The Role of a Polyphenol from *Myrothamnus flabellifolius* in the protection of membranes during desiccation - using Liposomes as a model membrane system.

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ABSTRACT

A deficiency of water is a common stress experienced by plants. Dehydration of plant tissue results in altered protein and lipid ultrastructure responsible for membrane stabilisation leading inevitably to the death of the plant. Some plants known as resurrection plants have evolved with the ability to survive dehydration to less than 2% water content, a property known as desiccation tolerance. Recently a polyphenol extracted from the leaves of a common African resurrection tree *Myrothamnus flabellifolius* Welw. has been isolated and characterised as 3,4,5-tri-*O*-galloylquinic acid. This study has investigated the role which this polyphenol may play in desiccation tolerance.

A model liposome system was prepared encapsulating a fluorescent marker, calcein. The release of calcein after desiccation and rehydration was used as a measurement of the disruption of liposome integrity. The system was validated using trehalose, which was found to maintain 29.2 ± 2.3 % of liposome structural integrity at a concentration of 30 μg /μg phospholipid when applied externally prior to desiccation. 3,4,5-tri-*O*-galloylquinic acid at a concentration 1μg /μg phospholipid was found to maintain 17.7 ± 2.0 % of liposome structural integrity after desiccation and rehydration at a concentration 30 times lower than that required by trehalose to maintain maximum structural integrity. The data suggested that 3,4,5-tri-*O*-galloylquinic acid maintained liposome structural integrity by interacting with the liposome membrane eliminating the phase transition from gel to liquid crystalline, a transformation responsible for structural degradation. It is possible that the interaction between 3,4,5-tri-*O*-galloylquinic acid and liposomal membrane lipids *in vitro* may be reflected *in vivo*. This polyphenol may thus play a role in maintenance of cells of *M. flabellifolius* under drought conditions allowing it to regenerate after dehydration.

LIST OF ABBREVIATIONS

A_{260}	Absorbance at wavelength 260nm
A_{280}	Absorbance at wavelength 280nm
calcein	3,6-dihydroxy-2,3-bis[*N,N`*-di(carboxymethyl)-aminomethyl]furan
Chol	Cholesterol
°C	Degrees centigrade
DPPC	Dipalmitoylphosphatidylcholine
DSC	Differential Scanning Calorimetry
EPC	Egg phosphatidylcholine
Fig.	Figure
g	Gravity
H_2O	Water
HCl	Hydrochloric acid
H` NMR	proton Nuclear Magnetic Resonance spectroscopy
HPLC	High Performance Liquid Chromatography
IR	Infra Red
KOH	Potassium Hydroxide
M. flabellifolius	*Myrothamnus flabellifolius*
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation Time of Flight
MeOH	Methanol
µg	Microgram
mg	Milligram
$MgCl_2$	Magnesium chloride
µl	Microlitre
ml	Millilitre
mM	Millimolar
mW	Milli Watts
MW	Molecular Weight
NaCl	Sodium chloride
PMSF	Phenylmethanesulfonylfluoride
PBS	Phosphate Buffered Saline

PC	Phosphatidylcholine
POPC	Palmitoyloleoylphosphatidylcholine
PS	Phosphatidylserine
rpm	Revolutions per minute
TEM	Transmission Electron Micrscopy
TLC	Thin layer chromatography
Tris-HCl	TRIS-(hydroxymethyl)aminomethane
T_m	Melting Temperature
UV	Ultraviolet Light
UV-Vis	Ultraviolet – visible light
v/v	volume / volume

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1. INTRODUCTION

1.1 Desiccation tolerance

The survival of plants is often determined by environmental factors including temperature variation and water availability. A deficiency of water is a very important stress experienced by plants, affecting their survival, growth and productivity [1, 2].

Dehydration of plant tissue results in changes in hydrophilic and hydrophobic interactions in cells, altered protein and lipid ultrastructure that are responsible for protein and structural integrity [3, 4, 5]. Both physical and chemical changes caused by dehydration result in irreparable changes in desiccation-sensitive tissues, inevitably leading to the death of the plant.

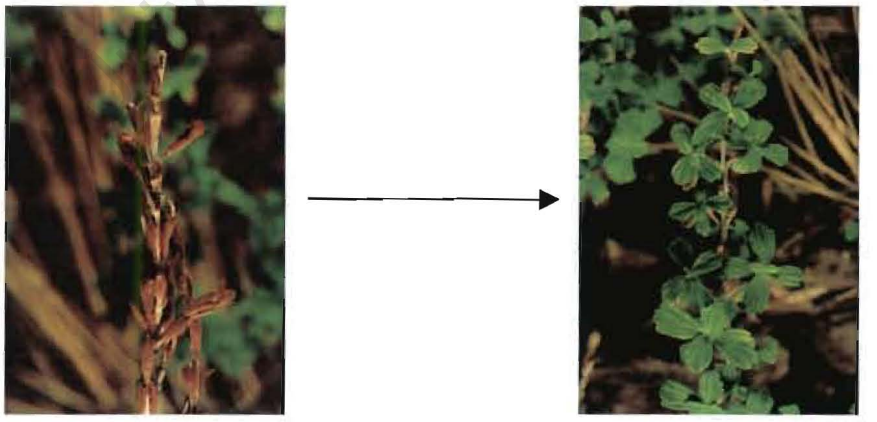
Some plants have evolved with the ability to survive with a relative water content of less than 2 % [6]. These plants have been found to accumulate solutes and specific proteins during dehydration. These include polyphenolics (including anthocyanins) sugars, antioxidants (ascorbate, peroxidase, glutathione and tocopherol) and LEA (late embryogenesis abundant) related proteins [7, 8]. It is thought that sugars and LEA proteins may replace the structural water in the cell, thus mimicking the behaviour of water molecules and maintaining the integrity of the cell structure [9, 10].

The ability to regenerate and regain vital metabolism without incurring any permanent damage after almost complete dehydration is known as desiccation tolerance [10, 11] and these plants are known as resurrection plants. An example commonly found in Southern Africa is *Myrothamnus flabellifolius*.

1.2 *Myrothamnus flabellifolius*

M. flabellifolius Welw. belongs to the family Myrothamnaceae of which there is a single genus, *Myrothamnus* Welw, but two different species, *Myrothamnus flabellifolius* Welw [12] and *Myrothamnus moschantus* Baill [13]. *M. flabellifolius* is a multi-stemmed homoiochlorophyllous (chloroplasts remain intact after desiccation) shrub which grows singly or in groups on rocky outcrops. *M. flabellifolius* has a woody stem, and differs from other resurrection plants in that it can grow up to 1.5 m high [14].

M. flabellifolius may exist in a dehydrated state for 5 to 6 months of a year, losing up to 95 % of its relative water content [15, 16]. This is accompanied by a loss of approximately 50 % of the cholorophyll content whilst the chloroplasts are maintained [17]. When *M. flabellifolius* is subjected to dehydration, its leaves become brown due to the accumulation of anthocyanins [18] and fold laterally along the stem [15, 12] so that only the abaxial surfaces are exposed to light. This state is readily reversed when water is available and metabolism is resumed. The leaves then unfold displaying their green adaxial surfaces (Figure 1.1).



Desiccated state Rehydrated state

Figure 1.1. *M. flabellifolius* Welw. in desiccated and rehydrated states [35]

M. flabellifolius has been found to accumulate solutes during desiccation, including hexose sugars [19] and polyphenols. These polyphenols have recently been extracted and purified [20]. Polyphenolics (including anthocyanins) have been found to protect membranes during desiccation. It has been shown that these amphipathic molecules migrate into the membranes of desiccation tolerant pollen and seed during dehydration [21, 22].

It is not known whether the polyphenols from *M. flabellifolius* play a role in maintaining membrane integrity.

1.2 Liposomes

Liposomes were originally described in 1965 [23]. Since then, owing to their similarity to cell membranes, liposomes have been used extensively as a model system to investigate the relationship between the structure and function of biological membranes.

Phospholipid molecules are the main structural component of all biological membranes and are used in the preparation of liposomes. The simplified structure of a typical phospholipid molecule showing its amphipathic nature i.e. having both hydrophilic and hydrophobic moieties, is shown in Figure 1.2.

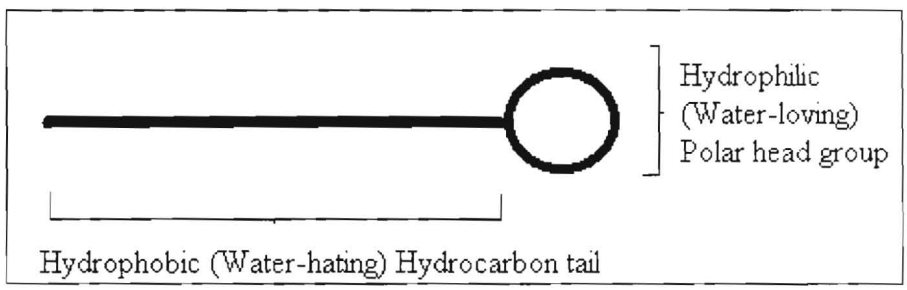


Figure 1.2. A simplified phospholipid.

Phosphatidylcholine (PC) is the most commonly occurring phospholipid in nature. (Figure 1.3)

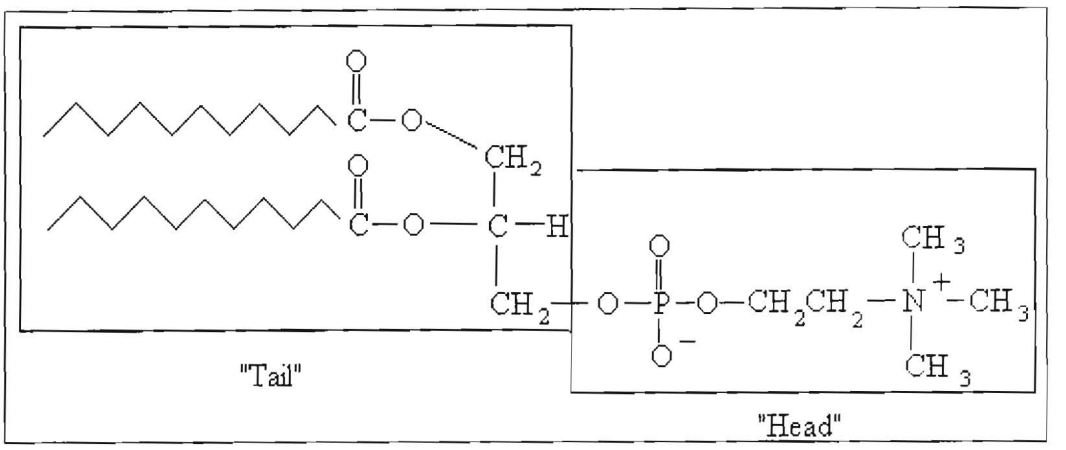


Figure 1.3. Phosphatidylcholine (lecithin)

Phosphatidylcholine can be readily extracted from Egg yolk and Soya beans in the form of lecithin, which contains a mixture of phosphatidylcholines, each possessing hydrophobic chains of different length and varying degrees of unsaturation. Phosphatidylcholine from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contains a higher proportion of fully saturated chains [24].

In aqueous solutions the polar head groups of phospholipids align themselves closely in planar sheets, or bilayers in order to minimise the unfavourable interactions between the long hydrophobic lipid chains and the bulk aqueous phase (Figure 1.4).

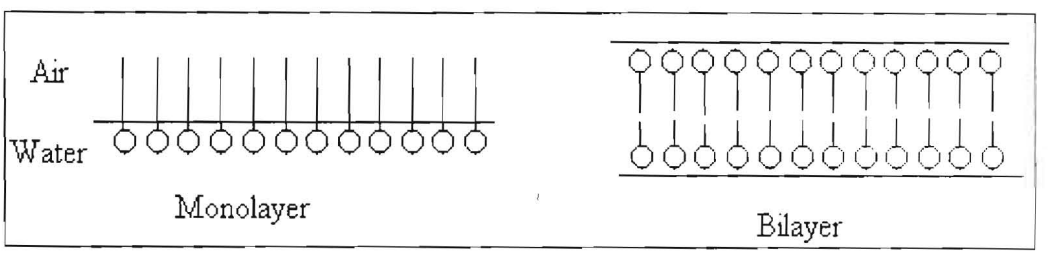


Figure 1.4. Layers formed by phospholipids

Phosphatidylcholine membranes may exist in either a gel phase or liquid crystalline phase depending on the transition temperature (T_m) of the phospholipid used (i.e. the temperature at which the hydrophobic phase transition occurs). Phases are characterised by packing properties, or motional freedom of the lipids. At low temperatures, lipids adopt a gel phase where molecules are closely packed together and rotational and lateral diffusion is slow. As temperatures increase the membrane structure changes from the tightly ordered "gel" or "Solid phase", to a liquid-crystal phase where the freedom of movement is higher, individual molecules are much further apart and rapid diffusion is observed. The temperature at which this transition takes place, the T_m, is determined by both the lipid headgroup and the acyl composition and is characteristic of a particular lipid species (Figure 1.4)

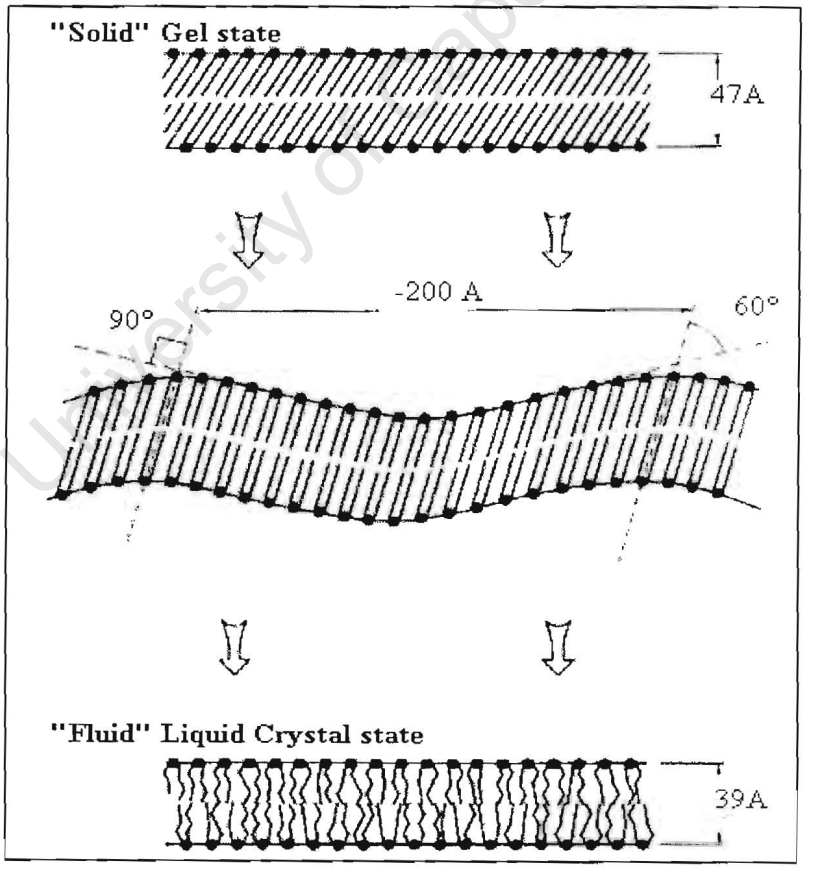


Figure 1.4. Phase transitions of phospholipid bilayers [24]

Liposomes are artificial, spherical, closed vesicles consisting of one, or more, phospholipid bilayers instead of a single phospholipid sheet. Liposomes may exist as either unilamellar (one bilayer) or multilamellar (multiple bilayers, as in an onion-type architecture) structures.

The composition of phosphatidylcholine liposomes can be varied to include charged lipids or sterols (e.g. cholesterol), which increase bilayer stability and fluidity. Cholesterol can be incorporated into membranes up to a 1:1 molar ratio (50 mol %) with phosphatidylcholine (PC). Cholesterol inserts itself into the membrane with its hydroxyl groups orientated towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer (Fig. 1.5).

