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Analysis of Quil A–phospholipid mixtures using drift spectroscopy

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Abstract

The aim of this study was to investigate molecular interactions between Quil A and phosphatidylcholine in the solid state using diffuse reflectance infrared Fourier-transform spectroscopy (DRIFTS). Analysis of the interactions was characterized on the different regions of phosphatidylcholine: hydrophobic chain, interfacial and headgroup regions. The spectra of the hydrocarbon region of phosphatidylcholine alone compared to that for the binary mixture of Quil A and phosphatidylcholine were similar. These findings suggest that Quil A did not cause conformational disorder of the fatty acyl chains of the phospholipid. In contrast, a shift in the wavenumber of the choline group and a broad band in this moiety indicate a modification of the phospholipid in the headgroup region due to interaction between Quil A and phosphatidylcholine. These results suggest possibly ionic interactions between the negatively charged glucuronic acid moiety of the Quil A molecule with the positively charged choline group. The findings could also be the result of conformational changes in the choline group because of the intercalation of sugar moieties in Quil A between the choline and phosphate groups due to hydrogen bonding. Shift of wavenumbers to lower values on the carbonyl group was observed suggesting hydrogen bonding between Quil A and phosphatidylcholine. The difference in degrees of wavenumber shift (choline > phosphate > carbonyl group) and observed broad bands indicated that Quil A preferentially interacted with phosphatidylcholine on the hydrophilic headgroup. Cholesterol influenced such interactions at relatively high concentration (60%, w/w).

Keywords: Quil A; Phosphatidylcholine; Cholesterol; Infra-red spectroscopy

1. Introduction

Lipid-based systems such as liposomes and solid lipid particles or implants are of great interest in the drug delivery research as a means of providing sustained or targeted release of incorporated drugs (Singh and O'Hagan, 2002; Kersten and Crommelin, 2003; El-Aneed, 2004; Demana et al., 2005). In the last two decades, lipid systems containing Quil A have become an important class of delivery systems for protein and antigen delivery in the immunological field (Morein et al., 1984; Barr and Mitchell, 1996; Myschik et al., 2006a,b). The Quil A molecule belongs to a class of saponins that are isolated from the cortex of the South American tree *Quillaja saponaria* Molina (Dalsgaard, 1974; Höglund et al., 1989; Kensil, 1996). The chemical structure of Quil A is a complex mixture of many structurally related bisdesmosidic triterpenoid glycosides (Higuchi and Komori, 1987; Kensil et al., 1991). The different components of Quil A, however, differ mainly in the composition of their sugar moieties (Higuchi and Komori (1987)). Quillaja saponins have a five-ringed quillaic acid backbone with two carbohydrate chains, consisting of two to five sugar units, attached to the 3′ and 28′ carbons of the quillaic acid molecule (Higuchi and Komori, 1987; Ronnberg et al., 1997). Therefore, Quil A consists of hydrophobic aglycone triterpene and hydrophilic regions due to sugar moieties with multiple hydroxyl groups. This structure results in Quil A behaving as a surfactant forming micelles in water at a concentration of 0.03% (Özel et al., 1989).

Quillaja saponins are potent adjuvants that have been used in veterinary vaccines since the 1970's (Horzinek and Mussgay, 1971; Höglund et al., 1989; Barr and Mitchell, 1996). However,

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this type of saponins is able to interact with cholesterol in biological membranes as a surfactant, with subsequent disruption of the bilayer and the formation of pores (Glauert et al., 1962). The resulting haemolysis limits the use of non-purified Quil A fractions in human medicine (Kensil, 1996; Barr et al., 1998). The toxicity of Quil A, however, is much reduced if it is bound in the colloidal particles such as immune-stimulating complexes (ISCOMs) or ring-like micelles because the appearance of free Quil A is largely avoided (Kersten, 1990; Sjölander et al., 1998; Morein and Bengtsson, 1999; Demana et al., 2004a). Colloidal particles such as ring-like micelles were found to be less toxic compared to ISCOMs despite the fact that the micelles contained relatively high amounts of Quil A (Demana et al., 2004a). Highly purified Quil A fractions with much reduced toxicity have been reported in the literature (Ronnberg et al., 1995) and human clinical trials on the vaccines containing Quil A fractions have now been conducted (Kersten and Crommelin, 2003; Sanders et al., 2005).

The complexation of Quil A with model and natural lipid membranes has been studied mainly by negative staining transmission electron microscopy (TEM) (Glauert et al., 1962; Özel et al., 1989; Kersten et al., 1991; Demana et al., 2004b; Myschik et al., 2006a,b). Recent studies have revealed that depending on the mass ratios of Quil A, cholesterol and phospholipid, different colloidal structures such as ISCOM matrices, lamellae (hexagonal array of ring-like micelles), ring-like micelles, worm-like micelles, and lipidic/layered structures are formed in aqueous media (Demana et al., 2004b). However, the optimum mass ratios of Quil A, cholesterol and phospholipid for the formation of these colloidal structures are dependent on the preparation method (Demana et al., 2004c; Lendemans et al., 2005a; Myschik et al., 2006a; Pham et al., 2006).

Apart from mainly TEM investigation into the interaction of Quil A with phospholipids, there are few other analytical techniques that have been used to characterize these systems (Myschik et al., 2006a,b). Moreover, there has been hardly any research done on the solid state characterization of Quil A–lipid systems. Attempts to investigate Quil A–lipid systems in the solid state have begun recently (Demana et al., 2005). Differential scanning calorimetry (DSC), X-ray powder diffraction (XRPD) and scanning electron microscopy (SEM) were the analytical techniques used to characterize such systems (Demana et al., 2005). Despite these attempts, specific molecular interactions between Quil A and lipids used such as phosphatidylcholine however, could not be provided. Understanding molecular interactions of Quil A–lipid solid mixtures is very important because such mixtures can be used in the form of pellets or implants as antigen controlled or sustained delivery systems (Demana et al., 2005; Myschik et al., 2006b). It has been demonstrated that increasing amount of cholesterol in an ISCOM pellet resulted in a release of modified albumin in a controlled manner (Demana et al., 2005).

Despite this growing interest in the potential of solid ISCOMs or other related Quil A–lipid mixtures for use as controlled antigen delivery systems (Demana et al., 2005; Myschik et al., 2006b), there has been no work reported in the literature on the specific molecular interactions of Quil A with phospholipids especially in the solid state. Therefore, the aim of this study was to investigate molecular interactions between Quil A and phosphatidylcholine with or without cholesterol in the solid state using diffuse reflectance Fourier-infra red spectroscopy (DRIFTS). Diffuse reflectance sampling, although not without problems, seems to offer some advantages when compared to other solid-state sampling methods for infrared spectroscopy (Dijiba et al., 2005). DRIFTS is suited for analysis of this type of investigation as it does not rely on externally added probes that may influence membrane properties, and it gives information on different parts of the lipid molecule simultaneously (Taylor and Smith, 1980; Mantsch and McElhaney, 1991; Brandenburg et al., 1999; Popova and Hincha, 2003). We have therefore been able to investigate Quil A–phospholipid interactions at the levels of the carbonyl, the phosphate, and the choline groups, in addition to the fatty acyl chains.

