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# Liquid chromatographic analysis of milk phospholipids with on-line pre-concentration

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**Abstract**

Two methods have been developed for the analysis of bovine milk phospholipid (PL) classes by NP-HPLC with evaporative light scattering detection. In the first method, a PVA-Sil guard column was used for the rapid determination of the major milk PL, phosphatidylethathanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM). In the second method, the guard column was used to pre-concentrate the PL, which were then transferred on-line onto a PVA-Sil analytical column by the use of column switching valves. This enabled separation of complete milk PL, including phosphatidylinositol (PI), phosphatidylserine (PS) and lysophosphatidylcholine (LPC).


*Keywords:* Milk; Food analysis; Pre-concentration; Phospholipids; Lipids

## 1. Introduction

Bovine milk lipids predominantly consist of triacylglycerols (>95%, w/w) and relatively small amounts of hydrocarbons, cholesteryl esters, cholesterol diacylglycerols, monoacylglycerols, free fatty acids and phospholipids [1]. The bovine milk phospholipids (PL) usually make up approximately 1% (w/w) of the total lipids. The actual amounts appear to vary, probably depending on the nutritional status of the cow, the state of lactation and other on-farm factors [2]. For US milk, values ranging from 0.20 to 1.00% (w/w) have been reported [1].

The most prevalent classes of bovine milk PL are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM), which account for about 90% (w/w) of the total [1]. The remainder consists of 3–5% (w/w) each of phosphatidylserine (PS) and phosphatidylinositol (PI) and trace amounts of lysophosphatidylcholine (LPC) and lysophosphatidylethoanolamine (LPE).

Many health benefits have been attributed to bovine milk PL as a group, as well as individual compounds [3,4]. In particular, SM, which is referred to as a tumour suppressor lipid, is a highly biological active compound that is associated with cell regulation [5]. Additionally, there is emerging evidence that bovine milk PL influence the physical functionality of dairy ingredients and food products. Phospholipids have been shown to impact on important functional properties of food products such as foaming [6], emulsification [7], gelation [8–11] and heat stability [12].

TLC has long been used for the analysis of PL [2]. Although excellent separations can be achieved using simple and relatively inexpensive equipment, TLC is tedious to perform. In recent years, HPLC has been successfully used for the separation and quantification of PL [13–20]. However, analysis of relatively low concentrations of PL, such as in milk lipids, requires concentration and removal of most of the triacylglycerols prior to HPLC [13]. Whilst this can be achieved by a technique such as TLC or solid-phase extraction, it adds to the complexity of the method and increases analysis time. In this paper, we report on the development of two HPLC methods for the analysis of milk PL. In the first method, the major milk PL (PE, PC and SM) are separated on a guard column only. In the second method, the PL are retained on the guard column before being transferred to an analytical column via column switching valves.

## 2. Experimental

### 2.1. Materials

Dichloromethane (DCM; ACS/HPLC grade) was obtained from Burdick & Jackson (stabilised with cy-

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clohexene; Muskegon, MI, USA), EM Science (HPLC grade) (stabilised with amylene; Gibbstown, NJ, USA) or APS Chemicals (Seven Hills, NSW, Australia). Methanol (MeOH; HiperSolv HPLC grade), 2,2,4-trimethylpentane (TMP; HiperSolv HPLC grade), 2-propanol (IPA; HiperSolv HPLC grade) and glacial acetic acid (approximately 100%; GAA) were obtained from BDH (Poole, UK). Triethylamine (99.5%; TEA), *N*-ethylmorpholine (approximately 99%; NEM) and formic acid (98–100%; FA) were obtained from Sigma–Aldrich (Milwaukee, WI, USA). All solvents used were of HPLC grade. The PL standards used in this work were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The purity of all these standards was greater than 99%. The following PC, PE, and PS synthetic standards were used: 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (6:0 PC); 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (8:0 PC); 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (12:0 PC); 1,2-distearoyl-*sn*-glycero-3-phosphocholine (18:0 PC); 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (6:0 PE); 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (12:0 PE); 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (18:0 PE); 1,2-dihexanoyl-*sn*-glycero-3-[phospho-L-serine] (sodium salt) (6:0 PS). L-α-Phosphatidylinositol ($Na^+$ salt) (PI) and sphingomyelin (SM) were obtained from bovine liver and milk, respectively, while L-α-lysophosphatidylcholine (LPC) was obtained from chicken egg.