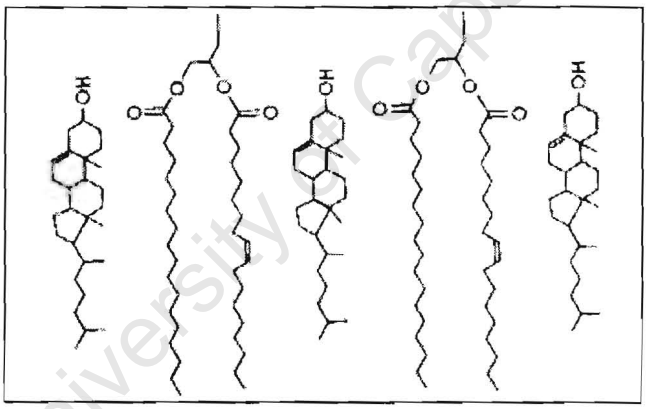


Figure 1.5. Position occupied by cholesterol in the liposome bilayer [24].

The presence of the rigid steroid nucleus reduces freedom of movement of the carbons in the first section of the phospholipid chains, while simultaneously creating space for a greater movement for the remaining carbons at the end of the chain. At a 1:1 ratio of phosphatidylcholine to cholesterol the molecules pack very efficiently into a regular linear structure. The overall effect of the addition of cholesterol to the membrane structure is a decrease in flexibility increasing the stability of the

liposomes. In this study phosphatidylcholine was combined with cholesterol in a 1:1 ratio to produce liposomes.

During synthesis of liposomes, fluorescent probes can be encapsulated in the aqueous phase and their release used as a measure of the degree of disruption of the membrane.

1.4 Polyphenols

Polyphenols are a group of aromatic molecules that possess hydroxyl groups. Polyphenols are common in nature and occur in the leaves, bark, fruit and flowers of most plants [25, 26]. Polyphenolic structures are diverse and include tannins, flavanoids, anthocyanins, alkaloids, carotenoids and phenolic acids. Some typical polyphenolic structures are shown below.

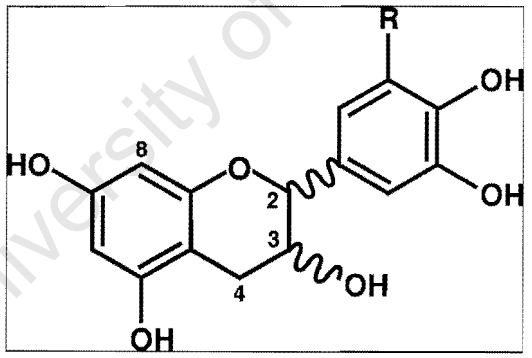


Figure 1.6. Flavan-3-ol. The basic structure of all flavanoids.

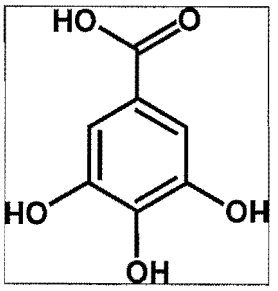


Figure 1.7. Gallic acid

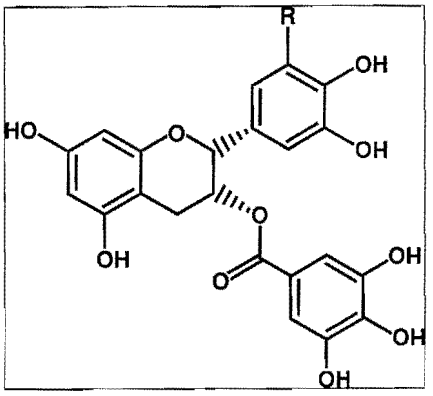


Figure 1.8. Epicatechin-3-O–gallate, the main polyphenolic compound found in tea.

Some polyphenols have been found to have antioxidant properties by acting as free radical scavengers [26, 27]. Epicatechin-3-O–gallate constitutes 15 to 30% of unfermented dried green tea and has been shown to efficiently scavenge pro-oxidants such as hydrogen peroxide, the superoxide anion radical [28], and the ascorbyl radical [29]. Japanese researchers have recently found that areas of Japan with the greatest tea consumption had the lowest death rate from cancer, in particular stomach cancer [30]. Similar research in France has shown that the drinking of red wine, which contains high levels of the polyphenol which has significantly reduced the occurrence of heart disease. This phenomenon has been named "The French paradox".

The ability of polyphenols to behave as antioxidants has been directly attributed to the presence of vitamins A, C and E and indirectly to the presence of anthocyanins. These molecules increase in concentration in certain plants under stress conditions [17]. Many plants have been shown to accumulate phenolic compounds in response to physiological stimuli and stress [31]. Phenolic acid esters and chlorogenic acids have been reported to protect lipids from peroxidation, [32] act as plant growth regulators to inhibit growth, and antagonise the action of plant hormones [33]. Polyphenols such as lignin and hydroxycinnamates have a structural function within plant cell walls [35]. It has been reported that 4-coumaric acid and ferulic acid link

lignin to polysaccharide polymers strengthening the cell wall, making it resistant to mechanical and enzymatic disruption [34]. Golovina *et al.* (1998) and Hoekstra *et al.* (1997) have shown that polyphenols are capable of protecting membranes during desiccation by migrating into the membranes of desiccation tolerant pollen and seeds during dehydration [12, 21]. Partitioning of the amphipathic molecules into the bilayer of the membrane alters the phase transition from gel to liquid crystalline, maintaining the integrity of the membrane.

Polyphenols have recently been extracted from the leaves of *M. flabellifolius* described in 1.2. The polyphenolic content in leaves of *M. flabellifolius* has been found to be significantly higher than in other desiccation tolerant plants and was found to increase further during desiccation [35]. It is not known whether these polyphenols contribute to the desiccation tolerant properties of this plant. In the present study, phosphatidylcholine /cholesterol liposomes have been used to determine whether polyphenols from *M. flabellifolius* maintain liposomal integrity in the model membrane system. Should this be the case, polyphenols may indeed play a role in plant desiccation tolerance.

2. MATERIALS AND METHODS

2.1.1 Solid materials

Phosphatidylcholine used for making liposomes was extracted from fresh egg yolk. Phosphatidylcholine (used as a TLC standard), sucrose, Ficoll and calcein were all purchased from SIGMA. Sigma-Aldrich Pty. Ltd. Johannesburg, South Africa.

Sodium chloride, potassium hydroxide and maltose (all AR grade) were purchased from Saarchem, Krugersdorp, South Africa.

Stachyose tetrahydrate and raffinose were purchased from ICN Biomedicals, Ohio, USA.

Polyphenol (3,4,5-tri-*O*-galloylquinic acid) was extracted and purified from leaves of *Myrothamnus flabellifolius.* (The extraction and purification protocol is outlined in Appendix 6.1)

2.1.2 Solvents

Twice distilled water was used in all experiments. Acetone, acetic acid, chloroform and methanol (UNIV AR) were all purchased from Saarchem, Krugersdorp, South Africa.

Ethanol, diethyl ether (GR) and hydrochloric acid (AR) were purchased from Merck, Darmstadt, Germany.

Petroleum ether (AR) was purchased from NT lab supplies Pty. Ltd, Johannesburg, South Africa.

2.2 Methods

2.2.1 Extraction of phosphatidylcholine from fresh egg yolk

Phosphatidylcholine (lecithin the main structural constituent of liposomes) was extracted from fresh egg yolk and purified using an aluminium oxide column [36].

Yolks from 3 fresh eggs weighing 48 g in total were combined with 100 ml of cold acetone and stirred at room temperature for 1 hour. The homogenate was filtered using a Buchner funnel with Whatmann #1 filter paper and the supernatant discarded. The remaining residue was washed three times with 20 ml cold acetone before being re-suspended in 50 ml of 95 % ethanol and left to stand at room temperature for one hour. The mixture was filtered, the filtrate kept and the supernatant re-extracted with 95 % ethanol. The filtrates from both extractions were combined and evaporated to dryness using a Buchi rotary evaporator. The residue was extracted twice with 30 ml petroleum ether. The extracts were combined, reduced to 20 ml using the rotary evaporator and left to cool at room temperature. After cooling, 100 ml cold acetone was added to the extract whilst stirring rapidly. The precipitate formed was allowed to settle overnight at room temperature, after which the supernatant was decanted and discarded. The precipitate was washed with cold acetone and excess solvent removed by evaporation. 1 mg of the crude phosphatidylcholine was dissolved in 1 ml of chloroform and a 10 µl aliquot applied to a Merck pre-coated Silica gel 60 (without fluorescent indicator) TLC plate for analysis and comparison with a commercial sample. A mobile phase of chloroform/methanol/water, 65:25:4 by volume was used and plates were stained using iodine vapour (Figure 3.1 - Results). The remaining crude phosphatidylcholine was transferred to a sample vial, weighed, purged with nitrogen and stored at –20 °C.

2.2.2 Purification of Crude Egg Yolk Phosphatidylcholine

75 g of neutral aluminium oxide was suspended in 80 ml of chloroform and applied to a silica gel column, 40 mm diameter, to a bed depth of 6 cm. The column was washed with three bed volumes of chloroform, then one of chloroform /methanol 9:1 (v/v). 0.5 g of crude phosphatidylcholine dissolved in 10 ml chloroform was applied to the column

and 10 ml fractions collected using a mobile phase of chloroform/methanol 9:1 (v/v). Thin-layer chromatography was used to check that the fractions were chromatographically pure. Fractions collected from the Alumina column were run in parallel with a commercial phosphatidylcholine standard and an aliquot of the original crude sample from the egg yolk (Figure 3.2 - Results).

Fractions 12 to 48 were determined using R_f values to be the most pure, pooled and the solvent removed using a Buchi rotary evaporator. The pure PC residue was dissolved in chloroform/methanol 2:1 at a final concentration of 25 mg/ml. The vial was purged with nitrogen to prevent oxidation during storage, sealed with Parafilm and stored at –20 °C. Final purified egg PC was analysed using TLC and compared to the standard sample (Figure 3.3 - Results).

TLC analysis confirmed that the purified phosphatidylcholine extracted from the pooled fractions collected from the alumina column was chromatographically pure.

2.2.3 Characterisation of purified phosphatidylcholine

The commercial and purified PC sample were analysed using MALDI-TOF (Figures 3.4 and 3.5 - Results) to compare purity and to determine the molecular mass of the purified PC obtained. From this data the lipid ratios of the hydrocarbon chains could be calculated.

Molecular mass analysis was carried out using a Voyager DE™-Pro (PerSeptive Biosystems) Matrix Assisted Laser Desorption/Ionisation - Time Of Flight (MALDI-TOF) mass spectrometer. Approximately 1 picomole of sample was dissolved in 1 µl of 0.1 M IAA matrix (trans-3-indoleacrylic acid).

2.3 Encapsulation of a fluorescent marker

2.3.1 Choice of fluorescent marker

Liposomes may be manipulated to encapsulate a required solute in the aqueous phase. Encapsulation of fluorescent probes is a useful tool when using liposomes as a model membrane system as simple techniques measuring the release of the probe reflect the degree to which the liposome structure is disrupted. Carboxyfluorescein is the fluorescent marker most commonly used with liposomes. In previous work using liposomes, [39] calcein was chosen in preference to carboxyfluorescein as it was found that carboxyfluorescein leached out of the liposomes more readily than calcein. Calcein has additional advantages over carboxyfluorescein in that it has an increased latency in liposomes due to its higher molecular weight and moreover does not experience pH-dependent quenching [40].

Calcein is a self-quenching fluorescent molecule. Its excitation and emission spectra overlap resulting in the transfer of electrons (fluorescence energy transfer) between adjacent calcein molecules at high concentrations. Fluorescent energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor molecules. The distance between calcein molecules within a liposome is very small and intact liposomes are, therefore, effectively non-fluorescent. Upon lysing the liposomal structure, calcein is released into solution, resulting in a decreased concentration, and thus an increase in the fluorescence.

2.3.2 Establishing linearity of fluorometer

The linearity of the Aminco SPF 500 fluorometer was tested. Excitation and emission wavelengths used were 490 nm and 520 nm with bandpaths of 2 and 4 nm respectively.

The fluorescence of dilutions of a 50 mM stock of calcein in PBS was measured to produce a calibration plot (Figure 3.6 - Results).

This showed that the fluorometer readings were linear for concentrations of calcein ranging from 1 x 10^{-4} % to 2 x 10^{-3} % (v/v) calcein stock in PBS.

2.4 Preparation of liposomes

Liposomes were prepared following the method of Szoka and Papahadjopoulos (1978) [41] Stock solutions of PC and cholesterol were prepared with concentrations of 25 mg /ml and 12.8 mg /ml respectively, in chloroform/ methanol 2:1 (v/v). 1 ml aliquots were purged with nitrogen to prevent oxidation and stored at –20 °C. 1 ml each of the PC and cholesterol aliquots were combined in a 50 ml pear-shaped flask and the solvent removed by rotary evaporation at 25 °C. The dry lipid film produced was kept under vacuum for 15 minutes and then kept under nitrogen for a further 15 minutes. 3 ml diethyl ether was added together with 1 ml of the aqueous phase (solutes in PBS, pH 7.4) to be encapsulated. The resulting mixture was sonicated at 0 °C under nitrogen until the layers became homogenous (2 -5 minutes). The use of nitrogen for purging is essential throughout sonication as sonicating small unilamellar liposomes in air has been reported to cause appreciable oxidative damage to the liposomes. Only very slight peroxidation has been recorded when preparations are kept under nitrogen [42]. After sonication, the flask was returned to the rotary evaporator, the solvent removed at 25 °C and after 15 minutes at 25 °C the temperature was reduced to 20 °C for a further 10 minutes. The liquid preparation frothed as the solvent was removed, became a viscous gel and then finally an aqueous suspension (Figure 2.1). This suspension was vortexed for 30 seconds before being sonicated for 5 minutes to produce small unilammelar liposomes (SUV). The liposome preparation was extruded through a 0.2 μm syringe

filter and mixed with 1 ml 30% Ficoll in a SW65 Beckman centrifuge tube. 2.5 ml 10% Ficoll was layered carefully on top, followed by 1 ml PBS. The resulting gradient was then centrifuged in a Beckman ultracentrifuge at 250 000 g for 30 minutes. Liposomes that banded at the 10 % Ficoll /PBS interface were collected.

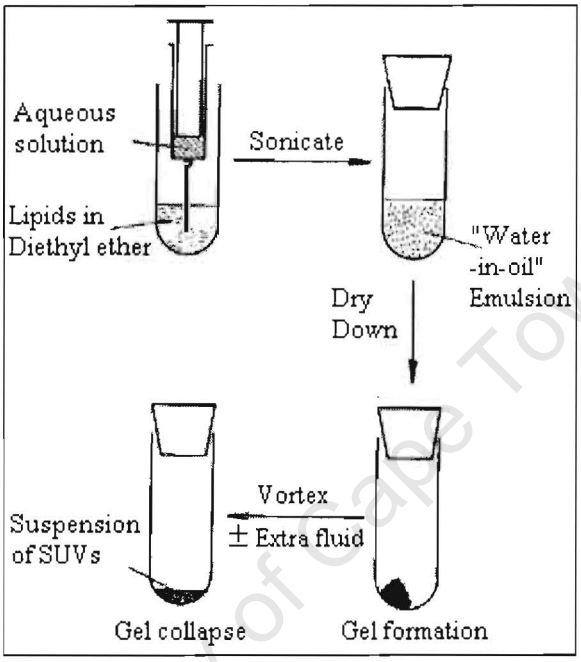


Figure 2.1. Stages in the preparation of liposomes by Reverse Phase Evaporation [43]

2.4.1 Purification of liposomes

Two methods have been used to remove excess aqueous phase and residual Ficoll from the liposome preparation. These are dialysis or purification using a Sephadex G50 chromatography column as used by Crowe et al [44, 45, 46].

Liposomes removed from the gradient were dialysed in a bag for against 2 l of PBS for 2 hours at 4 °C. PBS was changed for fresh solution after 2 hours twice and the third dialysis was left for 16 hours at 4 °C.

Alternatively liposomes were separated from excess calcein by passing the preparation through a Sephadex G50 column using PBS as the mobile phase. The fluorescence of the fractions collected was recorded. Plotting the data obtained (Fig. 3.7 - Results) showed that liposomes were separated from excess calcein, which was eluted after the liposome peak.

Liposomes purified using Sephadex G50 chromatography did not remain intact for as long a period upon storage at 4 °C as liposomes purified using dialysis. Liposomes purified by dialysis were stable for 10 days, whereas liposomes subjected to column purification lost structural integrity after only 4 days. This contrasts to a previous report [22] in which column chromatography yielded more stable liposomes. For this reason Sephadex G50 chromatography was abandoned as a method to remove excess calcein from liposomes.