2. Methods

2.1. Materials

Quil A was purchased from Superfos Biosector, Denmark. Cholesterol (purity approx. 99%) and L-α-phosphatidylcholine from egg yolk (purity approx. 99%) were purchased from Sigma Co., USA. Distilled deionised water having a conductivity of less than 0.1 μS (Milli-Q Water system, Millipore, Massachusetts, USA) was used throughout the study. All other chemicals and solvents were of at least analytical grade. The chemical structures of Quil A, cholesterol and phosphatidylcholine are shown in Fig. 1.

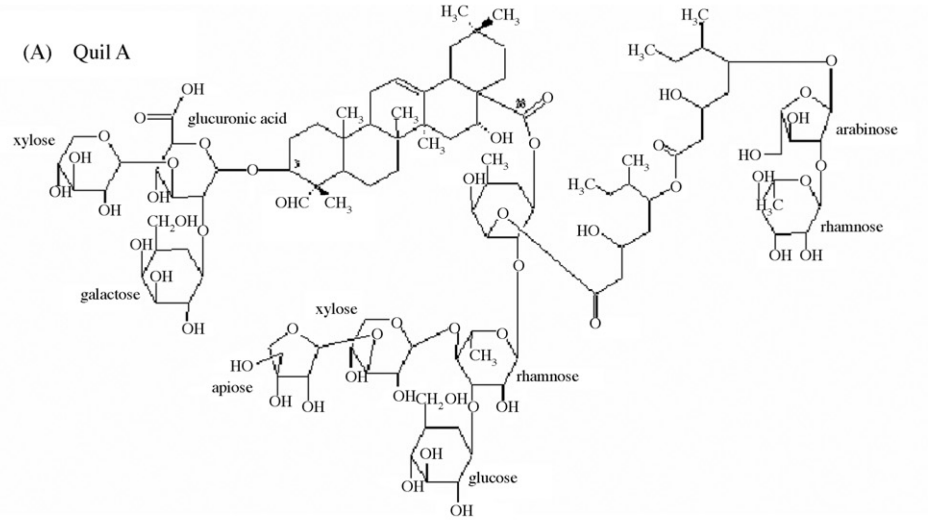
2.2. Preparation of lipid powder mixtures

Powders were prepared by either freeze-drying an aqueous dispersion of the lipids formed by hydrating a dried thin lipid film with an aqueous solution of Quil A or by physical mixing of lipid powders as described before (Demana et al., 2005). Table 1 summarizes compositions of the lipid powders investigated. Compositions were selected based on the pseudo-ternary phase diagram of aqueous mixtures of Quil A, cholesterol and phosphatidylcholine (Demana et al., 2004b). Based on the pseudo-ternary phase diagram, the selected mass ratios of lipids and corresponding formulations predominantly yield lipidic/layered structures, liposomes, ISCOMs, lamellae (hexagonal array of ring-like micelles) and worm-like micelles upon hydration (for formulations A–E, respectively) (Demana et al., 2004b). All formulations were prepared in triplicate.

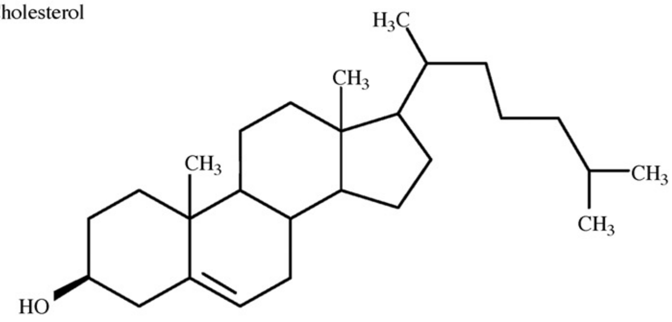
Table 1
Composition of the lipid powder mixtures used in the study (w/w, %)

Formulation	Quil A	Cholesterol	Phosphatidylcholine
A	70	–	30
B	–	60	40
C	40	20	40
D	30	60	10
E	70	30	–

(A) Quil A



(B) Cholesterol



(C) Phosphatidylcholine

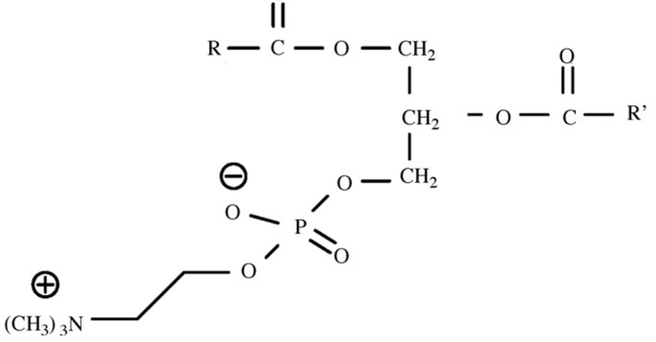


Fig. 1. Chemical structures of lipids used in this study: (A) purified Quil A fraction (Barr et al., 1998), (B) cholesterol and (C) phosphatidylcholine.

2.2.1. Preparation of lipid powder mixtures by freeze-drying of aqueous lipid dispersions

Required amounts of phosphatidylcholine and cholesterol were dissolved in 0.5 ml chloroform and evaporated to dryness at 45 °C for 1 h (Rotavapor R110, Büchi, Switzerland). The lipid films formed were hydrated for 2 h at room temperature with distilled water containing required amounts of Quil A. The total concentration of lipids in water was 6.7 mg/ml. The samples were subsequently freeze-dried for 48 h (Freezone 6, Model 79340, Labconco, Missouri, USA) at a condenser temperature of −82 °C and a pressure of less than 10^{-1} mbar. All samples were stored at 4 °C in a dessicator until required.

2.2.2. Preparation of lipid powder mixtures by physical mixing

Required amounts of Quil A, cholesterol and phosphatidylcholine were weighed (Sartorious Electronic Ultramicrobalance, Model 4431 MP8, Sartorious Gmbh, Gottingen, Germany) and mixed thoroughly by light trituration for a total period of 3 min using a mortar and pestle. Solid phosphatidylcholine was obtained by evaporation of chloroform to dryness overnight under vacuum (Speed Vac Concentrator, SVC-200H, Savant Instrument, USA). Various amounts of Quil A, cholesterol and phosphatidylcholine were weighed and mixed thoroughly by light trituration for a total period of 3 min with mortar and pestle. All samples were stored at 4 °C in a dessicator until required.

2.3. DRIFT spectroscopy

DRIFTS was performed on a Bio-Rad FTC 175C dynamic alignment FTIR spectrophotometer (Cambridge, USA) using diffuse reflectance accessory (Pike Technology, Madison, USA). The powder samples were diluted to 5% (w/w) in potassium bromide (Sigma Chemical Co.) and ground to uniform size in a mortar and pestle for 2 min. The powder surface was smoothed with a spatula. Samples were prepared and measured in triplicate. The powder samples were scanned in a sample cup containing approximately 100 mg of sample. The single-beam spectra were recorded against potassium bromide background in Kubelka–Munk units (Berbenni et al., 2001). Spectra were obtained from 16 iterative scans over the wavenumber range from 4000 to 500 cm^{-1}. Data was captured using a PC and Win-IR software.