The standards were delivered as dry powders. They were dissolved in MeOH/DCM (5:15, v/v) for the preparation of stock standard solutions. Some of the standards required gentle warming to enable complete dissolution. These stock standards were stored at −20 °C. Calibration standards were stored in amber-coloured glass HPLC vials with screw-top lids fitted with PTFE/silicone inserts. The diluent used to prepare the calibration standards was either DCM or TMP/IPA (3:2, v/v). The TMP/IPA diluent is reported to be an alternative lipid extractant [21]. The calibration drift for calibration standards prepared in the TMP/IPA diluent was considerably less than for the DCM diluent. The solubility of the 18:0 PE standard was reduced in the TMP/IPA diluent.

Fresh bovine milk samples were obtained from different dairy farms in Victoria (Department of Primary Industries, Kyabrum).

### 2.2. Purification of DCM

The Burdick & Jackson DCM was glass distilled to remove the cyclohexene stabiliser. The EM Science DCM was washed three times with high purity water to remove HCl by shaking vigorously in a separating funnel. The washed DCM was dried over anhydrous $Na_2SO_4$.

### 2.3. Preparation of eluents

The following eluents were filtered (Nylon 66 membrane, 0.45 μm; Supleco) prior to use and were degassed in-line by a vacuum degassing module:

(i) DCM (as-received or purified as above).
(ii) MeOH or IPA containing 14.3 mM of TEA and FA.
(iii) TMP/IPA (980:20, v/v) containing 7.2 mM of TEA and FA. The TEA and FA were dissolved in IPA before addition to TMP.
(iv) MeOH containing 24 mM of NEM and 16.4 mM of GAA.
(v) TMP/IPA containing 48 mM of NEM and 32.8 mM of GAA. The NEM and GAA were dissolved in IPA before addition to TMP.

### 2.4. Extraction of milk lipids

The milk lipids were extracted using a modified Bligh and Dyer method [22]. In brief, a milk sample (10 g) was mixed (Vortex, 2 min) with MeOH (10 mL) and DCM (5 mL) in a polyethylene centrifuge tube (50 mL, BioCorp Aust., Huntingdale, Vic., Australia). DCM (5 mL) and NaCl (0.1 g) was then added and mixed (Vortex, 30 s). After centrifuging (1780 × *g*, 20 min, 0 °C), the mixture was partitioned into two distinct solvent layers separated by a white gelatinous layer. The volumes of the top and bottom layers were approximately 20 and 7.5 mL, respectively, with the residual DCM incorporated in the gelatinous layer. The top layer was siphoned off to waste and the tube was placed in a freezer for 20 min to allow the gelatinous layer to be moved after hardening. The bottom layer (DCM) was collected. A DCM wash (5 mL) was added to the gelatinous layer, mixed (Vortex, 1 min) and centrifuged (1780 × *g*, 10 min, 0 °C). The tube was returned to the freezer (15 min) before the DCM was collected and combined with the original extract. This washing step recovered approximately 20% of the lipids. The use of the centrifuge reduced the lengthy solvent partitioning step and allowed a rapid re-extraction of the interfacial layer [17]. A similar procedure, employing small centrifuge tubes and short spin times (1 min) has been described elsewhere [23]. The final volume of the lipid samples was adjusted to 10 mL. The concentrations of the total lipids and total PL in the samples were 39.9 ± 4.7 and 0.26 ± 0.03 mg/mL, respectively. The lipid samples were filtered with a Nylon syringe filters (25 mm × 0.45 μm; Supelco, Milwaukee, WI, USA) and stored (at −20 °C) in amber-coloured glass HPLC vials with screw-top lids fitted with PTFE/silicone inserts until analysed. The lipid extracts were prepared in duplicate.