2.4.2 Electron microscopy of Liposomes

Purified liposomes were analysed using electron microscopy, after staining with uranyl acetate and lead citrate. Carbon-coated copper grids were floated on a 20 µl sample of fresh liposomes for 10 minutes. The grids were then washed five times by flotation on a drop of water and were then stained with 2 % uranyl acetate followed by 1 % lead citrate. Liposomes were viewed and photographed using a Zeiss EM109 transmission electron microscope (Figure 3.8 - Results).

2.4.3 Disruption of liposome structure using Triton-X-100

The actual encapsulation of calcein in dialysed liposomes was investigated. 1 µl dialysed liposomes (approximately 10 nmoles of lipid) was mixed with 1 ml PBS and the solution transferred to a 1 ml quartz fluorimeter cuvette. The fluorescence of the

sample was measured and remeasured after the addition of 10 µl of 10 % Triton-X-100, a detergent which disrupts liposomal structural integrity (Figure 2.2) [39]. An increase in fluorescence from zero to 0.67 (arbitrary units) was recorded. This correlated to an encapsulation efficiency of 1.08 %, which was in agreement with the value reported previously.

1 2 3

Figure 2.2. Increase in fluorescence of liposomes in solution, before, during, and after the addition of 10 % Triton-X-100. [52] Whereas no fluorescence was visible from intact liposomes containing calcein (1), fluorescence occurred immediately on the injection of Triton-X-100 (2). A dramatic increase in fluorescence was observed as the liposomes were lysed and calcein was released into the surrounding solution (3).

In order to determine whether 1 % of Triton-X-100 was the optimal concentration for lysing liposomes, the fluorescence was recorded before, and then after the addition of increasing volumes of 10 % Triton-X-100 to a solution of liposomes (Figure 3.9 – Results). The plot obtained showed that up to a volume of 10 µl, a linear increase in fluorescence was recorded, after which the further addition of Triton X-100 resulted in no increase in fluorescence, thus confirming this volume was sufficient to lyse liposomes present in the solution.

2.4.4 The effect of time on Fluorescence

Sales [39] allowed liposomes to stand for 20 minutes after the addition of Triton-X-100 before reading the increase in fluorescence to allow all liposomes to lyse. Not only was fluorescence observed immediately after the addition of Triton, but fluorescence is also known to have a relatively short half-life. To determine the optimum period between Triton addition and fluorescence determination the fluorescence of calcein released from liposomes after the addition of Triton-X-100 was determined as a function of time (Figure 3.10 - Results). The data indicated a decrease in fluorescence with time. To confirm that this was due to fluorescent decay of released calcein and not the presence of either PBS or Triton, the fluorescence of a 50 mM solution of calcein with and without Triton was measured as a function of time (Figure 3.11 - Results).

2.5 Measurement of phase transition

Phase transitions and fluidity of phospholipid membranes affect the physical properties of the liposome, significantly affecting the stability and their behaviour in biological systems. A Perkin-Elmer DSC7 calorimeter was used to obtain phase transition data of liposomes prepared with and without 3,4,5-tri-*O*-galloylquinic acid. Samples (10 – 20 mg) were weighed in aluminium sample pans of known mass. Sealed pans were used with desiccated liposomes, while vented pans were used with hydrated liposome samples. A scanning rate of 5.0 °C min^{-1} was used between 25 and 60 °C according to the procedure used by Crowe et al [47].

2.6 Protocol for investigation of protection of liposomes against desiccation

Release of calcein could be used as a method to determine the leakage from liposomes with or without the addition of potential membrane protective agents. The technique used was as follows:

1µl aliquots of liposomes were added to labelled Eppendorf tubes containing increasing concentrations of test material or PBS buffer as a control to a final volume of 50 µl. Each tube was vortexed to mix liposomes with the external solution, centrifuged on a bench-top centrifuge to ensure that the mixture was at the bottom of the sample tube, and left to stand for 2 hours to allow equilibrium to be reached. All concentrations prepared were analysed in triplicate. Liposomes were desiccated overnight using a speedy-vac set at 30 °C in order to simulate desiccation. Desiccated liposome preparations were rehydrated, by the addition of 50 µl of Milli-Q water, followed by 950 µl PBS buffer to obtain a final volume of 1 ml. The initial fluorescence was recorded. 10 µl of 10 % Triton-X-100 was added, the cuvette was covered with Parafilm and inverted 3 times and the fluorescence remeasured. Using equation 1 followed by equation 2 the percentage structural integrity maintained could be calculated.

Equation 1.

$$\% \text{ Leakage} = \frac{\text{Fluorescence of rehydrated sample}}{\text{Maximum fluorescence of sample (after addition of Triton-X-100)}} \times 100$$

Equation 2.

% Structural integrity maintained = 100 - % Leakage

3. RESULTS AND DISCUSSION

3.1. Results from extraction of phosphatidylcholine from fresh egg yolk (2.2.1)

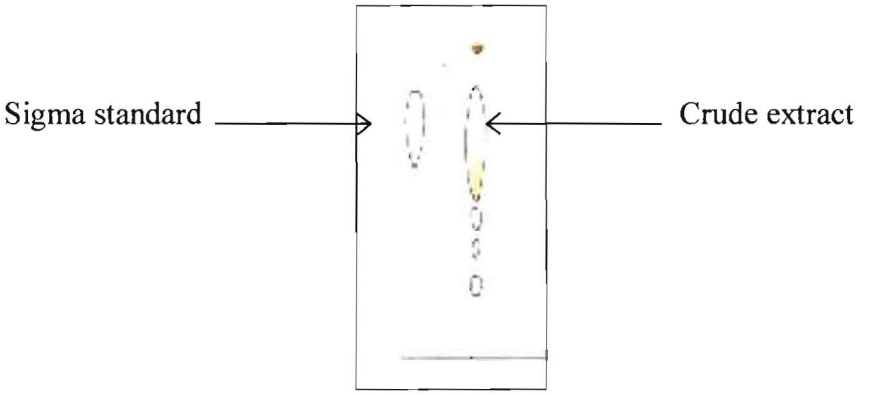


Figure 3.1. TLC plate comparing crude egg yolk PC to commercial PC.

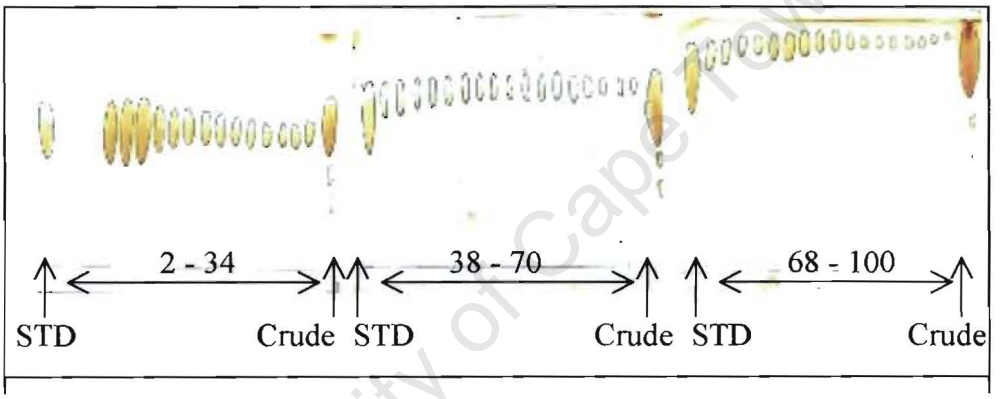


Figure 3.2. TLC plates showing every second fraction collected from the alumina column.

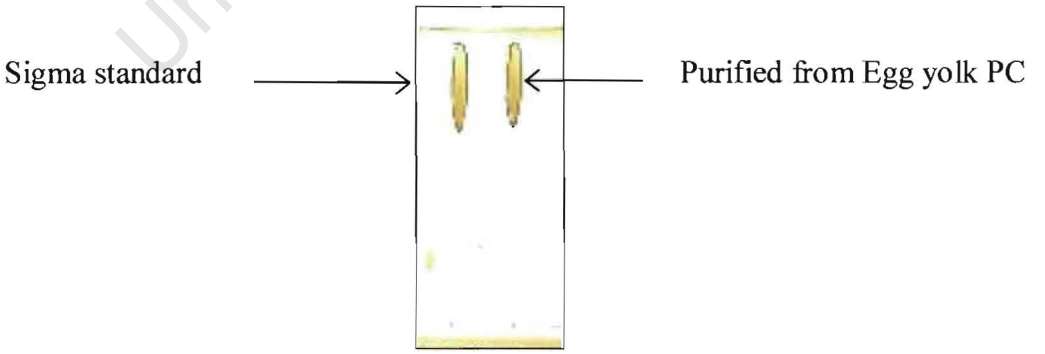


Figure 3.3. Comparison of crude egg PC purified on alumina column with a sample of egg yolk PC purchased from Sigma using TLC.

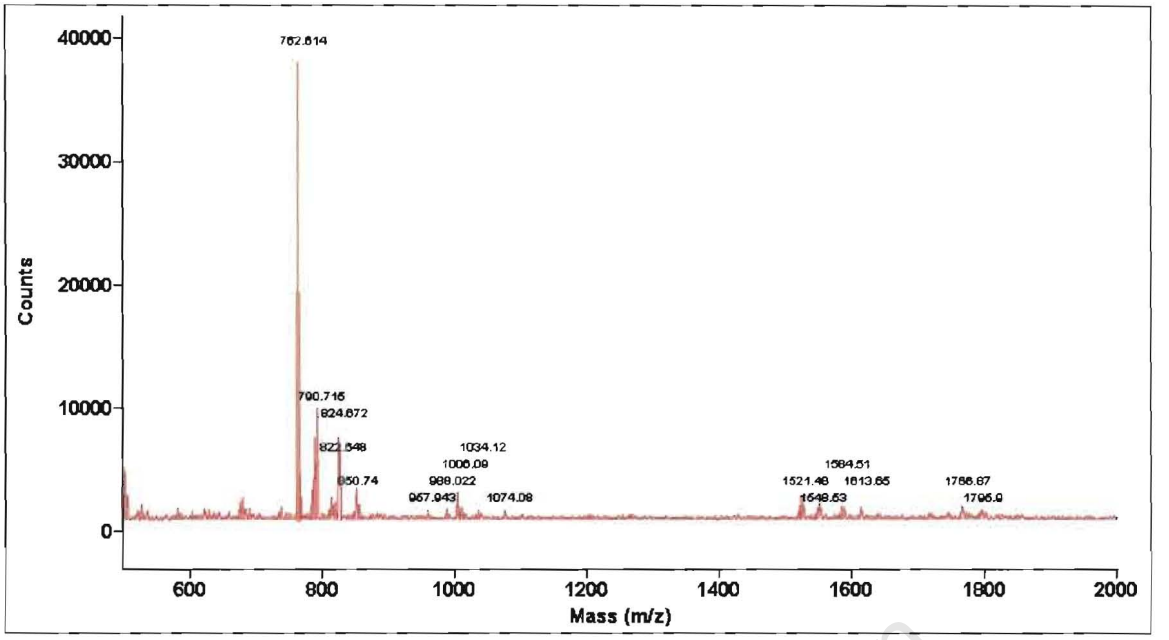


Figure 3.4. MALDI-TOF spectra for commercial PC from egg yolk

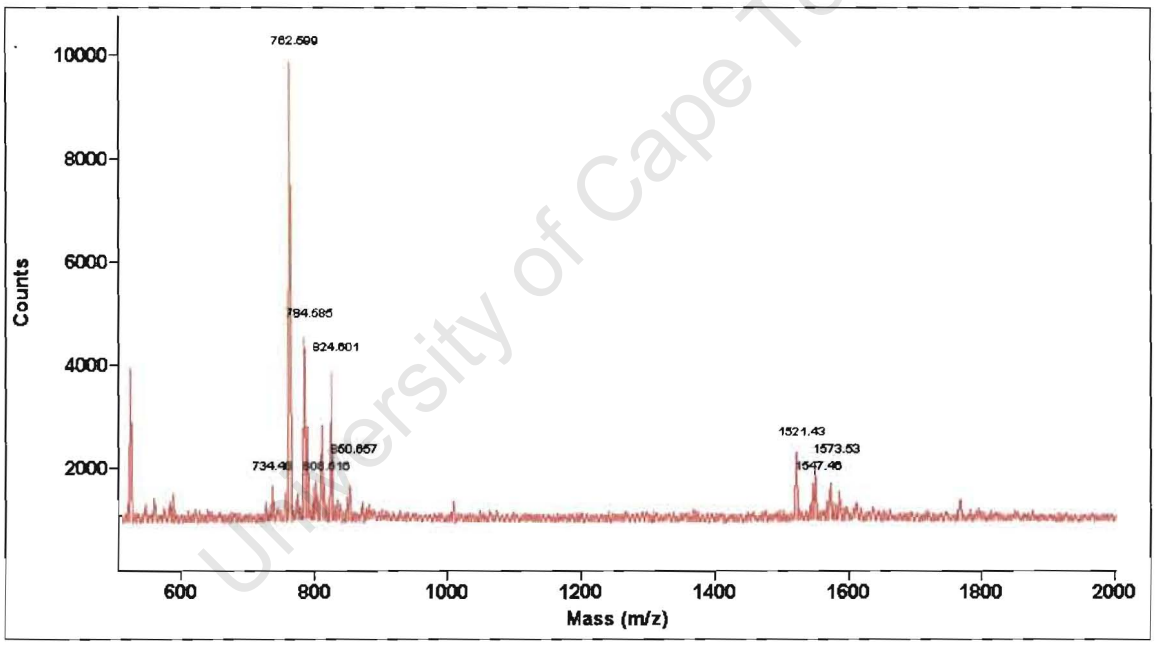


Figure 3.5. MALDI-TOF spectra obtained for PC purified from egg yolk.

MALDI- TOF confirmed that the phosphatidylcholine purified from fresh egg yolk was pure when compared to the standard purchased from Sigma. Results obtained show that the extracted PC consisted of four species with different acyl chains attached to the backbone of the molecule. Major monomeric peaks were observed at m/z of 762.60, 784.58 and 808.62 with a ratio of 5.25: 1.5: 1, and dimer peaks were observed at 1521.43,

at 1521.43, 1547.46 and 1573.53. Molecular mass of purified PC ranged between 762 and 808 g mol^{-1}. Identical peaks were observed for purified PC and the commercial standard analysed. The following hydrocarbon chain combinations would account for the major species observed: $C_{16:0}$: $C_{16:0}$ (peak at 762 m/z), $C_{18:1}$, $C_{18.2}$ (peak at 784 m/z) and $C_{18:1}$, $C_{20:4}$ (peak at 808 m/z).

Natural lecithin is reported to consist mainly of palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) linoleic acid ($C_{18:2}$) and arachidonic ($C_{20:4}$) [37]. The results are in agreement with the results from the MALDI- TOF spectra obtained for the purified PC. PC is reported to have an average molecular mass of 770 $gmol^{-1}$ [38]. Molecular mass obtained from MALDI spectra for purified PC ranged between 762 and 808 $gmol^{-1}$. The ratio of the peak heights from the spectra obtained indicates that the purified PC consist mainly of dipalmitoylphosphatidylcholine (C_{16} C_{16}) commonly abbreviated to DPPC.