3. Results and discussion

3.1. DRIFT spectra of phosphatidylcholine, cholesterol and Quil A

The DRIFT spectra of phosphatidylcholine, cholesterol and Quil A are shown in Fig. 2. The assignment of DRIFT bands for phosphatidylcholine and cholesterol is summarized in Table 2. According to the molecular structure of phospholipids, vibrational spectroscopy presents various peaks which can be classified into three regions: the hydrophobic chain involving the C–H stretching modes and CH$_2$ scissoring, the interfacial region consisting of C=O stretching, and the polar headgroup region comprising of PO$_2^-$ and ($^+$N(CH$_3$)$_3$) asymmetric stretching bands respectively (Bush et al., 1980; Akutsu, 1981; Mantsch and McElhaney, 1991; Sailer et al., 1997; Popova and Hincha, 2004; Tantipolphan et al., 2006).

The positions of various bands in the spectrum of egg phosphatidylcholine (Fig. 2) are similar to those reported by others (Verma and Wallach, 1973; Hincha and Crowe, 1996; Popova and Hincha, 2004; Cacela and Hincha, 2006). The intense bands are usually found at wavenumbers of about 2850 and 2920 cm^{-1} in the C–H stretching region and are assigned to symmetric and antisymmetric methylene stretching, respectively (Mantsch and McElhaney, 1991; Popova and Hincha, 2004; Cacela and Hincha, 2006). The band at 2956 cm^{-1} is attributed to terminal methyl asymmetric stretching (Cameron et al., 1980; Mantsch and McElhaney, 1991). The C–H stretching band arising from the –CH=CH– moiety is located at about 3010 cm^{-1} (Mendelsohn et al., 1984; Mantsch and McElhaney, 1991). The CH$_2$ scissoring peaks are centered at a peak of about 1468 cm^{-1} with a shoulder peak at 1460 cm^{-1} (Hincha and Crowe, 1996).

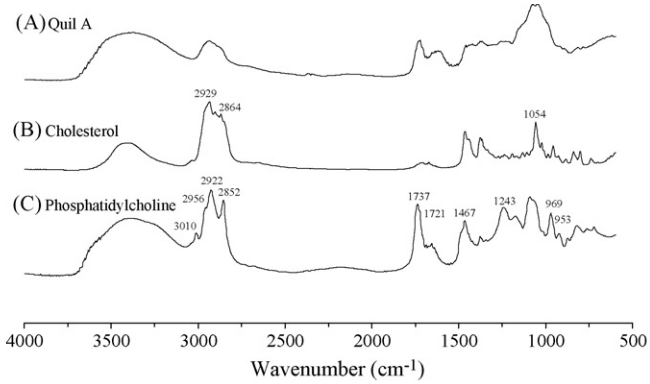


Fig. 2. DRIFT spectra of lipid powders investigated in this study: (A) Quil A, (B) cholesterol and (C) phosphatidylcholine.

Table 2
Assignment of DRIFT bands observed in phosphatidylcholine and cholesterol powder

Band position (cm^{-1})	Reference	Assignment
Phosphatidylcholine		
3010	3010[a,b]	–CH=CH stretching
2956	2956[a,b]	–C–H asymmetric
2922	2920[b,c]	–CH$_2$ antisymmetric
2852	2850[a,b,c,d]	–CH$_2$ symmetric
1737	1738[e,f,g]	–C=O stretching
1721	1720[e]	–C=O stretching
1467	1468[c,h]	–CH$_2$ scissoring
1243	1247[e]	–P=O asymmetric
969	968[d,e]	–$^+$N(CH$_3$)$_3$ asymmetric
Cholesterol		
2929	2935[i,j,k]	–CH$_2$ symmetric
2864	2868[i,k]	–CH$_3$ asymmetric
1054	1055[c]	–C–O–C symmetric

a: Mantsch and McElhaney (1991), b: Mendelsohn et al. (1984), c: Umemura et al. (1980), d: Popova and Hincha (2004), e: Popova and Hincha (2003), f: Cacela and Hincha (2006), g: Bush et al. (1980), h: Hincha and Crowe (1996), i: Faiman (1977), j: Hattab et al. (2006), k: Daghbouche et al. (1997).

The C=O stretching band for dry egg phosphatidylcholine was located at 1737 cm^{-1} with a weak shoulder at 1721 cm^{-1} (Fig. 2). This is consistent with literature in which dry egg phosphatidylcholine showed a well-defined peak at about 1738 cm^{-1} and a weak shoulder peak at lower wavenumbers of approximately 1720 cm^{-1} (Popova and Hincha, 2003; Cacela and Hincha, 2006). It is well known that the higher wavenumber band component at about 1740 cm^{-1} is due to non-hydrogen-bonded C=O groups whereas the lower wavenumbers are as a result of hydrogen-bonded C=O groups (Blume et al., 1988; Lewis and McElhaney, 1998; Arrondo and Goni, 1998; Popova and Hincha, 2004; Cacela and Hincha, 2006). The observation of C=O stretching at lower wavenumber of 1721 cm^{-1} (Fig. 2) would therefore imply that phosphatidylcholine used in this study was not completely anhydrous. Moisture content on the phosphatidylcholine used in this study was however, not investigated. Similar results on egg phosphatidylcholine in the dry or solid state have also been reported in the literature in which the lower wavenumbers observed for C=O stretching were attributed to hydrogen bonding due to residual water molecules trapped in the phospholipid (Popova and Hincha, 2003).

For the polar headgroup of dry egg phosphatidylcholine, the band associated with asymmetric P=O stretching vibration is located at about 1261 cm^{-1} (Cacela and Hincha, 2006), 1250 cm^{-1} (Popova and Hincha, 2004) or 1247 cm^{-1} (Popova and Hincha, 2003). In this study, the asymmetric P=O stretching vibration of egg phosphatidylcholine was found to be approximately 1243 cm^{-1} (Fig. 2). The different values reported in the literature above 1240 cm^{-1} reflect various degrees of hydration of dry phospholipid molecules (Cacela and Hincha, 2006). This is because numerous studies have shown that hydration shifts the asymmetric P=O stretching vibration to lower wavenumbers (Crowe et al., 1988; Wong et al., 1989; Hübner and Blume, 1998; Lewis and McElhaney, 1998; Popova and Hincha, 2003). The main band associated with the other polar headgroup of phosphatidylcholine (i.e. ($^+N(CH_3)_3$)), was found at 969 cm^{-1} with a weak shoulder peak at about 953 cm^{-1} (Fig. 2). These results are consistent with literature for the anhydrous egg phosphatidylcholine in which the main peak was observed at about 968 cm^{-1} with a weak shoulder peak at about 955 cm^{-1} (Grdadolnik and Hadzi, 1993; Popova and Hincha, 2003, 2004). When $^+N(CH_3)_3$ is involved in dipolar interactions, the position of the band at about 968 cm^{-1} is shifted to higher wavenumbers (Akutsu, 1981; Matsuzaki et al., 1991; Popova and Hincha, 2003, 2004).

