alpha\text{-lecithin/l sample solution]}$$

4. Determination of choline

Reagents

1. Glycine
2. Magnesium sulfate, $MgSO_4 \cdot 7H_2O$, A.R.
3. Sodium hydroxide, 5 mol/l
4. Test-Combination L-α-Lecithin, bottles 3-5

Preparation of solutions

I. Buffer
(Glycine, 0.2 mol/l; Mg^{2+}, 10 mmol/l; pH 8.0):
Dissolve 1.50 g glycine and 0.25 g $MgSO_4 \cdot 7H_2O$ with approx. 80 ml redist. water, adjust to pH 8.0 with sodium hydroxide (5 mol/l) and fill up to 100 ml with redist. water.
The buffer is stable for 3 months at +4°C.

II. Further reagents prepare as described for the determination of L-α-lecithin.

Procedure

Pipette into cuvettes	blank	sample
buffer (I) sample solution redist. water solution 3 suspension 4	1.00 ml - 1.00 ml 0.10 ml 0.02 ml	1.00 ml 0.10 ml 0.90 ml 0.10 ml 0.02 ml
mix and incubate for 10 min. Read absorbances (A_1) of the solutions. Addition of		
solution 5	0.05 ml	0.05 ml
mix and incubate for 30 min at 20-25°C. Read absorbances (A_2) of the sample and the blank immediately one after another. Calculate the result according to the general formula (molecular weight of choline hydroxide, $C_5H_{15}NO_2$ = 121.2).		

Calculation

$$c = \frac{2.17 \times 121.2}{\varepsilon \times 1 \times 0.1 \times 1000} \times \Delta A \text{ [g choline hydroxide/l sample solution]}$$

5. Further applications

The method may also be used in the examination of pharmaceuticals and in research when analyzing biological samples (see Ref. 2). For details of sampling, treatment and stability of the sample see Ref. 3.

References

1 Beutler, H.-O. & Henniger, G. (1981) Enzymatische Bestimmung von Lecithin, Swiss Food 3, 27-29
2 Diedrich, K., Hepp, S., Welker, H., Krebs, D., Beutler, H.-O. & Michal, G. (1979) Die enzymatische Lecithin-Bestimmung im Fruchtwasser zur Beurteilung der fötalen Lungenreife, Geburtshilfe und Frauenheilkunde 39, 849-856.
3 Schiefer, S. & Beutler, H.-O. (1985) in Methods of Enzymatic Analysis (Bergmeyer, H. U, ed.) 3rd ed., vol. VIII, pp. 87-104, Verlag Chemie, Weinheim Deerfield Beach/Florida, Basel.
4 Möllering, H. & Bergmeyer, H. U. (1974) in Methoden der enzymatischen Analyse (Bergmeyer, H. U., Hrsg.) 3. Aufl., Bd. 2, S. 1861-1862, Verlag Chemie, Weinheim and (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) 2nd ed., vol 4, pp. 1813-1815, Verlag Chemie, Weinheim, Academic Press, Inc. New York and London.

©1987

BOEHRINGER MANNHEIM GMBH
Biochemica

787.1223.2.537 209