3.2 Standardisation of techniques

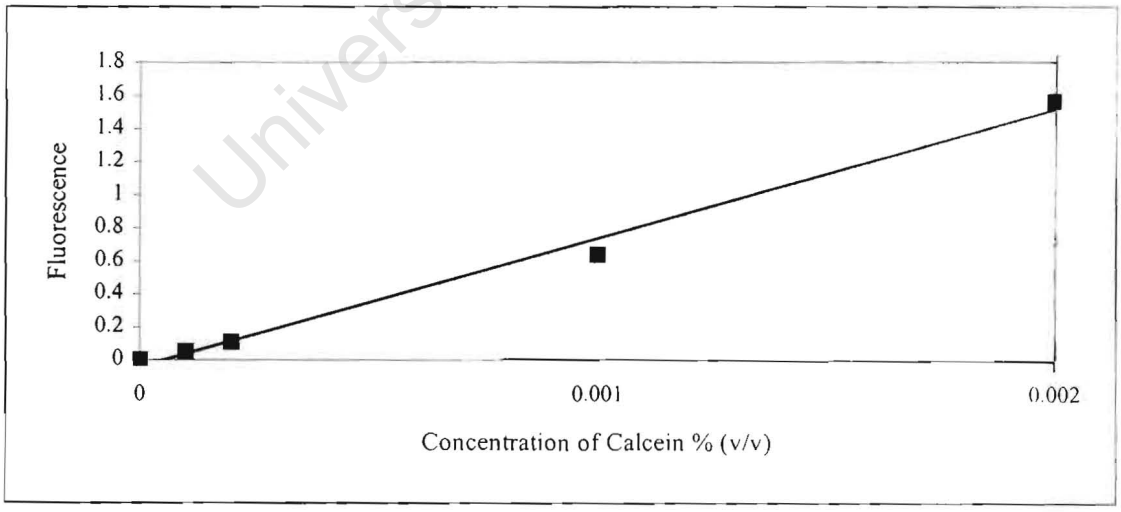


Figure 3.6. Calibration of fluorescence of calcein concentrations

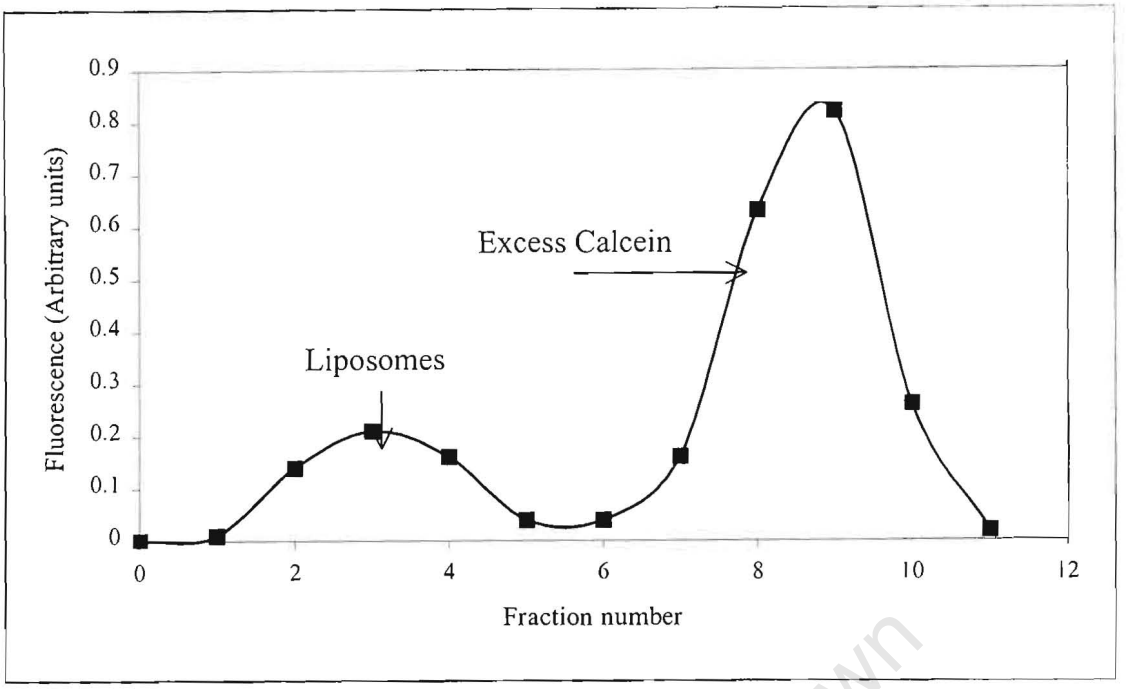


Figure 3.7. Separation of excess calcein from liposomes using a Sephadex G50 column.

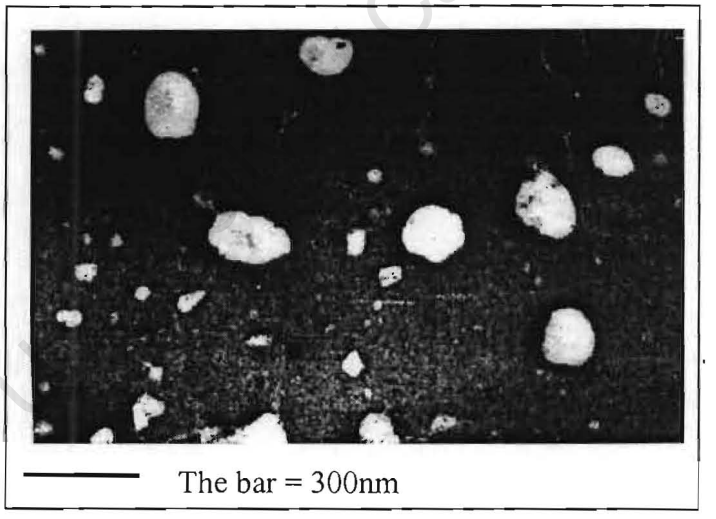


Figure 3.8. Transmission electron micrograph of liposomes. The magnification used was 45 000 times.

Electron microscopy confirmed the presence of liposomes in the preparation. Liposomal diameters have been reported to range from 25 nm to many micrometers, their size being determined by the maximum possible crowding that the hydrophilic headgroups will tolerate as the curvature in the inner layer increases with decreasing

radius. Liposomes have been categorised according to their size and are known as small unilamellar vesicles (SUVs), multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs) [48].

A significant variation in liposomal diameter was evident from the electron micrograph. Smaller liposomes photographed had an average diameter approximately 80 nm while the larger liposomes shown had a diameter approximately 200 nm. Guiot and Baudhuin (1984) [49] state that "in suspensions of monolamellar liposomes, standard deviations of the particle radius between 30 and 100 % are not unusual, hence measurements of bulk properties in liposome suspensions may give estimates reflecting essentially the occurrence of a small proportion of large particles".

Liposomes consisting of egg phosphatidylcholine /phosphatidylserine (PC/PS) extruded through a 200 nm pore filter have previously been shown to have an average diameter of 89 ± 6 nm [44]. By careful control of solvent removal it is possible to produce liposomes by reverse phase evaporation as small as 60 nm [48] while large unilamellar vesicles are reported to have average diameters of around 100 nm [50]. Using the distribution of the mean radius (140 nm) of liposomes observed under electron microscopy, the encapsulation efficiency of 50 mM calcein was calculated and was found to be 1.06 ± 0.18 %.

A disadvantage of producing small unilamellar liposomes is that their encapsulation of an aqueous phase is low, usually 0.2 % to 1 % per mole of lipid, with an efficiency of 0.1 to 1 % of the required material entrapped. This is due to sonication during the preparation rendering small vesicles thermodynamically unstable [51].

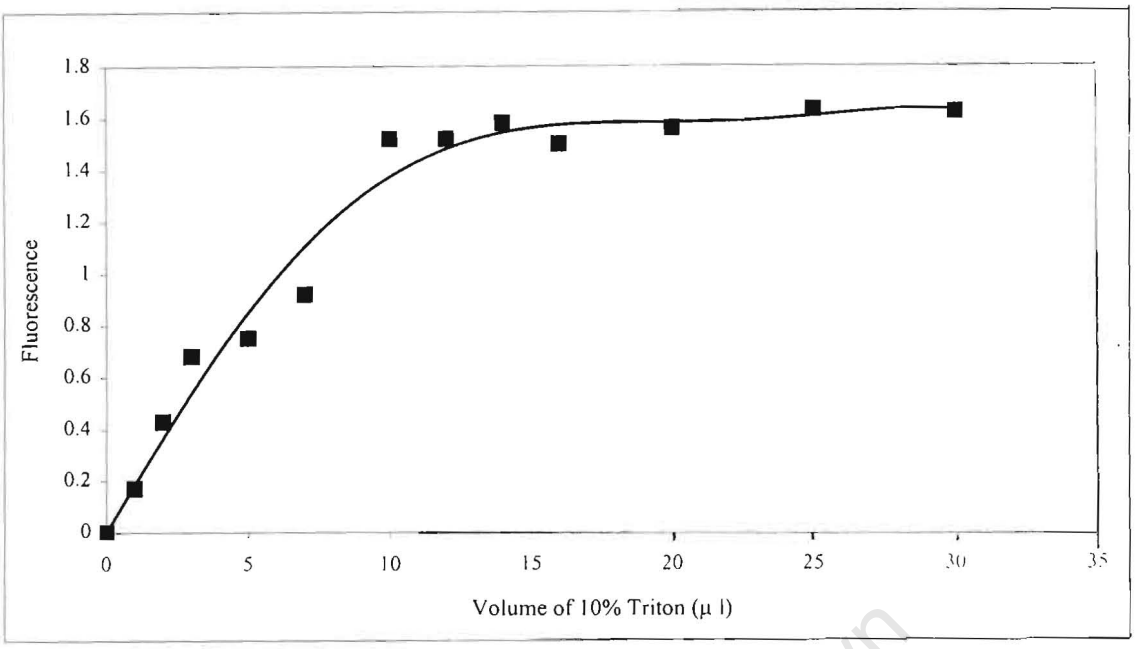


Figure 3.9. Determination of the optimum concentration of Triton-X-100 required to lyse liposomes.

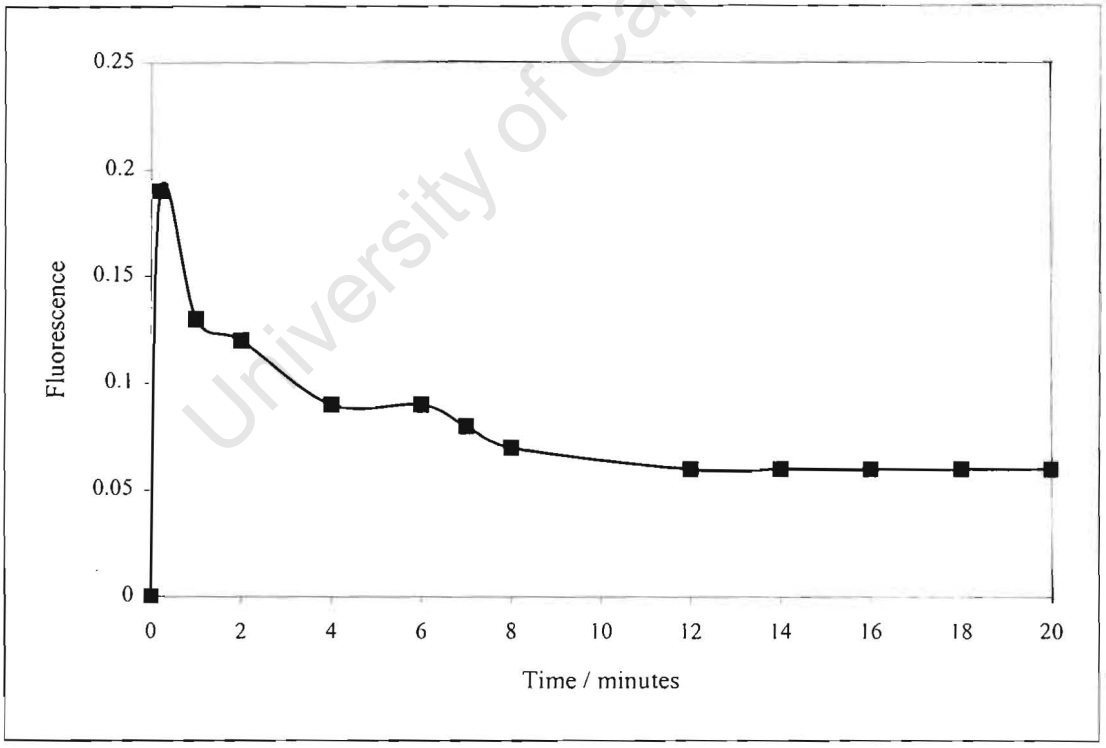


Figure 3.10. Decrease of fluorescence of calcein with time after release from liposomes after the addition of Triton-X-100.

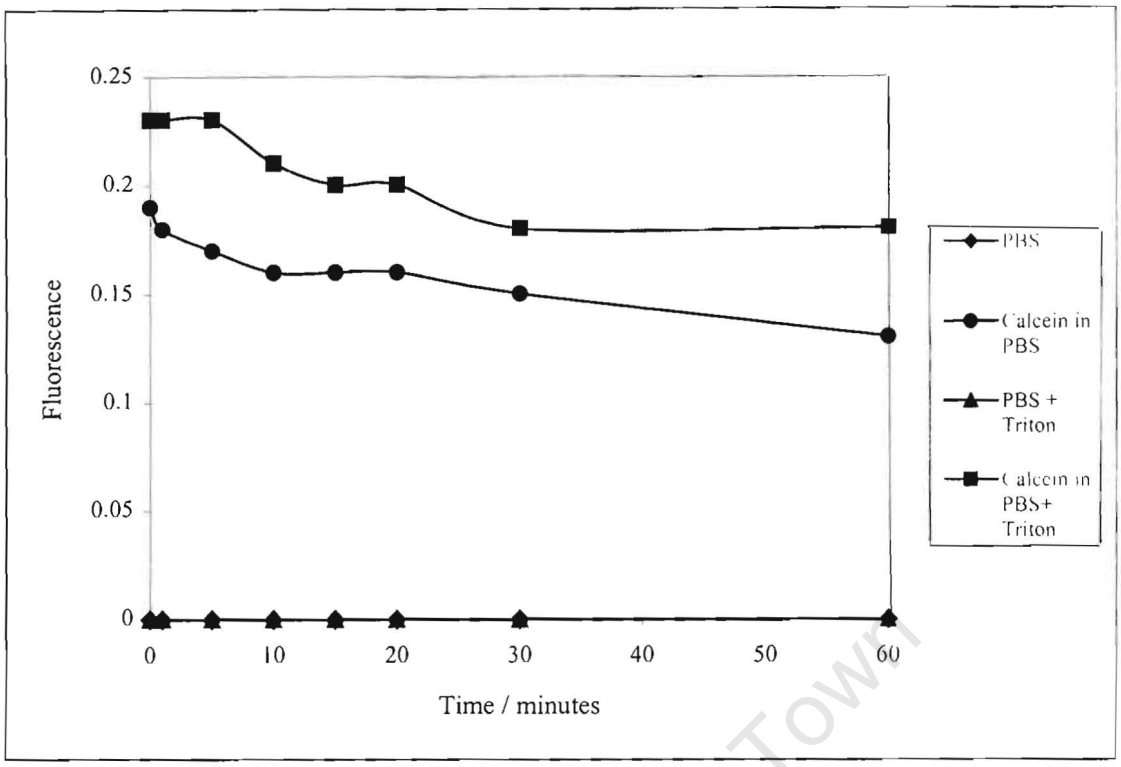


Figure 3.11. Decrease in fluorescence with time of a solution of calcein with and without Triton and fluorescence of PBS with and without Triton.

The results showed an increase in fluorescence of the calcein in PBS after the addition of Triton-X-100. Since no fluorescence was observed when Triton-X-100 was added to PBS alone, this increase in fluorescence was therefore not due to fluorescing properties of Triton-X-100, but may be due to a changed environment decreasing the quenching of calcein. As fluorescence decreased with time and the addition of Triton-X-100 resulted in the spontaneous loss of liposome integrity, it was decided that the fluorescence of lysed liposomes would be read 10 seconds after the addition of Triton.

3.3. Maintenance of liposome structural integrity during dehydration

Liposomes, prepared according to section 2.4, were used to determine the potential of the polyphenol 3,4,5-tri-*O*-galloylquinic acid to maintain liposome structural integrity

during desiccation, according to the protocol outlined in section 2.6. In an attempt to reproduce results obtained from previous experimental work with liposomes of similar lipid composition [39], to establish the authenticity of the liposome preparation, liposomes were desiccated overnight in the presence of trehalose before being rehydrated and the change in calcein fluorescence determined.

3.3.1 Maintenance by external trehalose.

Trehalose up to a concentration of 40 μg /μg phospholipid was added to hydrated liposomes, which were then desiccated with a speedy-vac overnight. The internal temperature of the sample was monitored with a thermocouple and found to be between 28 °C and 31 °C throughout the desiccation process. This agreed with data reported previously [39]. Desiccated liposomes were then rehydrated and the samples analysed for calcein leakage to determine the percentage of liposome structural integrity maintained (Figure 3.12).