The spectrum of cholesterol shown in Fig. 2 is consistent with other studies (Daghbouche et al., 1997; Williams et al., 1998; Hattab et al., 2006; Laugel et al., in press). It shows two regions of strong bands which are the C–H stretching modes between 2700 and 3100 cm^{-1} and the CH_2 scissoring bands between 1400 and 1500 cm^{-1} (Faiman, 1977). The strong bands at 2864 and 2929 cm^{-1} are due to symmetric C–H stretching as they are found in the region between 2850 and 2935 cm^{-1} (Daghbouche et al., 1997; Hattab et al., 2006; Laugel et al., in press). Cholesterol presents also an intense band between 1000 and 1100 cm^{-1} (Williams et al., 1998), and more specifically at about 1055 cm^{-1} (Umemura et al., 1980). This band was found at 1054 cm^{-1} (Fig. 2), and it corresponds to the C–O–C stretching (Roberts et al., 1958; Umemura et al., 1980). The stretching in the region wavenumbers of about 3500–3600 cm^{-1} is due to the OH group (Williams et al., 1998; Hattab et al., 2006; Laugel et al., in press).

The infra-red spectrum of Quil A has not been previously reported in the literature. The spectrum of Quil A depicts a broad band at wavenumber range between 2700 and 3100 cm^{-1} (Fig. 2). In numerous vibrational spectroscopic studies, this region is attributed to C–H stretching for variety of lipid molecules (Faiman, 1977; Casal et al., 1980; Naumann et al., 1987; Mantsch and McElhaney, 1991; Brandenburg and Seydel, 1998; Castano and Desbat, 2005). In contrast to phosphatidylcholine and more especially cholesterol, there is relatively a lack of number of well-defined sharp peaks both in the C–H stretching region (2700 and 3100 cm^{-1}) and the fingerprint region (500–1500 cm^{-1}) in the case of Quil A molecule (Fig. 2). Several distinct peaks in the C–H stretching region is known to depict a highly defined crystal structure, and this region becomes smoothed if the molecule is in the amorphous state (Crowe, 2002). Similarly, drug-free solid dispersions were found to exhibit relatively broad peaks in the region between 500 and 4000 cm^{-1} compared to the dispersions containing drug (Vippagunta et al., 2002). This observation was also attributed to the amorphous nature of the drug-free solid dispersions. Therefore, the lack of number of sharp distinct peaks in the C–H stretching region and also in the fingerprint region observed for Quil A suggests that this molecule exists largely in the amorphous solid state. These findings support recent studies using XRPD and DSC analytical techniques in which solid Quil A was demonstrated to be amorphous (Demana et al., 2005).

The broad band between 3200 and 3600 cm^{-1} in Quil A is most likely due to multiple OH groups present in the sugar moieties of the Quil A molecule (Fig. 1). Similar findings with other glycolipids, pure sugars and cyclodextrins have been reported in which the broad band stretching in this region was due to the presence of OH groups in the sugar moieties (Brandenburg and Seydel, 1998; Williams et al., 1998; Crowe, 2002; Wolkers et al., 2004).

3.2. Interaction of Quil A with the fatty acyl chains of phospholipid

The C–H stretching vibration in the region of 2700–3100 cm^{-1} for pure phosphatidylcholine and Quil A/phosphatidylcholine mixture (70:30%, w/w) is shown in Fig. 3A. In phosphatidylcholine, it is well known that the CH_2 antisymmetric mode at about 2920 cm^{-1} and the CH_2 symmetric stretching at 2850 cm^{-1} are the strongest bands (Umemura et al., 1980; Kodati et al., 1994; Cannon et al., 2003) in this region. The band at 2850 cm^{-1} however, is the most used spectral band for determining lipid hydrocarbon chain disorder (Casal et al., 1989; Paré and Lafleur, 1998; Popova and Hincha, 2004; Cacela and Hincha, 2006). The frequency of this band is very sensitive to acyl chain conformation (Popova and Hincha, 2003). In the gel or solid state, the fatty acyl chains are tightly packed in all-trans conformation. Disruption of the regular chain packing by either melting or dissolution is accompanied by an increase in the wavenumber of the CH_2 symmetric stretching band at 2850 cm^{-1} (Casal et al., 1980; Kodati et al., 1994; Popova and Hincha, 2004; Cacela and Hincha, 2006). Similar effects have also been observed for the antisymmetric band at 2920 cm^{-1} following disruption of the regular chain packing (Mendelsohn et al., 1984; Mantsch and McElhaney, 1991).

On the addition of Quil A to phosphatidylcholine, the position at 2852 cm^{-1} (CH_2 symmetric) was unaffected (Fig. 3A). Similar findings were also observed for the CH_2 antisymmetric band at 2922 cm^{-1} (Fig. 3A). The lack of change in the wavenumbers of these bands suggests that the addition of Quil A to phosphatidylcholine at a concentration of 70% (w/w) resulted in no conformational disorder of the fatty acyl chains. The CH_2 scissoring bands at wavenumber region between 1450 and 1480 cm^{-1} were also investigated (Fig. 3B) to assess the order/disorder characteristics of the acyl chain packing (Umemura et al., 1980; Hincha and Crowe, 1996; McMullen et al., 2000). The peak stretching band of the CH_2 scissoring bands for most diacyl phospholipids including egg phosphatidylcholine is normally found at a wavenumber of about 1468 cm^{-1} (Umemura et al., 1980; Mantsch et al., 1981; Hincha and Crowe, 1996; McMullen et al., 2000) with a weak shoulder peak at about 1460 cm^{-1} (Hincha and Crowe, 1996). The results demonstrated in Fig. 3B (i) show that the band for pure phosphatidylcholine was observed at 1467 cm^{-1} with a shoulder peak at 1459 cm^{-1}. These values are clearly very close to the reported data in the lit-