### 2.5. Instrumentation

Chromatography was performed on an Agilent (Palo Alto, CA, USA) HP 1050 series HPLC instrument consisting of a quaternary pump, four-channel solvent degasser, autosampler fitted with a 400 μL (0.50 mm i.d.) extended volume loop and 35900 ADC interface. Column switching was performed with two dual-position (6- and 10-port) electrically operated high pressure EV700 series Rheodyne col-

Table 1
Gradient program for analysis of milk phospholipids on PVA-Sil guard column

| T (min) | A (%) | B (%) | C (%) | D (%) |
|---|---|---|---|---|
| 0.0 | 60 | 0 | 40 | 0 |
| 5.0 | 60 | 0 | 40 | 0 |
| 10.0 | 45 | 15 | 5 | 35 |
| 11.0 | 45 | 15 | 0 | 40 |
| 12.0 | 60 | 0 | 40 | 0 |
| 14.0 | 60 | 0 | 40 | 0 |

Flow rate 1.0 mL/min. Refer to Fig. 2. Eluents: (A) DCM; (B) IPA containing 7.2 mM TEA and FA; (C) TMP containing 7.2 mM TEA and FA; (D) MeOH containing 7.2 mM TEA and FA.

umn switching valves (Select-Pro Fluid processors, Alltech, Deerfield, IL, USA). Both switching valves were controlled via closure contacts on the pump and autosampler. Detection was performed with a Polymer Labs (Amherst, MA, USA) PL-ELS 1000 evaporative light scattering detector (ELSD). The ELSD nebuliser and evaporator temperatures were set at 40 and 100 °C, respectively, while the nitrogen (high purity) flow rate was set at 1.5 L/min. Instrument control, data acquisition and processing were performed with ChemStation software for LC (Rev A, 06.01).

A PVA-Sil (150 mm × 3.0 mm i.d.) analytical column (YMC, Japan) and one of the following guard columns was used: PVA-Sil (23 mm × 4.0 mm i.d.) threaded guard column cartridge (YMC, Japan); Waters (Milford, MA, USA) Resolve Pak guard insert (3.0 mm × 4.6 mm i.d.) and Alltech Econosil 10 μm guard cartridge (7.5 mm × 4.6 mm i.d.).

### 2.6. Analysis on guard column

A milk lipid sample (20–100 μL) was injected onto a PVA-Sil guard column. The simple lipids were washed off the guard column with DCM/TMP (60:40, v/v) for 5.0 min, before the PL were eluted off the guard column with a gradient program (Table 1). The flow rate was 1.0 mL/min.

### 2.7. Pre-concentration and analysis of phospholipids on coupled system

The quaternary HPLC pump was used for washing the simple lipids from the guard column, transferring the PL onto the analytical column and eluting the PL from the analytical column. The following procedure was employed: a milk lipid sample (100–300 μL) was injected onto the guard column (Fig. 1a). After washing off the simple lipids with DCM/TMP (60:40, v/v) for 1.5 min at the rate of 1.0 mL/min, the pump was stopped and the flow from the guard column diverted to the analytical column using the six-port switching valve (SV1) (Fig. 1b). The flow rate was then increased to 1.0 mL/min over 4 min, and the ELSD baseline was allowed to stabilise for 1 min. The flow was then diverted through the guard column and onto the analytical column using the 10-port switching valve (SV 2) (Fig. 1c). At the end of each run, the pump flow was reduced and both switching valves were returned to their initial positions.