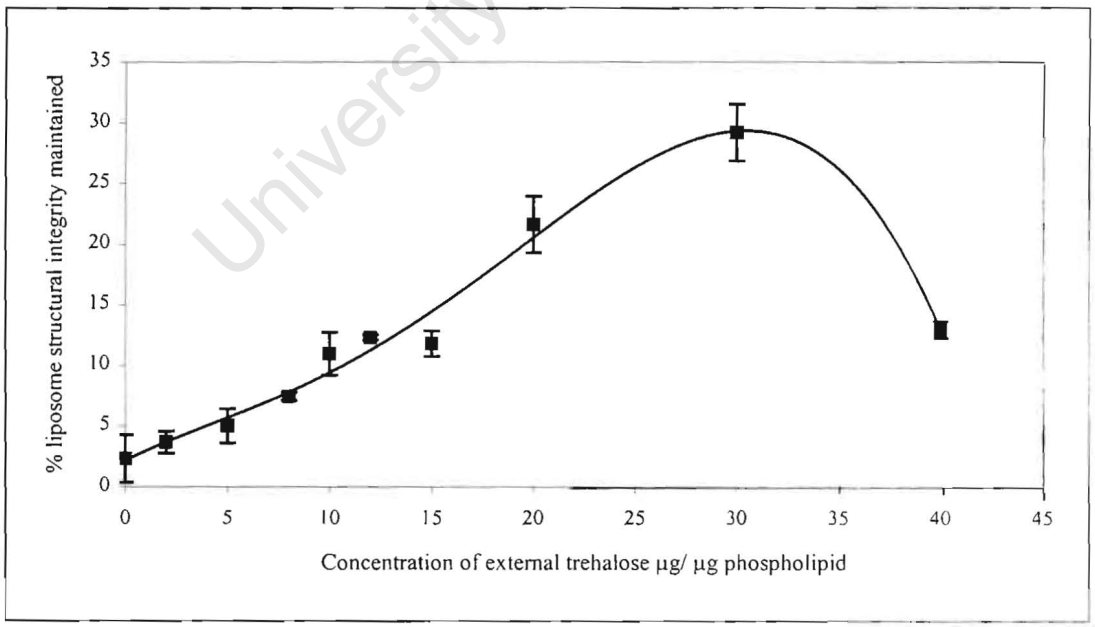


Figure 3.12. The effect of trehalose concentration on the maintenance of structural integrity of liposomes desiccated in the presence of external trehalose. Invisible error bars are where the deviation of results obtained was smaller than the symbol used.

The maximum structural integrity maintenance of 30 % was observed at a concentration of 30 μg trehalose per μg PC. Above this concentration, maintenance of liposome integrity decreased. A comparable maximum structural maintenance of 26 % was reported by Crowe et al with POPC/PS sonicated liposomes and an external trehalose concentration of 3.9 μg trehalose /μg phospholipid [47] Although the lipid composition differed, the preparative technique was similar to that used in this study. Crowe et al [53] reported that, at high concentrations, sugars have a destabilising effect on the membrane due to an asymmetric distribution. This might explain the decrease in the maintenance of structural integrity observed above 30 μg trehalose /μg phospholipid. Furthermore, Crowe et al [53] stated that many discrepancies occur when comparing the stability of dry liposomes. Factors responsible for these discrepancies include size, charge, the sugars used and the dry-mass ratio of sugar to lipid, buffers and concentration of buffer used. Thus smaller sonicated vesicles are comparatively unstable and retain no more than 70 % of trapped solute after drying, even with extremely high concentrations of sugars. Very large liposomes are similarly unstable. A small amount of charged lipid in the bilayer significantly increases the stability. The comparative efficacy of the sugar used varies with the size of the vesicles. The dry-mass ratio between the stabilising sugar and the lipid rather than the concentration of either the lipid or the sugar in bulk solution is important in the preservation during freeze-drying [9].

The present results did not agree with those previously obtained where a maximum maintenance of 70 % was observed using 15 μg trehalose /μg [39]. However, this may have been due to the inclusion of the charged lipid stearylamine. Although the conditions used in the present study were similar to those used previously [39], the results obtained are not in complete agreement. Liposomes used in this study were

approximately 80 nm in diameter whereas those used previously were reported to be 100 nm in diameter. This difference in size could account for the differences in the maintenance of structural integrity recorded.

Dehydration and subsequent rehydration of liposomes have been found to cause alterations in the membrane structure, leading to irreparable damage and complete leakage of an entrapped aqueous phase. Liposomes have been successfully maintained after desiccation and rehydration in the presence of certain sugars, of which trehalose, a non-reducing disaccharide is particularly effective [44]. Liposomes lyophilised in the presence of trehalose did not leak their contents i.e. the membrane integrity was preserved, and under electron microscopy, liposomes were seen to exist as vesicles embedded in trehalose [44].

Leakage of liposomes desiccated with and without sugars has been used to investigate the role of sugar molecules in the protection of cells during dehydration. The "glass formation hypothesis" suggests that sugar molecules replace lost water molecules, thereby reducing structural degradation and damage that occurs due to a change in solute concentrations. Concentrated solutions of sugar characteristically form glasses (vitrification) which offer resistance to desiccation since they not only have a lower water vapour pressure than crystalline solids, but also a higher viscosity which prevents chemical diffusion and promotes metabolic dormancy, thus conferring stability [54].

More recently it has been suggested that the formation of a glass together with a depression of the phase transition temperature (T_m) between the gel and liquid crystalline states of the dry lipid is required for membrane stabilisation [4, 55]. It has been proposed that trehalose substitutes the water of hydration through interactions with the hydrophilic phosphate headgroups [9]. Direct interaction between the sugar and the polar headgroup of phosphatidylcholine results in a depression of the transition temperature of the lipid and its maintenance in the liquid crystalline state, even in the absence of water [4]. Ensuring that the lipids do not pass through the gel to liquid crystalline phase transition at any stage is necessary to stabilise the phospholipid vesicles [9] (Figure 3.13).

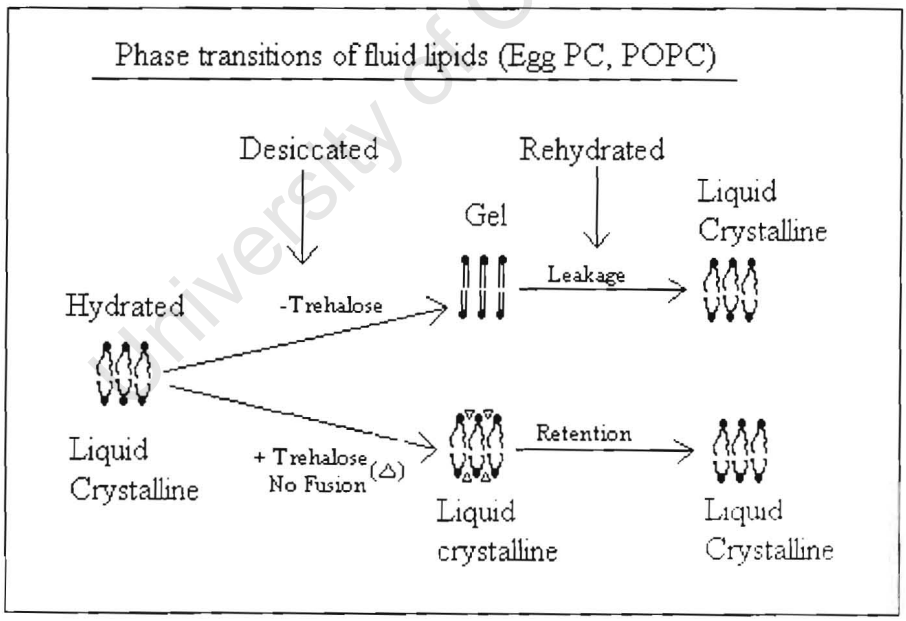


Figure 3.13. Phase transitions of fluid lipids such as Egg Phosphatidylcholine with and without trehalose during desiccation and subsequent rehydration [9]

3.3.2 Maintenance by internal and external trehalose.

It has been shown that incorporation of trehalose both internally and externally increased the maintenance of structural integrity of liposomes desiccated and rehydrated, compared with the presence of external trehalose alone [39, 47, 56]. It has been reported that liposomes containing 12 μg trehalose /μg PC were found to show approximately 20 % increase in maintained structural integrity when desiccated and rehydrated in the presence of an external concentration of 12 μg trehalose /μg PC [39], 12 μg trehalose /μg PC was used internally as this concentration was optimum when applied externally. As 30 μg trehalose /μg PC provided maximum maintenance in the present study, we unsuccessfully attempted to prepare liposomes containing 30 μg trehalose/ μg PC. Liposomes containing 3 μg trehalose /μg PC were, however, successfully prepared. Aliquots of these liposomes were added to increasing concentrations of external trehalose, desiccated, and the liposome structural integrity maintained was calculated (Figure 3.14).

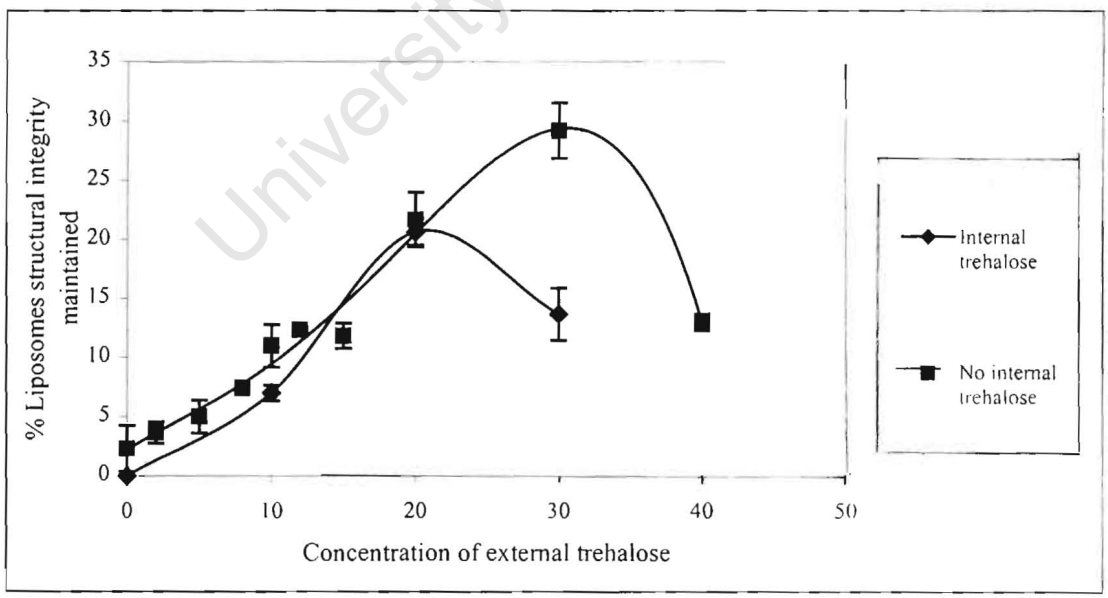


Figure 3.14. Maintenance of structural integrity of liposomes containing 3 μg trehalose /μg PC with increasing concentrations of external trehalose.

The encapsulation of trehalose (3 μg /μg PC) decreased maintenance of liposome integrity. Maximum maintenance of structural integrity with liposomes containing 3 μg /μg PC with external trehalose of 30 μg /μg PC was 20.7 ± 1.1%, while that seen with external trehalose alone at 30 μg /μg PC was 29.2 ± 2.3%. However, in the presence of internal trehalose maximum maintenance of structural integrity was observed at a lower external concentration of 20 μg /μg PC.

Since it has been suggested that only a small amount of trehalose is required internally to increase liposome stability [53] a range of lower internal trehalose concentrations was investigated. Concentrations ranging from 0.1 mg to 0.8 mg trehalose were added to the aqueous phase during the preparation to produce final internal concentrations of 0.004, 0.008, 0.016, 0.024, 0.032 μg trehalose /μg PC respectively. These liposomes were desiccated overnight using the concentrations of external trehalose used previously. After rehydration the % structural maintenance was calculated.

The trend was similar to that seen using an internal trehalose concentration of 3 μg trehalose /μg PC (Fig. 3.3) for all concentrations of internal trehalose used. The addition of lower concentrations of internal trehalose had no apparent effect on the maintenance of liposomal structural integrity (data not shown). These results were in agreement with the data of Crowe and Crowe (1988) where internal trehalose concentrations of 0.06M, 0.125M and 0.5M had no additional effect on the maintenance of liposome structural integrity by external trehalose [53]. Since encapsulation of internal trehalose did not augment liposome structural integrity, desiccation in the presence of external trehalose was used as the standard by which the possible protective effects of putative protectants was compared.

3.3.3 Maintenance by Oligosaccharides

Many oligosaccharides have been tested as a putative protectant molecules for liposomes against desiccation and rehydration [39, 46]. All have shown some degree of maintenance during desiccation, although none have been reported to be as effective as trehalose. We initially determined the purity of the sugars used, by confirming that the melting point for each oligosaccharide was close to published values (Table 1).

Oligosaccharide	Experimentally determined Melting point °C	Melting point °C
Trehalose	96 – 98	96.5 – 97.5 [57]
Sucrose	179 – 181	160 – 186 [57]
Raffinose	79 – 81	80 [57]
Maltose	103 – 105	102 – 103 [57]
Stachyose	167 – 169	170 [58]

Table 1. Melting points of oligosaccharides used in desiccation experiments. All the melting point values were found to be close to published values thereby confirming the purity of the oligosaccharides.

Liposomes were desiccated in the presence of different concentrations of four disaccharides. Concentrations were applied externally to 30 μg trehalose /μg PC since above this concentration trehalose (Fig. 3.12) was found to destabilise liposomes. Liposome structural integrity maintained was determined for all oligosaccharides (Fig. 3.15)

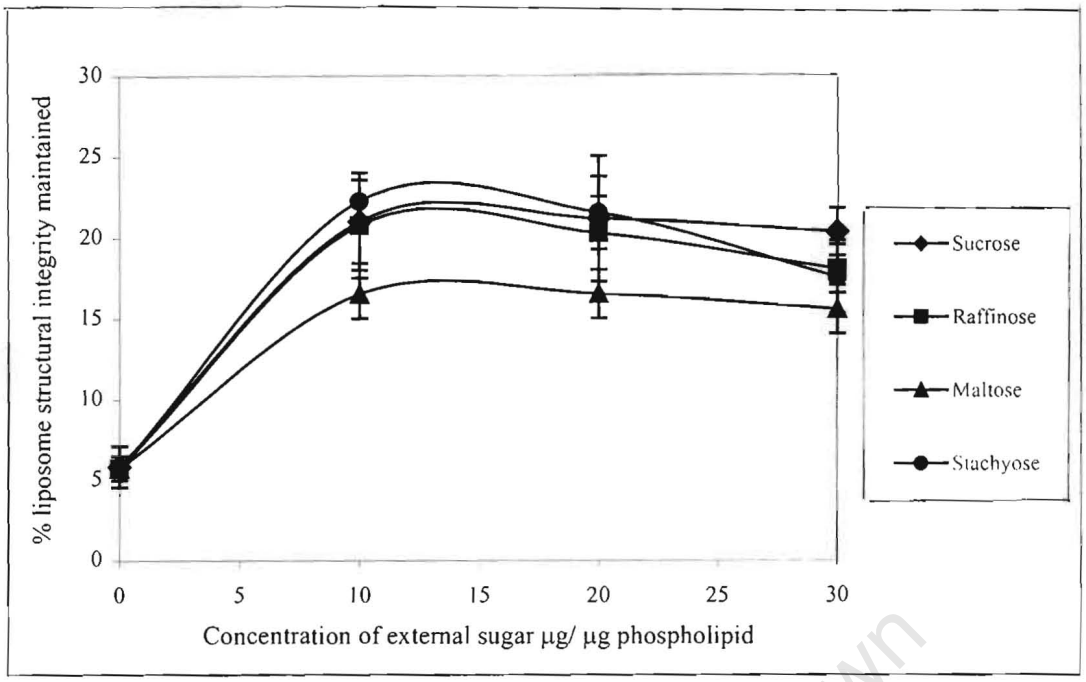


Figure 3.15. Maintenance of liposome structural integrity by oligosaccharides

Maximal maintenance of the four oligosaccharides tested was observed at an external concentration of 10 µg /µg PC. Increasing the external concentration did not increase the maintainance of the liposome structural integrity. Stachyose maintained liposome structural integrity by a maximum of 22.25 ± 1.75 %, sucrose by 21.0 ± 2.57 % and raffinose by 20.75 ± 3.25 %. Maltose was the least effective, maintaining only 16.5 ± 1.5 % at 10 µg /µg PC. The results for trehalose were similar to these oligosaccharides at an external concentration of 20 µg /µg PC, but was significantly higher (29.2 ± 2.3 %) at an external concentration of 30 µg /µg PC.