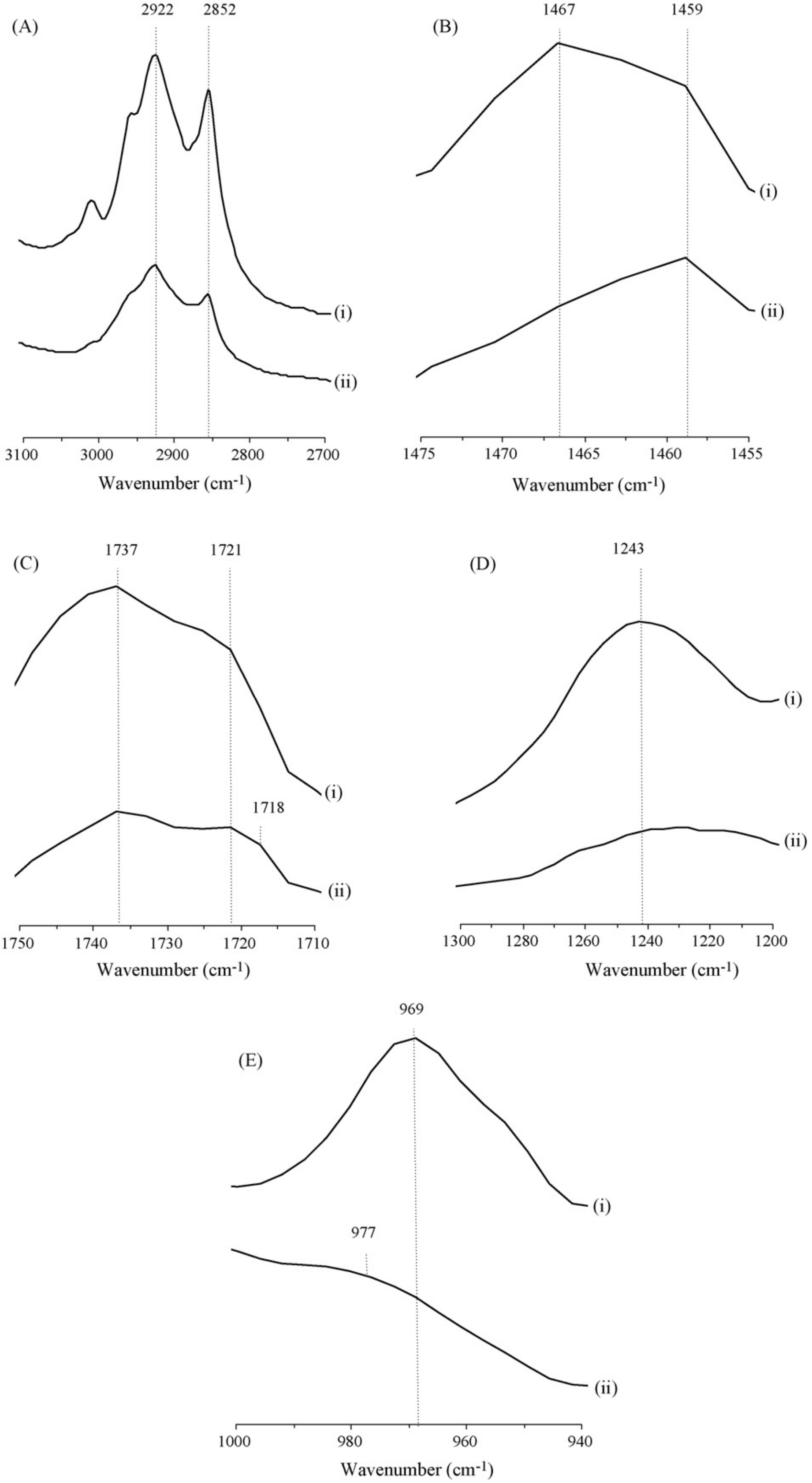


Fig. 3. DRIFT spectra of (i) phosphatidylcholine and (ii) Quil A–phosphatidylcholine mixture (70:30%, w/w) at (A) C—H stretching, (B) CH_2 scissoring, (C) C═O stretching, (D) P═O stretching and (E) $^{+}N(CH_3)_3$ stretching modes.

erature. The wavenumbers of these bands did not change when Quil A was added to phosphatidylcholine at a concentration of 70% (w/w) (Fig. 3B (ii)). These results therefore confirm again that this molecule did not cause conformational disorder of the fatty acyl chains. Minor alterations in the spectrum of binary mixtures of Quil A/phosphatidylcholine are likely to originate solely from the different ratios of characteristic peaks from each component in the lipid mixtures at particular concentration in agreement with other similar studies (Tantipolphan et al., 2006).

3.3. *Interaction of Quil A with the carbonyl group region of phospholipid*

The results for the interaction of Quil A and phosphatidylcholine on the carbonyl group are presented in Fig. 3C. In the presence of Quil A, the band at 1737 cm^{-1} for C=O stretching was not shifted. However, the band at 1721 cm^{-1} has become broader with an appearance of a weak shoulder peak at 1718 cm^{-1} (Fig. 3C (ii)). The shoulder peak was not observed in the spectrum of phosphatidylcholine without Quil A. These results therefore suggest an interaction between the C=O group of phosphatidylcholine and Quil A. Such interaction may be due to hydrogen bonding between the OH groups present in the sugar moieties of Quil A molecule and the C=O group of phospholipid. Other investigators have previously attributed hydrogen bonding between the C=O group of phosphatidylcholine and the OH groups of the sugar moieties in trehalose, sucrose and oligosaccharides (Crowe, 2002; Wolkers et al., 2004; Cacela and Hincha, 2006).

3.4. *Interaction of Quil A with the polar head groups of phospholipid*

The polar head groups of phospholipid assessed for interaction with the Quil A were the asymmetric P=O and $^+N(CH_3)_3$ stretching bands. The peak asymmetric P=O stretching band for phosphatidylcholine alone was located at a wavenumber of about 1243 cm^{-1} (Figs. 2 and 3D). However, in the presence of Quil A this band was relatively much broader and the peak appears to have shifted to lower wavenumbers (Fig. 3D (ii)). The broad band observed implies extensive hydrogen bonding due to interaction of possibly multiple OH groups of the sugar moieties of Quil A with the P=O group of the phospholipid.

For the choline group, the peak asymmetric ($^+N(CH_3)_3$) stretching band was found at 969 cm^{-1} (Figs. 2 and 3E). The addition of Quil A to phosphatidylcholine however, resulted in a spectrum which is clearly different from that of phosphatidylcholine alone (Fig. 3E (ii). The peak band at 969 cm^{-1} observed for pure phosphatidylcholine was distorted in the presence of Quil A. This sharp peak became relatively a much broad band. Furthermore, the stretching band had shifted to higher wavenumbers with a centre peak of about 977 cm^{-1}. These findings demonstrate strong interactions between Quil A and choline group. An upshift of wavenumber of the asymmetric ($^+N(CH_3)_3$) stretching band for egg phosphatidylcholine in the presence of low amounts of sucrose have been reported recently (Cacela and Hincha, 2006). The results obtained in such studies indicated that the sugars interacted with the choline groups of dry phosphatidylcholine (Cacela and Hincha, 2006). The authors suggested that the shifts in the choline band positions observed could be the result of conformational changes in the choline group because of the intercalation of sugar molecules between the choline and phosphate groups due to hydrogen bonding. Similar conclusions to those described above (Cacela and Hincha, 2006) with regard to hydrogen bonding can therefore also be applicable in this study since Quil A has many sugar moieties such as glucose, xylose, galactose, rhamnose, etc. (Fig. 1). However, Quil A is a negatively charged molecule (Kersten et al., 1991; Lendemans et al., 2005b) due to presence of a glucuronic acid in its structure. Therefore, it is possible that ionic interactions between the negatively charged glucuronic acid moiety of Quil A and the positively charged choline group of phosphatidylcholine could have contributed to broad stretching of the asymmetric ($^+N(CH_3)_3$) band.