3.3.4 Maintenance by 3,4,5-tri-*O*-galloylquinic acid

A polyphenolic compound, 3,4,5-tri-*O*-galloylquinic acid has recently been isolated from the leaves of the desiccation tolerant plant *M. flabellifolius*. 3,4,5-tri-*O*-galloylquinic acid was the major polyphenolic compound extracted. It was found to constitute approximately 30 % w/w of the dry leaf [21].

3,4,5-tri-*O*-galloylquinic was added in increasing concentrations to liposomes prior to desiccation and subsequent rehydration and the liposome structural integrity maintained was determined (Figure 3.16).

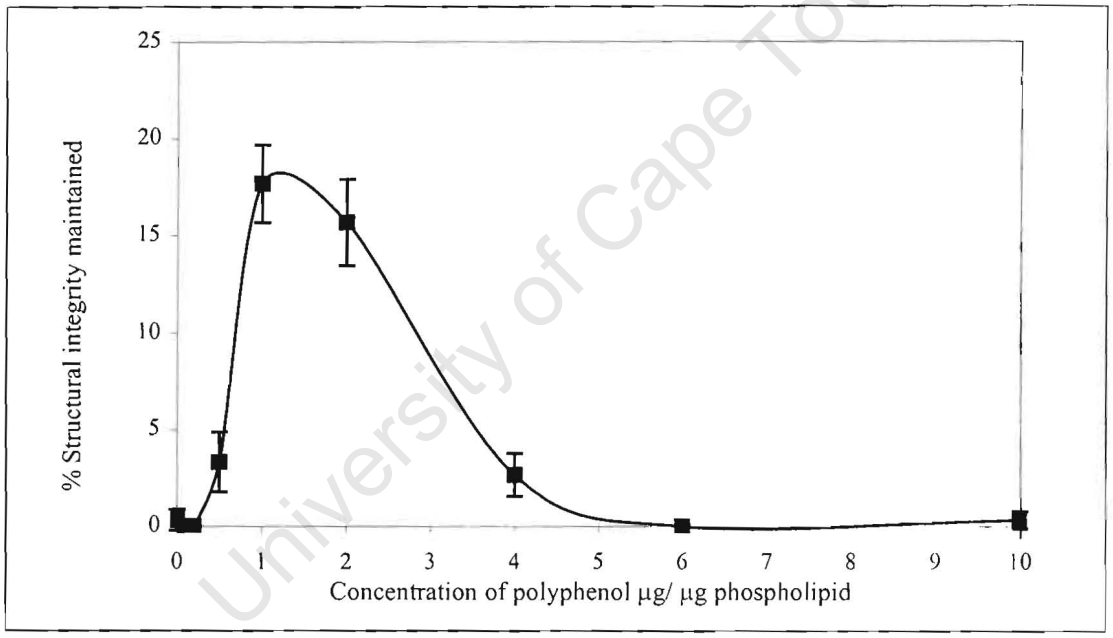


Figure 3.16. Maintenance of liposome structural integrity by 3,4,5-tri-*O*-galloylquinic acid extracted from the leaves of *M. flabellifolius*.

The results showed a maximum structural integrity maintenance of 17.7 ± 2.0 %. This maximum maintenance was in the concentration range between 1 and 2 µg /µg PC. Although the maximum structural integrity maintained (17.7 ± 2.0 %) was lower than that achieved with trehalose (29.2 ± 2.3 %) only 1 µg 3,4,5-tri-*O*-galloylquinic acid /µg

phospholipid was required to achieve this level of protection, compared with 30 μg /μg PC for trehalose.

Between 2 and 4 μg 3,4,5-tri-*O*-galloylquinic acid / μg PC the maintenance observed decreased and above 5 μg 3,4,5-tri-*O*-galloylquinic acid / μg PC no maintenance was observed. The decrease in structural maintenance with a concentration of 3,4,5-tri-*O*-galloylquinic acid greater than 2 μg/ μg PC might be explained by the amphipathic properties of 3,4,5-tri-*O*-galloylquinic acid and that at higher concentrations it preferentially forms micelles, no longer interacting with the liposomal membrane.

To ensure that the polyphenol 3,4,5-tri-*O*-galloylquinic acid did not contribute to the fluorescence of the calcein present after liposome degradation, the fluorescence of 3,4,5-tri-*O*-galloylquinic acid alone, and in the presence of Triton-X-100 was determined. No fluorescence was observed with or without Triton-X-100, confirming that 3,4,5-tri-*O*-galloylquinic acid had no fluorescing properties (Results not shown). The results also confirmed that fluorescence recorded in the previous experiment (Figure 3.5) was due only to loss of liposomal structural integrity, resulting in the release of calcein, and not the presence of 3,4,5-tri-*O*-galloylquinic acid or its interaction with Triton-X-100. To investigate whether the apparent loss of maintenance of structural integrity above 2 μg /μg PC was not caused by lysis of liposomes in the presence of 3,4,5-tri-*O*-galloylquinic acid, 3,4,5-tri-*O*-galloylquinic acid solution or Triton-X-100 was added to freshly dialysed liposomes (Figure 3.17).

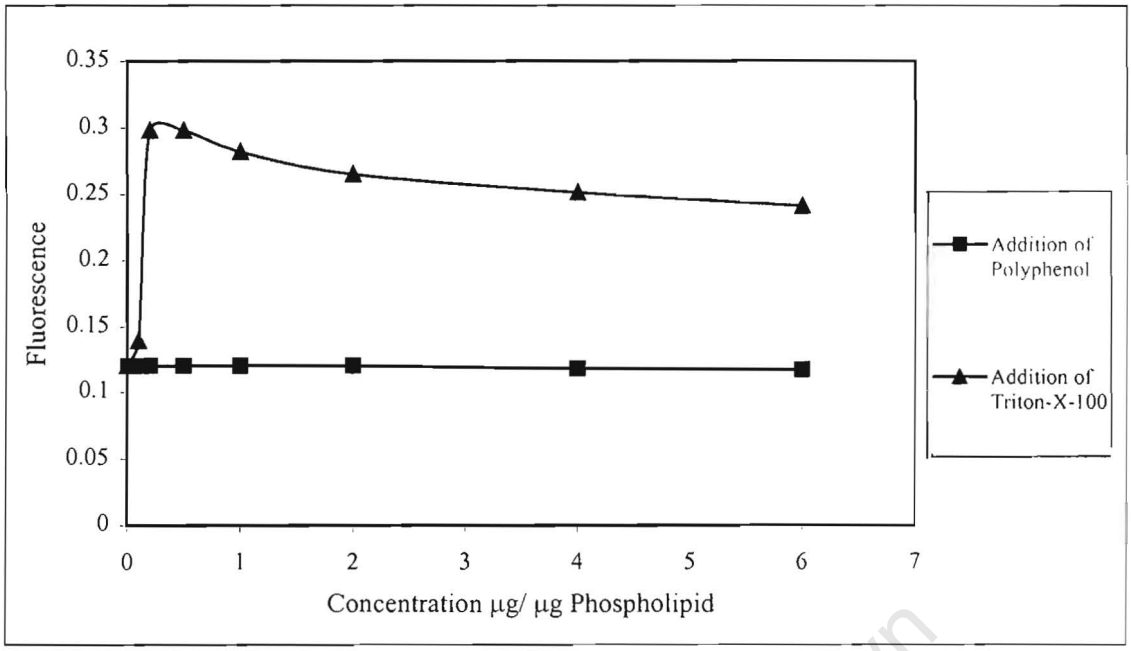


Figure 3.17. The effect of 3,4,5-tri-*O*-galloylquinic acid and Triton-X-100 on freshly dialysed liposomes.

The results showed that liposomes were only lysed upon the addition of Triton-X-100 as shown by increased fluorescence in the cuvette. 3,4,5-tri-*O*-galloylquinic acid had no effect on intact liposomes confirming that the increased fluorescence seen previously was due only to loss of structural integrity of liposomes during desiccation and rehydration. Since 3,4,5-tri-*O*-galloylquinic acid was capable of protecting liposomes during desiccation and rehydration, this suggests the polyphenol interacted with the phospholipid bilayer.

Commercial arbutin and gallic acid were combined with liposomes at concentrations similar to those used with 3,4,5-tri-*O*-galloylquinic acid and desiccated overnight. These particular commercial polyphenols were chosen as arbutin is the main sugar that accumulates in the leaves of *M. flabellifolius* during desiccation [36] and gallic acid is the main structural component of 3,4,5-tri-*O*-galloylquinic acid. The results (Figure 3.18) were compared with those found with 3,4,5-tri-*O*-galloylquinic acid.

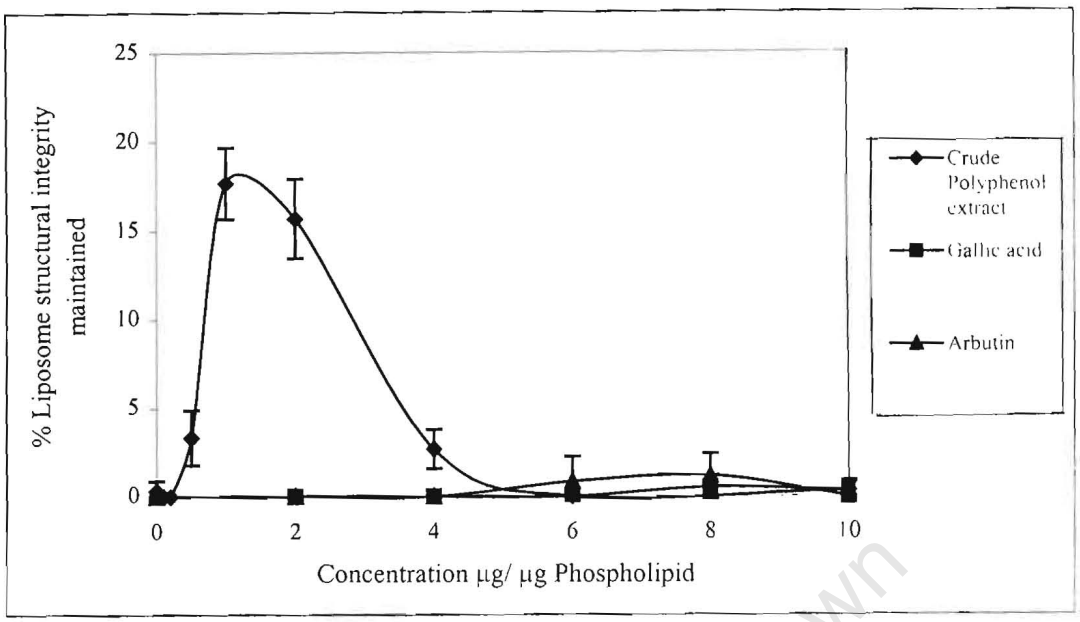


Figure 3.18. Maintenance of liposome structural integrity by 3,4,5-tri-*O*-galloylquinic acid and commercial polyphenols.

The results showed that the commercially available polyphenols, arbutin (4-hydroxyphenyl-beta-glucopyranoide) and gallic acid had no significant effect on maintaining structural integrity of liposomes after desiccation and rehydration (Figure 3.18). Gallic acid has been reported to have no affinity for egg PC liposomes [59]. An affinity factor is calculated by division of the amount of polyphenol incorporated by the amount added to the liposomal solution [59]. This suggests that unlike 3,4,5-tri-*O*-galloylquinic acid extracted from *M. flabellifolius*, no interaction occurs between gallic acid and the liposomal membrane. Hincha *et al.* (1999) have shown that unless the chloroplast lipid monogalactosyldiacylglycerol (MGDG) is included in the liposomal membranes, arbutin destabilises phosphatidylcholine liposomes during desiccation and rehydration. When lipid composition of liposomes consisted of more than 15 % MGDG, arbutin was capable of behaving as a cryoprotectant and reduced liposome

leakage by 55%. Arbutin when used alone, leads to complete leakage of an entrapped marker [23].

These results are in agreement with the data in the present study.

3.4 Interaction between 3,4,5-tri-*O*-galloylquinic acid and liposomes

A range of 3,4,5-tri-*O*-galloylquinic acid concentrations were added to 1 µl of freshly dialysed liposomes and centrifuged on a bench top centrifuge for 1 minute. The absorption at 260 nm of the supernatant was compared with the absorption of identical concentrations of 3,4,5-tri-*O*-galloylquinic acid (Figure 3.19).

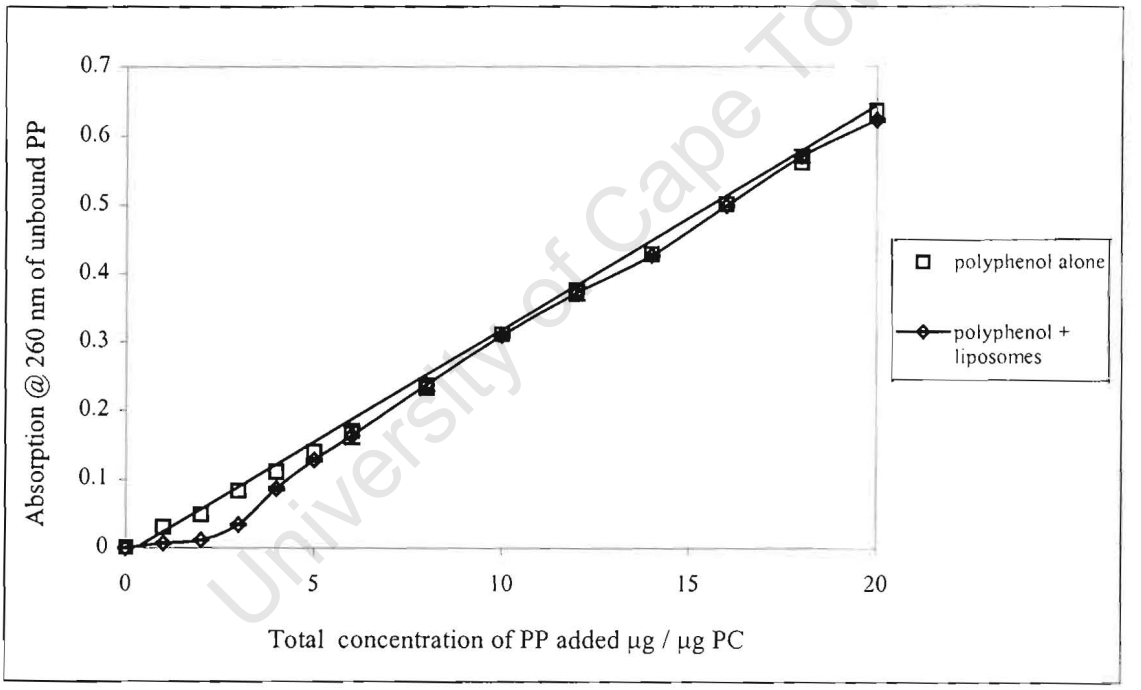


Figure 3.19. Interaction of 3,4,5-tri-*O*-galloylquinic acid with liposomes. Invisible error bars are where the deviation of results obtained was smaller than the symbol used.

In a liposome binding assay involving the removal of liposomes by centrifugation, addition of 3,4,5-tri-*O*-galloylquinic acid to liposomes resulted in no 260 nm

absorption of the supernatant up to 2 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC. Between 2 μg and 4 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC absorption of the supernatant fraction was observed. At 5 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC and above, the absorption of the supernatant fraction was identical to that measured for 3,4,5-tri-*O*-galloylquinic acid alone. This suggested that 3,4,5-tri-*O*-galloylquinic acid interacted with liposomes up to 4 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC but that no interaction occurred above 5 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC. Since 3,4,5-tri-*O*-galloylquinic acid molecules are amphipathic they presumably interchelated into the phospholipid membrane when present at low concentration. As the concentration of 3,4,5-tri-*O*-galloylquinic acid increased, binding to the liposome membrane reached saturation, and an increase in absorption of the supernatant fraction was observed, as free 3,4,5-tri-*O*-galloylquinic acid molecules were present in solution. Above 5 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC the concentration in solution was high enough that a critical micellar concentration was reached, and 3,4,5-tri-*O*-galloylquinic acid formed micelles. 3,4,5-tri-*O*-galloylquinic acid bound to the liposomal membrane was released to preferentially bind to micelles of 3,4,5-tri-*O*-galloylquinic acid formed. Above 5 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC the absorption for both plots was identical (Fig 3.19) Suggesting that no 3,4,5-tri-*O*-galloylquinic acid was bound to the liposome and was completely in solution as micelles and that binding is reversible. If 3,4,5-tri-*O*-galloylquinic acid remained bound to liposomes at high concentration the plot of polyphenol with liposomes would have been lower than observed for 3,4,5-tri-*O*-galloylquinic acid alone.