Although Quil A is a negatively charged glycolipid molecule, dipolar interactions have been reported in the literature between neutral glycolipids and phosphatidylcholine (Popova and Hincha, 2004). In egg phosphatidylcholine in the solid state, digalactosyldiacylglycerol (a neutral glycolipid molecule) was reported to shift the peak asymmetric ($^+N(CH_3)_3$) stretching band to higher wavenumbers and hence indicating dipolar interactions between the sugars and choline (Popova and Hincha, 2004). Therefore, dipolar interactions are also more likely to occur between the sugars moieties of Quil A and the choline group. An increase in the wavenumber of asymmetric ($^+N(CH_3)_3$) peak after hydration of phosphatidylcholine has also been attributed to dipolar interactions between the water molecules and the choline group (Akutsu, 1981; Grdadolnik et al., 1991; Pohle et al., 2004).

3.5. *Interaction of Quil A with phospholipid and cholesterol*

As described in Section 1, it is well known that the interaction of Quil A with phospholipid and cholesterol in aqueous media produce different colloidal structures such as ISCOMs, ring-like micelles, lamellae (hexagonal array of ring-like micelles) and worm-like micelles (Demana et al., 2004b; Myschik et al., 2006a,b). In the absence of cholesterol, none of these colloidal structures are formed suggesting the significant role this molecule plays in the formation of such particles. The interaction of Quil A with phospholipid and cholesterol was therefore investigated in this study albeit in the solid state (Fig. 4).

Fig. 4A shows spectra in the C–H stretching region of ternary mixtures composed of Quil A with phosphatidylcholine and cholesterol. Spectra of pure phosphatidylcholine and binary mixtures composed of cholesterol and phospholipid are also shown for comparison purposes. The positions at 2852 and 2922 cm^{-1} are generally the same in all mixtures suggesting that Quil A did not cause disorder of the fatty acyl chain packing even in the presence of cholesterol. However, there is slightly a broad band at 2852 cm^{-1}, and a small shift at 2922 cm^{-1} to higher wavenumbers for lipid mixture with 60% (w/w) cholesterol in the presence of Quil A and phosphatidylcholine (Fig. 4A (iv)). This observation implies a slight disorder

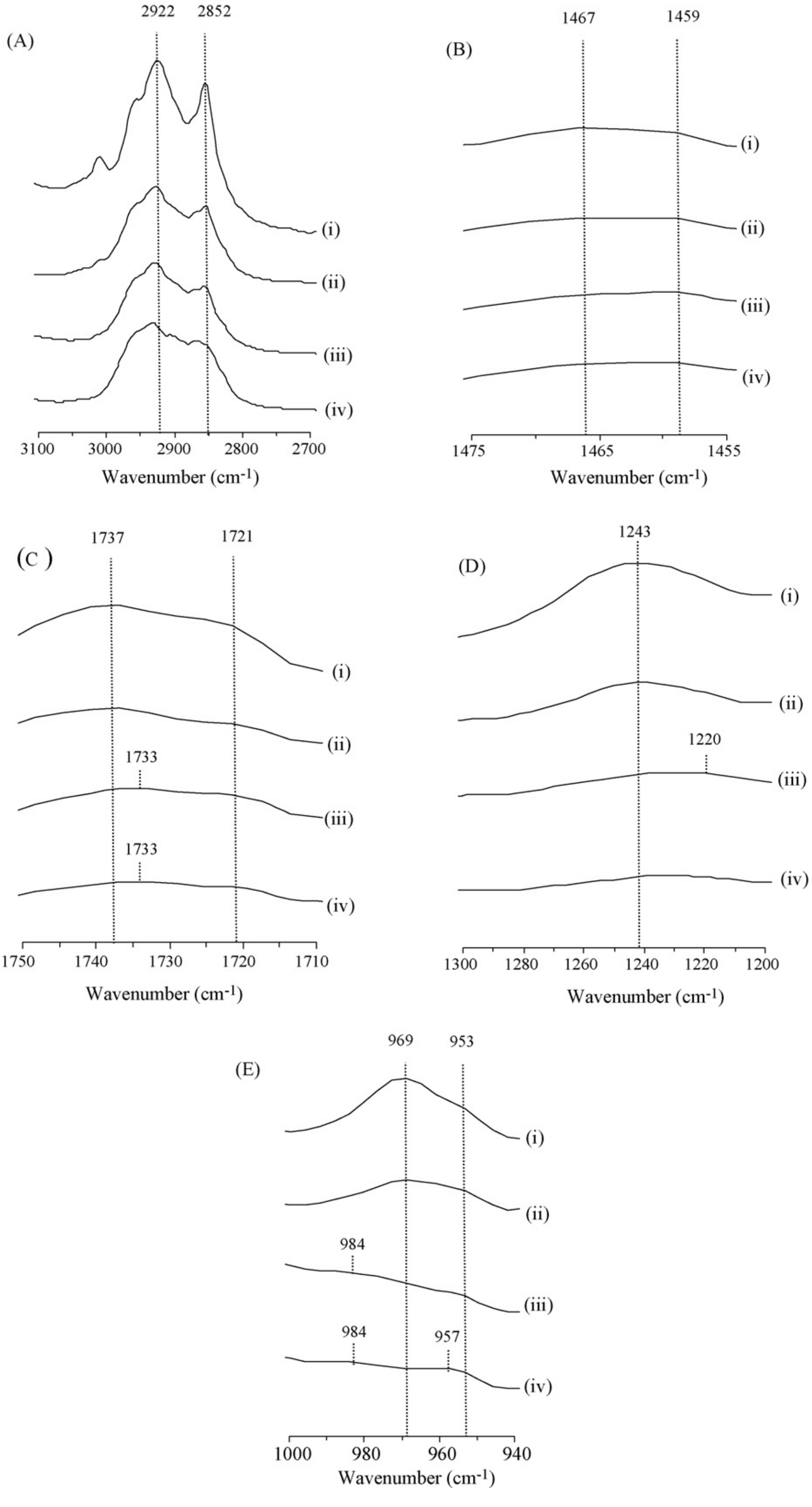


Fig. 4. DRIFT spectra of (i) phosphatidylcholine (ii) cholesterol–phosphatidylcholine mixture (60:40%, w/w) (iii) Quil A–cholesterol–phosphatidylcholine mixture (40:20:40%, w/w) and (iv) Quil A–cholesterol–phosphatidylcholine mixture (30:60:10%, w/w) at (A) C—H stretching, (B) CH_2 scissoring, (C) C═O stretching, (D) P═O stretching and (E) $^{+}N(CH_3)_3$ stretching modes.

of phosphatidylcholine fatty acyl chains possibly resulting from different conformation of Quil A molecule within phospholipid at a cholesterol concentration of 60% (w/w) although the positions for the CH_2 scissoring peaks at 1467 and 1459 cm^{-1} appear to be the same (Fig. 4B).

As described earlier, the position for C=O stretching at 1737 cm^{-1} was found not to have changed in the presence of Quil A, although noticeable effects were observed at lower wavenumbers (Fig. 3C). On the other hand, the interaction of Quil A with both phosphatidylcholine and cholesterol on the C=O group at 1737 cm^{-1} or lower wavenumbers (1721 cm^{-1}) was not very clear (Fig. 4C). On closer examination, the inclusion of cholesterol in the presence of Quil A and phosphatidylcholine appears to result in the band at 1737 cm^{-1} shifting to lower wavenumbers of about 1733 cm^{-1} (Fig. 4C (iii) and (iv). This observation suggests that when cholesterol is included in the mixture, there is possibly different conformation or orientation of Quil A molecule on the carbonyl group region of phosphatidylcholine.

The results for the asymmetric P=O stretching in ternary mixtures of Quil A, phosphatidylcholine, and cholesterol are presented in Fig. 4D. Similar results as those described above for C=O stretching were observed. Although the spectrum of Quil A/phosphatidylcholine mixture (Fig. 3D (ii)) looks similar to that containing both Quil A, phosphatidylcholine and cholesterol, there is however, a ‘flat’ broad band in the region between 1239 and 1220 cm^{-1} in the spectrum of the mixture containing cholesterol on closer examination, particularly on Fig. 4D (iii). This result was probably due to cholesterol effect in the mixture containing Quil A and phosphatidylcholine although the concentration of the lipids used were not the same.

The asymmetric ($^{+}N(CH_3)_3$) stretching band appears to be strongly distorted in the presence of both Quil A and cholesterol (Fig. 4E (iii and iv)). The main band at 969 cm^{-1} has resulted in a broad band with a peak at about 984 cm^{-1}. This is clearly demonstrated with a formulation containing high concentration of cholesterol in the presence of both Quil A and phosphatidylcholine (60%, w/w) (Fig. 4E (iv)). With this formulation, the shoulder peak at 953 cm^{-1} has also shifted to higher wavenumber of about 957 cm^{-1}(Fig. 4E (iv)). A shift to higher wavenumbers in this region is characteristic of dipolar interactions (Akutsu, 1981; Grdadolnik et al., 1991; Pohle et al., 2004), and therefore cholesterol appears to be influencing such interactions when it is mixed together with Quil A and phosphatidylcholine. It may also be possible that the OH group of cholesterol is involved in the conformational changes in the choline group due to reported intercalation of sugar molecules (Quil A has sugar moieties) between the choline and phosphate groups due to hydrogen bonding (Cacela and Hincha, 2006). The DRIFT analysis on the differences observed resulting from different mass ratios of Quil A, cholesterol and phosphatidylcholine in the solid state may therefore explain partly the different colloidal structures that are known to form upon hydration. It would however, be of paramount importance to analyze spectra of the hydrated formulations as to investigate the effect of hydration on the molecular interaction of Quil A, cholesterol and phosphatidylcholine. In general, the results demonstrated in Fig. 4 appear to indicate the importance of cholesterol in the mixtures containing phosphatidylcholine and cholesterol, and hence in ISCOMs formation. However, the influence of cholesterol in these lipid mixtures was not very clear in the present study. This could be possibly due to preparation method and/or limitation of the analytical equipment used. The use of NMR or possibly Raman spectroscopy (Sujak et al., 2005; Tantipolphan et al., 2006) and dialysis preparation method (Lendemans et al., 2007) could therefore be used in future to complement the current data.

3.6. *Effect of preparation methods on the DRIFT spectra of Quil A/phosphatidylcholine mixtures*

The degree of molecular interaction between phospholipid and cholesterol was found to vary depending on the preparation method used to prepare the lipid mixtures (Tantipolphan et al., 2006). XRPD and DSC analytical techniques were recently used to analyze Quil A–phospholipid mixtures prepared by freeze-drying of aqueous lipid dispersions and physical mixing methods (Demana et al., 2005). It was found that the method of freeze-drying aqueous lipid dispersions was better in promoting molecular interaction compared to the method of physical mixing the lipids (Demana et al., 2005). Comparisons between the two different preparation methods were also made in this study but using IR spectroscopic technique as this method of analysis is known to be capable of showing subtle differences at molecular level due to absorption bands characteristic of specific functional groups (Umemura et al., 1980; Mantsch et al., 1981; Levin, 1984).

The DRIFT spectra of Quil A/phosphatidylcholine mixture prepared by the methods of freeze drying aqueous lipid dispersions and physical mixing are shown in Fig. 5. No significant modifications could be obtained in the hydrophobic regions at 2922, 2852 and 1459 cm^{-1} (Figs. 5A and B) suggesting that Quil A did not induce disorder of the fatty acyl chains of phosphatidylcholine irrespective of the preparation method. Differences between the two preparation methods are however, observed on the C=O stretching regions (Fig. 5C). Although both preparation methods show spectra of the C=O stretching peaks at 1737 and 1721 cm^{-1}, only the mixture prepared by freeze-drying aqueous lipid dispersion reveals a weak shoulder peak at 1718 cm^{-1} (Fig. 5C (i)) suggesting an interaction between Quil A and phosphatidylcholine. These results therefore imply good mixing of Quil A and phosphatidylcholine if such mixtures are prepared by the method of freeze-drying aqueous lipid dispersion and hence support recent findings using other analytical techniques such as XRPD and DSC (Demana et al., 2005).

The spectra at the asymmetric P=O stretching for both preparation methods look similar (Fig. 5D) Differences were however, found on the spectra at the asymmetric ($^{+}N(CH_3)_3$) stretching regions (Fig. 5E). The peak at 969 cm^{-1} was observed to have shifted to a higher wavenumber of about 977 cm^{-1} on the mixtures prepared by the method of freeze-drying aqueous lipid dispersion (Fig. 5E (i)). In contrast, the peak at 969 cm^{-1} was not changed for mixtures prepared by physical mixing suggesting less interaction between Quil A and phosphatidylcholine. This result again suggests that good mixing of Quil A with phos-