Maintenance of liposome structural integrity during desiccation and rehydration was observed in the concentration range of 0.5 μg to 4 μg 3,4,5-tri-*O*-galloylquinic acid

/µg PC (Fig. 3.16) above which the level of maintenance decreased. This concentration range correlated with the data presented in figure 3.19.

Maximum structural integrity maintained by 3,4,5-tri-*O*-galloylquinic acid has been found to be 17.7 ± 2.0 % (Figure 3.16) which suggests that approximately 82 % of entrapped calcein was released due to liposome degradation.

The experiment was repeated except that liposomes were desiccated after the addition of 3,4,5-tri-*O*-galloylquinic acid. After rehydration and centrifugation the absorption at 260 nm of the supernatant was determined (Figure 3.20).

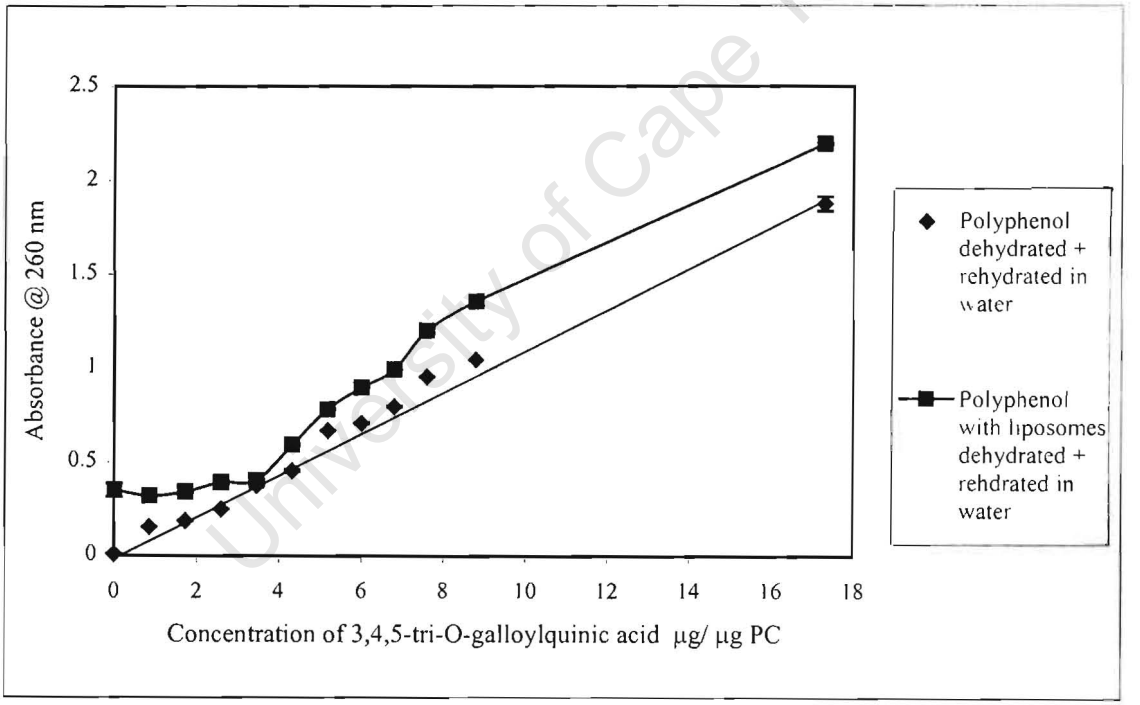


Figure 3.20. Effect of liposomes on the absorption of 3,4,5-tri-*O*-galloylquinic acid desiccated and rehydrated.

A plot of a 3,4,5-tri-*O*-galloylquinic acid solution desiccated and rehydrated alone is shown in Fig 3.20 for comparison.

Some 260 nm absorption of liposomes was observed in the absence of 3,4,5-tri-*O*-galloylquinic acid. This could be explained by the presence of small non-centrifugable light scattering particles in solution. No increase in the absorption of the supernatant fraction was observed to a concentration of approximately 3 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC. Between 3.8 μg and 8 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC absorption of the supernatant increased. Above 8 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC the absorption of the supernatant fraction observed was equal to that with no liposomes plus the absorption contributed by light scattering. The results suggested that below 3.8 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC no 3,4,5-tri-*O*-galloylquinic acid was free in the supernatant and was bound to the liposome fragments. As the concentration of 3,4,5-tri-*O*-galloylquinic acid increased a saturation point was reached at 3.8 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC above which 3,4,5-tri-*O*-galloylquinic acid was present free in solution. Above 8 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC the concentration of free 3,4,5-tri-*O*-galloylquinic acid was sufficiently high that 3,4,5-tri-*O*-galloylquinic acid was not present in liposome fragments and instead was present only in solution as micelles. 3,4,5-tri-*O*-galloylquinic acid bound to the liposomes was released to preferentially bind to micelles. The plots (Fig. 3.20) of 3,4,5-tri-*O*-galloylquinic acid alone and 3,4,5-tri-*O*-galloylquinic acid with liposome fragments are parallel. The absorption measured for 3,4,5-tri-*O*-galloylquinic acid with liposome fragments was equal to that of 3,4,5-tri-*O*-galloylquinic acid alone plus that absorption due to light scattering. The range of concentrations (0.5 to 4 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC) again corresponded with the region where maintenance of liposome structural integrity by 3,4,5-tri-*O*-galloylquinic acid was observed (Fig. 3.16). Maximum maintenance was observed between 1 and 2 μg 3,4,5-tri-*O*-galloylquinic acid / μg PC, decreasing

between 2 and 4 μg 3,4,5-tri-*O*-galloylquinic acid / μg PC and no maintenance above 5 μg 3,4,5-tri-*O*-galloylquinic acid / μg PC.

These data suggested that at low concentrations 3,4,5-tri-*O*-galloylquinic acid maintained liposome structural integrity by interacting with the liposomal membrane. As the concentration of 3,4,5-tri-*O*-galloylquinic acid increased a saturation point was reached, where 3,4,5-tri-*O*-galloylquinic acid preferentially formed micelles releasing molecules bound to the liposome, and leading to a loss of liposome structural integrity. Results obtained in these binding studies with and without dehydration are identical and seem to indicate that 3,4,5-tri-*O*-galloylquinic acid binds to intact liposome and structurally compromised liposomes in the same manner.

Nam-Won Huh et al. [60] investigated the interaction of polyphenols with bilayers. Tannic acid, which contains five di-gallic acid residues covalently linked to a central D-glucose, was found to increase the adhesion between PC bilayers. These authors concluded that tannic acid collapses the fluid space of PC bilayers because it is amphipathic and can partition into the bilayers` interfacial region. Tannic acid molecules are long enough to span the interbilayer space and, because they contain several gallic acids they can partition simultaneously into opposing bilayers, interacting with the lipid headgroups and covering the bilayer surface. It has been suggested that polyphenols form interbilayer bridges stabilised by increased adhesion from van der Waals interactions between opposing bilayers. Electrostatic interactions between π electrons in the phenol ring and trimethylammonium groups on PC headgroups decrease hydration repulsion between bilayers. Hydrogen bonds between the H-bond donating moieties on the polyphenol and H-bond accepting groups in the bilayer also contribute to bilayer stabilisation [60].

Since 3,4,5-tri-*O*-galloylquinic acid has a similar structure to the gallic acid moieties on tannic acid it is possible that the aforementioned interaction may also occur between 3,4,5-tri-*O*-galloylquinic acid and both PC and liposomes.

Gubernator et al. investigated the effect of alkylresorcinols, natural amphiphilic, compounds on liposomal permeability [61]. It was found that the effect of amphiphilic compounds on the liposomes depended on their localisation symmetry. When alkylresorcinols were applied to liposomes internally and externally, liposomes remained stable for 40 days, whereas control liposomes remained intact for only 10 days [61]. However, when the same compounds were applied externally only, liposomes were stable to a saturation point, after which an increase in the applied concentration resulted in an increase in membrane permeability correlating with a decrease in membrane stability. It was found that when these compounds were present in the external solution to the liposomes they would incorporate into the membrane and preferentially localise in the outer monolayer. When the number of alkylresorcinol molecules exceeded the limiting value the formation of non-bilayer structures was triggered and an increase in the membrane permeability was observed [61].

3.5 Effect of 3,4,5-tri-*O*-galloylquinic acid on lipid phase transition.

Since 3,4,5-tri-*O*-galloylquinic acid has been shown to maintain liposome structural integrity after desiccation and rehydration, it is possible that this compound affected the temperature of the phase transition between the liquid crystalline and the gel phases during desiccation (Fig 3.13). This possibility was investigated using Differential Scanning Calorimetry (DSC).

DSC accurately measures both the temperature at which lipid bilayers undergo a phase transition and the enthalpy of the transition. Two sample pans, one as a

reference and the other containing the sample are heated at an identical set rate. The sample pan requires more energy to heat to the same degree as the reference pan due to conformational changes occurring within the sample, and this required energy is recorded. It can therefore be used to detect phase separation of different lipid species within a mixture.

The DSC plot for desiccated liposomes is shown in Figure 3.21.

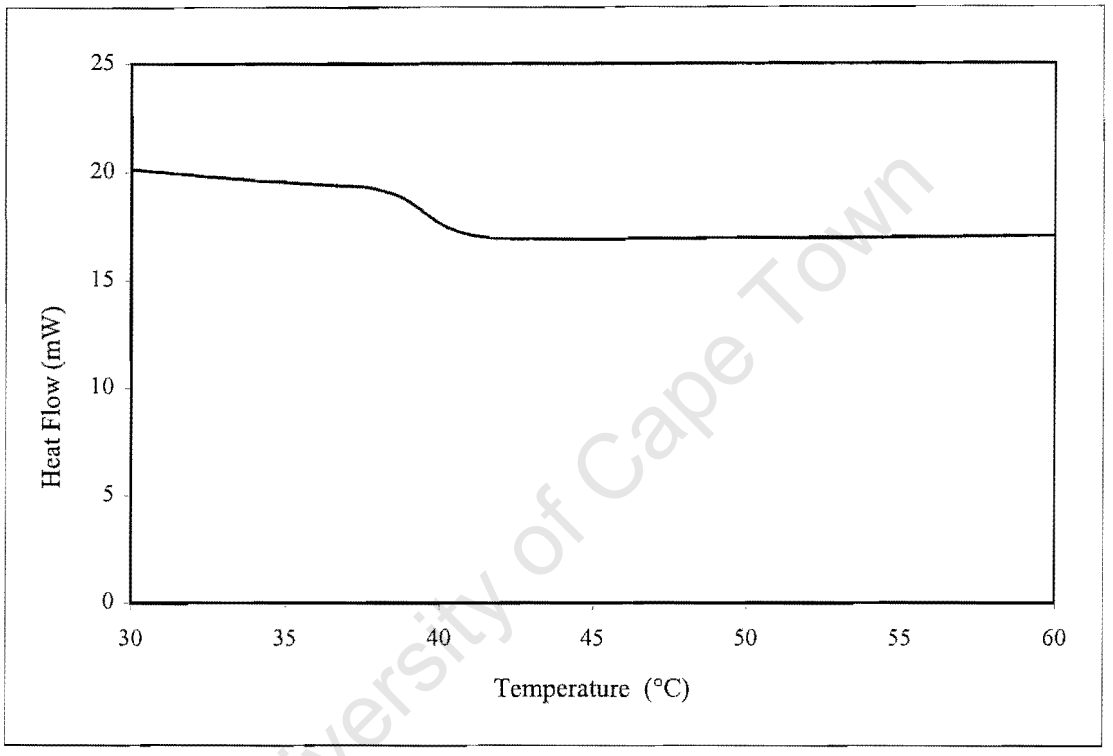


Figure 3.21. Differential Scanning calorimetry plot obtained for desiccated liposomes.

No transition was observed. This is in agreement with the findings of Chapman and Dodd (1971) who reported that it was difficult to interpret DSC data when dry mixtures of phosphatidylcholine together with cholesterol were investigated. However, the authors suggested that if the system was examined in the presence of excess water, a clearly defined interaction was observed [43].

DSC was therefore used to determine the T_m of hydrated liposomes (Figure 3.22).

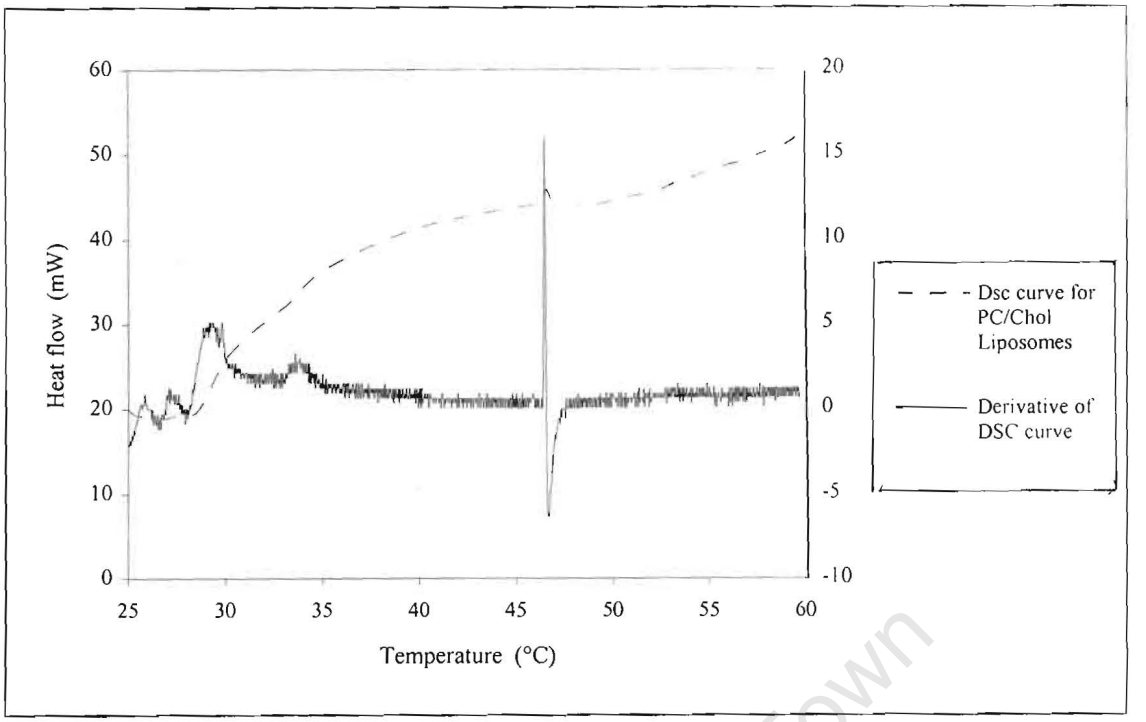


Figure 3.22. DSC curve for 1:1 Phosphatidylcholine/cholesterol liposomes.

Figure 3.22 shows the plot of heat flow versus temperature. A derivative of this data emphasises an event occurring at approximately 46 °C. The presence of this event in hydrated liposomes and its absence in desiccated liposomes is in agreement with the data reported by Chapman and Dodd (1971) [43]. Mabrey *et al.* (1978) [27] studied phase transitions in PC /cholesterol liposomes and reported that, with 20 mol % cholesterol in the membrane, a broad transition occurred between 41.0 and 41.6 °C. The temperature of this transition increased to 45.6 °C when the cholesterol concentration increased to 33 mol %. It remained detectable up to a cholesterol concentration of 50 mol % [27]. The transition observed at 46 °C with liposomes containing 50 mol % cholesterol in the present study agreed with these findings.

To determine the effect of 3,4,5-tri-*O*-galloylquinic acid on the phase transition of hydrated liposomes, the experiment was repeated with 1 μg polyphenol /μg PC, the previously determined optimum concentration for maintaining structural integrity

after desiccation and rehydration (Figure 3.16). The results for this experiment are shown in Figure 3.23.