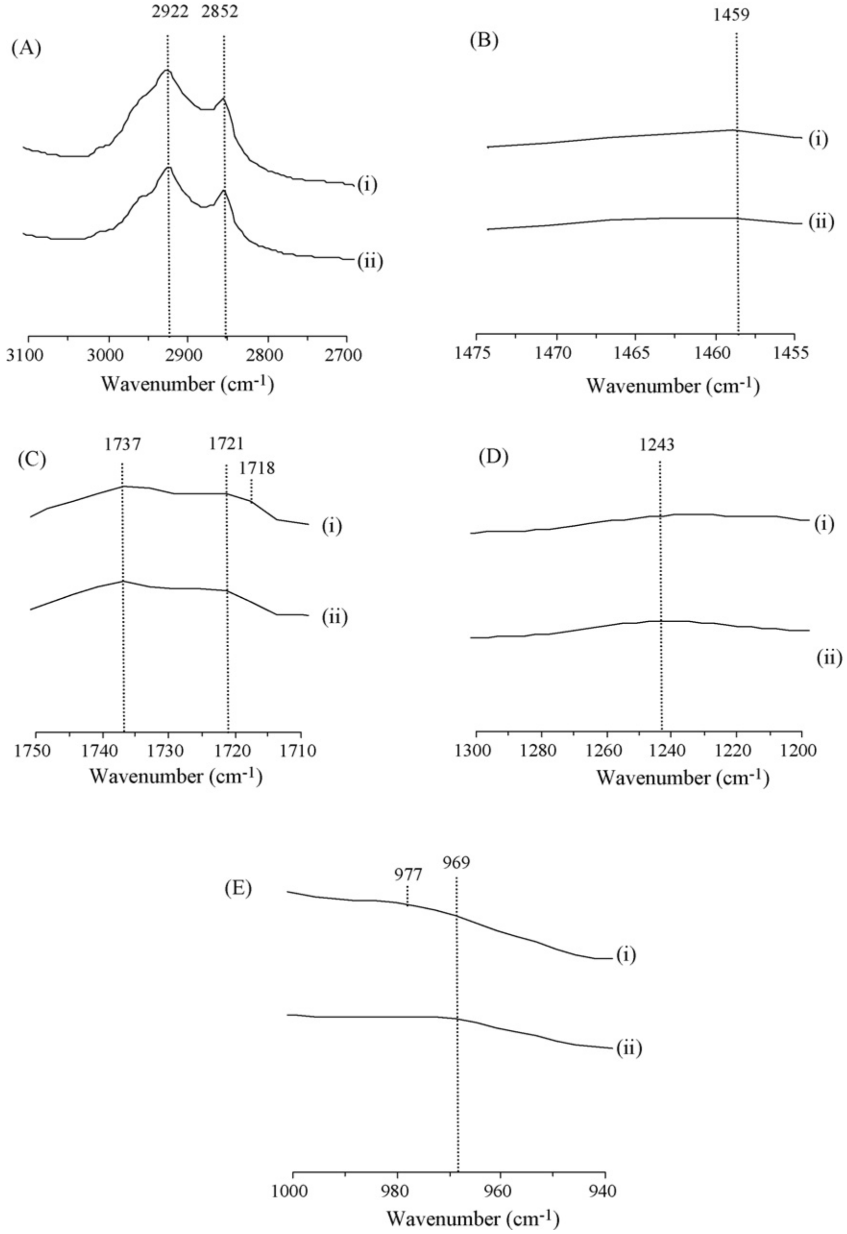


Fig. 5. DRIFT spectra of Quil A–phosphatidylcholine mixture (70:30%, w/w) prepared by (i) freeze-drying aqueous lipid dispersion and (ii) physical mixing methods at (A) C—H stretching, (B) CH_2 scissoring, (C) C═O stretching, (D) P═O stretching and (E) $^+N(CH_3)_3$ stretching modes.

phatidylcholine is obtained if the mixtures are prepared by the method of freeze-drying aqueous lipid dispersion compared to the physical mixing method described in this study.

3.7. Application of Quil A–lipid systems

The application of information gained in this study has an important role in the formulation of vaccine delivery systems, their physical and chemical stability. It was demonstrated that there is a strong interaction between phospholipids and Quil A involving polar headgroups, the C═O group and to a lesser extent the hydrocarbon chains. The effect of cholesterol in the lipid mixtures was also shown (Fig. 4), albeit to a lesser extent. Since various fractions of Quil A are currently available which differ mainly in their sugar headgroups (Ronnberg et al., 1997), it is therefore possible to optimise vaccine formulations by selecting Quil A molecules based on their appropriate polar headgroups properties. Similarly, positively charged choline group was suggested to interact with the negatively charged Quil A moiety in the current study. This information on electrostatic interactions would also be relevant in optimising vaccine formulations considering recent studies which have shown formation of positively charged ISCOMs (Lendemans et al., 2005b). This type of ISCOMs has an important role in DNA-vaccine technologies, and other poly-anionic antigens (Lendemans et al., 2005b).

The physical and chemical stability of ISCOM formulations and related colloidal systems could be controlled if there is sufficient knowledge in understanding molecular interactions at specific functional groups as investigated in the current study. Various forces such as hydrogen bonding and electrostatic interactions would play a role in the stability of such systems. Recent studies have shown that whilst DOTAP, a cationic phopsholipid, can be incorporated with Quil A and cholesterol to form cage-like particles, it appeared to be unsuitable to prepare cationic equivalents of ISCOMs (Lendemans et al., 2007). Therefore, information gained in the current study would contribute significantly in formulation development in Quil A–lipid systems such as ISCOMs, ring- and worm-like micelles, lipidic particles and layered structures, lamellae, and even helices as recently described in the literature (Demana et al., 2004b, 2005; Myschik et al., 2006a).

The application of information gained in this study would also be helpful in the evaluation of biological properties of vaccine systems. Phosphatidylcholine and cholesterol are often used as models of natural membranes in biological systems. Ronnberg et al., 1997 found less toxicity with Quil A after modification of carbohydrate moiety. Since the current study shows involvement of possibly polar groups in part of Quil A, understanding molecular interactions of Quil A with these lipids would therefore be relevant to elucidate toxicity, adjuvant activity and other biological properties of vaccine/adjuvant formulations.

4. Conclusions

This study shows that Quil A interacts with specific moieties of phosphatidylcholine. The most pronounced effects were found on the phospholipid headgroup particularly the choline group. This was possibly due to ionic interactions between the negatively charged glucuronic acid moiety of Quil A and the positively charged choline group of phospholipid molecules. The interactions could have also resulted from conformational changes in the choline group because of the intercalation of sugar moieties of Quil A molecules between the choline and phosphate groups due to hydrogen bonding. The interaction of Quil A with carbonyl group of phosphatidylcholine was also demonstrated. These results also suggested hydrogen bonding between the sugar headgroups of Quil A molecules with the carbonyl group of phosphatidylcholine. No considerable changes in the spectra of phosphatidylcholine and Quil A/phosphatidylcholine mixtures were observed in the hydrophobic regions implying that Quil A did not cause conformational disorder on the fatty acyl chains of phosphatidylcholine.

Inclusion of cholesterol at a concentration of 20% (w/w) was found not to considerably change spectra of Quil A with phosphatidylcholine. There were however, some changes in the spectra of Quil A with phosphatidylcholine at a cholesterol concentration of 60% (w/w). These results could be due to different conformation or orientation of the Quil A molecules on the phosphatidylcholine at high cholesterol concentration. It was also demonstrated that the preparation method of freeze-drying aqueous lipid dispersion was a better mixing technique to produce solid mixtures compared to the physical mixing method described in this study. Differences of the molecular interactions of Quil A on the phospholipid revealed in this study using DRIFT spectroscopy would optimize such formulations because these solid mixtures are currently being investigated as potential antigen or vaccine controlled delivery release systems in form of Quil A–lipid pellets or ISCOM implants (Demana et al., 2005; Myschik et al., 2006b).

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