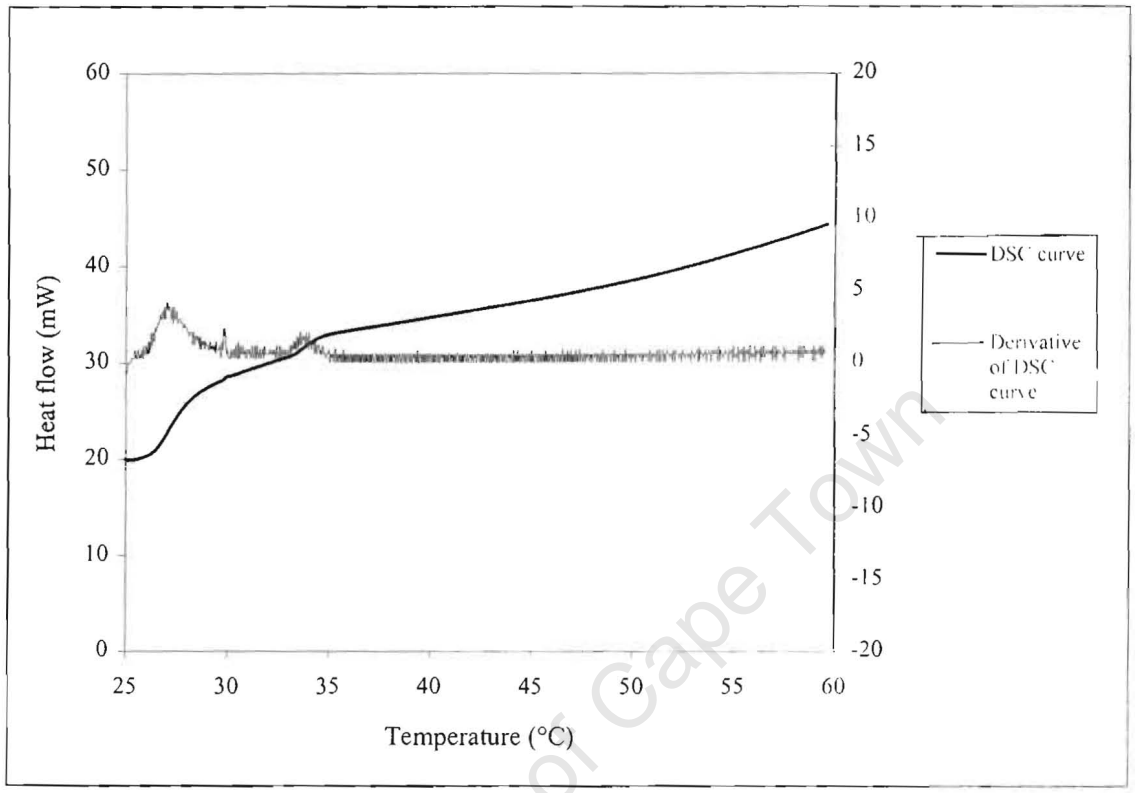


Figure 3.23. DSC plot of hydrated liposomes with 1 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC.

The DSC plots showed that no phase transition occurred as observed with hydrated liposomes alone. A derivative curve failed to show any fluctuations in heat flow in the range 40 – 50 °C. This would suggest that the presence of 3,4,5-tri-*O*-galloylquinic acid eliminated the phase transition of the liposome lipid bilayer, or that it was too small to be detectable.

DSC was repeated using the identical mass of 3,4,5-tri-*O*-galloylquinic acid in solution (Figure 3.24). No transition was observed.

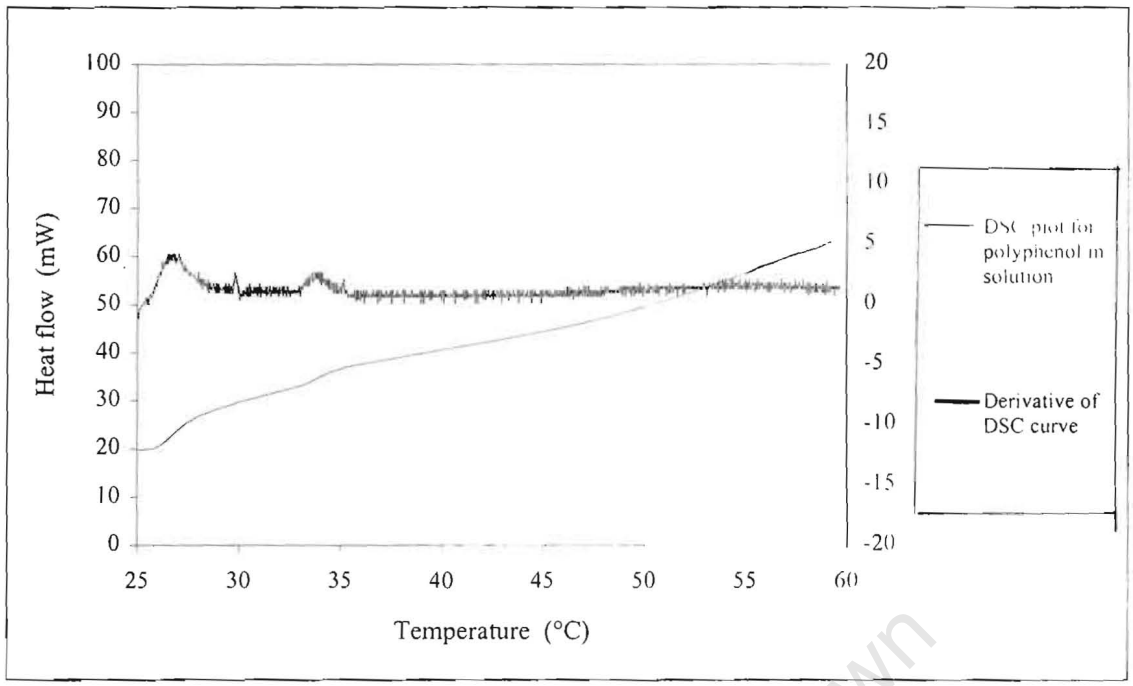


Figure 3.24. DSC plot including derivative curve for 3,4,5-tri-*O*-galloylquinic acid in solution.

In all three plots using hydrated samples (Figures 3.22, 3.23 and 3.24), peaks were observed between 25 and 35 °C. These may be due to the presence of water and reflect a heating effect. In contrast, no such peaks were found in desiccated liposomes. It is unlikely that these peaks are in any way related to phase transition.

CONCLUSIONS

A liposome preparation of 1:1 w/w phosphatidylcholine /cholesterol encapsulating calcein, a fluorescent marker, provided a model system for investigating the role of 3,4,5-tri-*O*-galloylquinic acid in the maintenance of liposomal membrane structural integrity after desiccation and rehydration. This model system was validated using externally applied trehalose which was found to maintain 29.2 ± 2.3 % of the membrane structural integrity at a concentration of 30 μg trehalose /μg PC.

The polyphenol 3,4,5-tri-*O*-galloylquinic acid extracted from leaves of the desiccation tolerant bush *M. flabellifolius* was applied externally to liposomes prior to desiccation and rehydration. This polyphenol in concentrations between 0.5 μg and 4 μg 3,4,5-tri-*O*-galloylquinic acid /μg phosphatidylcholine maintained liposomal integrity with a maximum maintenance of 17.7 ± 2.0 % at a concentration of 1μg 3,4,5-tri-*O*-galloylquinic acid /μg phosphatidylcholine. Above 5 μg 3,4,5-tri-*O*-galloylquinic acid /μg PC did not stabilise the integrity of the liposomal membrane. This maintenance was shown to be due to the entire 3,4,5-tri-*O*-galloylquinic acid molecule as no maintenance was achieved using commercial gallic acid, the major structural constituent of 3,4,5-tri-*O*-galloylquinic acid. Evidence of binding between liposomes and 3,4,5-tri-*O*-galloylquinic acid was observed to a saturation point, after which the free 3,4,5-tri-*O*-galloylquinic acid molecules in solution possibly preferentially adopted a micellar structure and molecules bound to liposomal membranes were released. Interactions between both hydrated and desiccated-rehydrated liposomes and 3,4,5-tri-*O*-galloylquinic acid were shown to correlate exactly with the concentration range at which 3,4,5-tri-*O*-galloylquinic acid maintained liposome structural integrity. At higher concentrations

there was no evidence of either an interaction or maintenance of structural integrity. Evidence obtained by DSC indicated that the interaction between the liposome membrane bilayer and 3,4,5-tri-*O*-galloylquinic acid eliminated the phase transition between gel and liquid crystalline phase. The mechanism whereby 3,4,5-tri-*O*-galloylquinic acid stabilises liposomal membrane structural integrity would thus appear to be by the elimination of this transition by interacting with the liposomal membrane in manner similar to that of trehalose. The data suggest that the interaction between 3,4,5-tri-*O*-galloylquinic acid and liposomal membrane lipids *in vitro* may be reflected *in vivo*. This polyphenol may play a role in maintenance of the membrane structural integrity of cells in *M. flabellifolius*. 3,4,5-tri-*O*-galloylquinic acid has been found to be stored in specialised vacuoles when leaves are hydrated [35]. It is conceivable that on dehydration these vacuoles become unstable releasing 3,4,5-tri-*O*-galloylquinic acid which consequently stabilise the cell membrane. Increases in the free 3,4,5-tri-*O*-galloylquinic acid in the cytoplasm might reverse the mechanism allowing 3,4,5-tri-*O*-galloylquinic acid to be released into micelles.

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6. APPENDICES

6.1 Extraction of polyphenols from *Myrothamnus flabellifolius*

Polyphenols were extracted and purified by a colleague using the following method [50]. 5g of crushed desiccated leaves were placed in a round-bottomed flask and extracted by sequentially refluxing at 60 °C for 1 hour with 100 ml of each of the following solvents: heptane (twice), 70 % v/v MeOH:H_2O (twice) and once with distilled water.

Extracts were filtered through Whatmann #1 filter paper and the residue dried at 50 °C for 10 minutes and re-extracted. Aliquots of extracts were analysed using a Novaspec UV-Vis spectrophotometer at 280nm and by TLC on pre-coated cellulose plates using butanol: Acetic acid: water v/v 4: 1: 5 as the mobile phase. The methanol/ water polyphenol extract was separated using cellulose column chromatography and purified by HPLC using a C18 column. Purified polyphenol was characterised as 3,4,5-tri-*O*-galloylquinic acid by ^{1}H NMR and MALDI-TOF spectroscopy. The chemical structure was determined (Figures a and b)

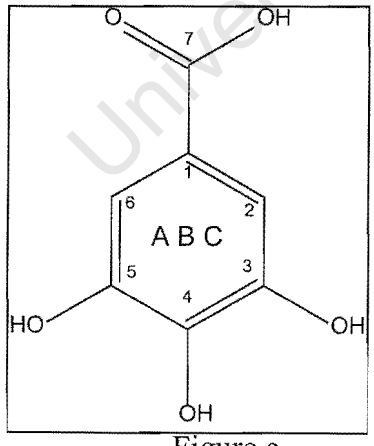


Figure a

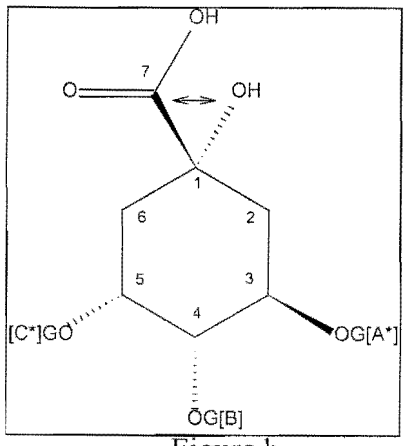
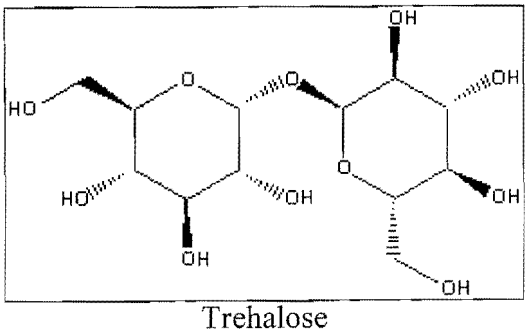


Figure b

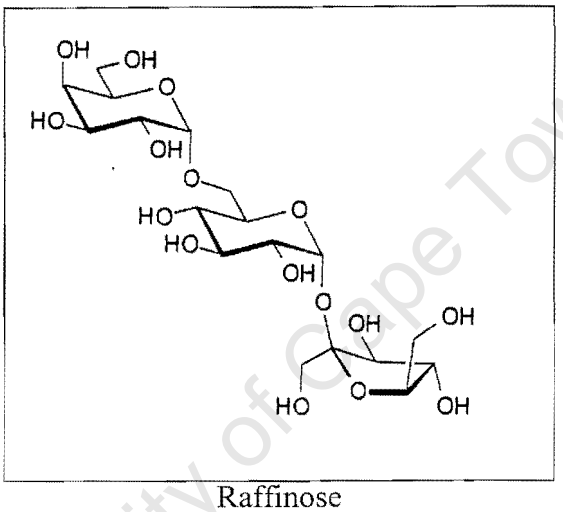
Figure a. Gallic acid (3,4,5-trihydroxybenzene)

Figure b. 3,4,5-tri-*O*-galloylquinic acid. (G=Galloyl group linked through C7)

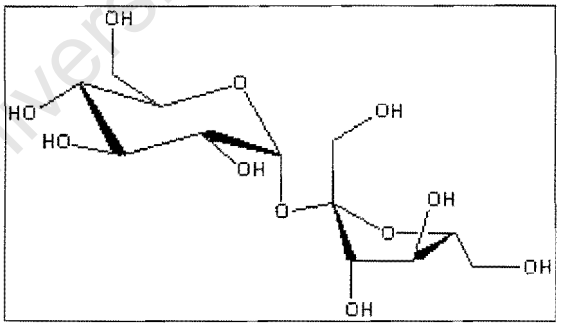
6.2 Chemical structures.



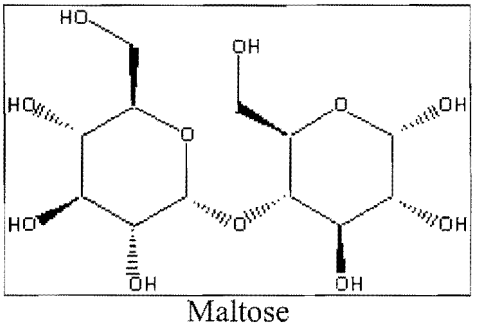
Trehalose



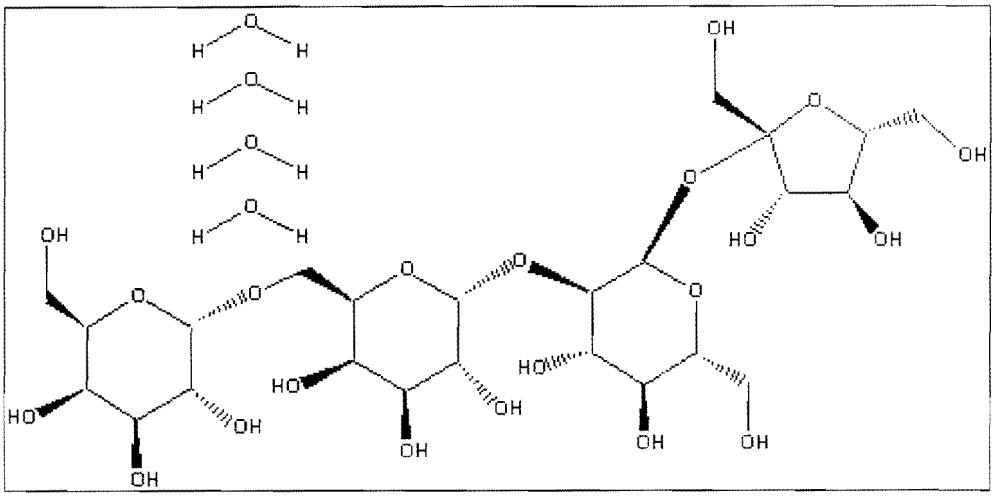
Raffinose



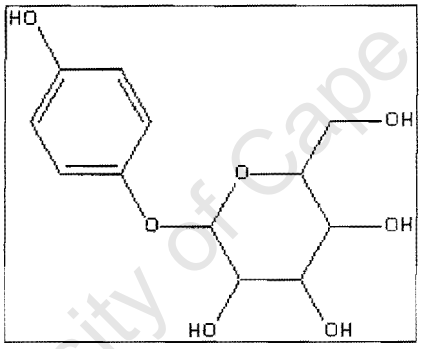
Sucrose



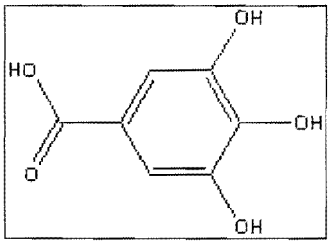
Maltose



Stachoyse tetrahydrate



Arbutin



Gallic acid

Structures available online from : http://chemfinder.cambridgesoft.com