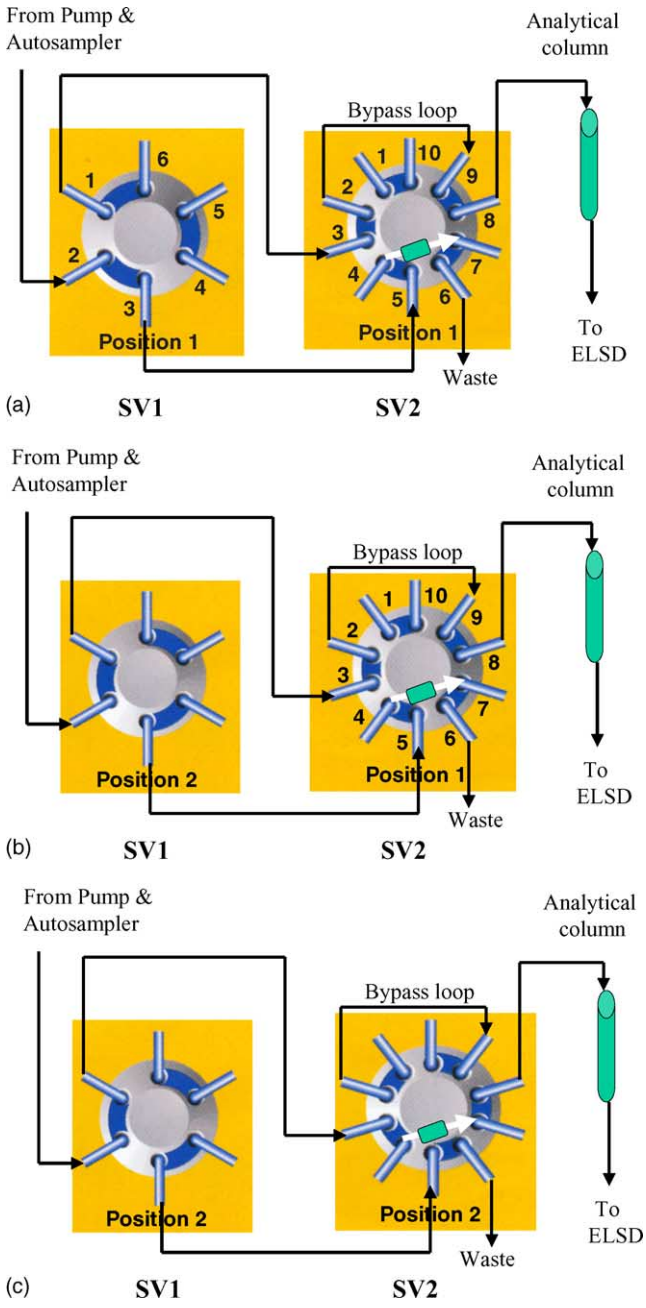


Fig. 1. Operation of switching valves for removal of simple lipids on a PVA-Sil guard column and transfer of pre-concentrated phospholipids onto an analytical column. (a) Injection of lipid extract onto guard column and removal of simple lipids with non-polar eluent. (b) Equilibration of analytical column and stabilisation of ELSD baseline at operational flow rate. (c) Transfer of phospholipids from guard column onto analytical column. The switching valve schematics are used with the permission of Rheodyne LLC.

### 2.8. Investigations with different eluent modifiers

The gradient and SV program for use with eluents containing TEA/FA modifiers is shown in Table 2. The gradient

Table 2
Gradient and switching valve (SV) program for the pre-concentration and analysis of milk phospholipids on PVA-Sil guard and analytical columns with eluents containing TEA/FA modifiers

| *T* (min) | A (%) | B (%) | C (%) | D (%) | Flow | SV1 | SV2 |
|---|---|---|---|---|---|---|---|
| 0.0 | 50 | 0 | 50 | 0 | 0.3 | 1 | 1 |
| 0.1 | 50 | 0 | 50 | 0 | 1.0 | | |
| 1.5 | 50 | 0 | 50 | 0 | 1.0 | | |
| 1.6 | 50 | 0 | 50 | 0 | 0.0 | | |
| 1.7 | 60 | 20 | 20 | 0 | 0.0 | 2 | 1 |
| 5.7 | 60 | 20 | 20 | 0 | 1.0 | | |
| 6.0 | 60 | 20 | 20 | 0 | 1.0 | 2 | 2 |
| 26.0 | 50 | 10 | 5 | 35 | 1.0 | | |
| 27.0 | 60 | 20 | 20 | 0 | 1.0 | | |
| 30.0 | 60 | 20 | 20 | 0 | 1.0 | | |
| 30.1 | 50 | 0 | 50 | 0 | 0.3 | 1 | 1 |

Refer to Fig. 3. Eluents: (A) DCM; (B) IPA containing 14.3 mM TEA and FA; (C) TMP containing 7.2 mM TEA and FA; (D) MeOH containing 14.3 mM TEA and FA.

Table 3
Gradient and switching valve (SV) program for the pre-concentration and analysis of milk phospholipids on PVA-Sil guard and analytical columns with NEM/GAA modifiers only in the MeOH eluent

| *T* (min) | A (%) | B (%) | C (%) | D (%) | Flow | SV1 | SV2 |
|---|---|---|---|---|---|---|---|
| 0.0 | 60 | 40 | 0 | 0 | 0.3 | 1 | 1 |
| 0.1 | 60 | 40 | 0 | 0 | 1.0 | | |
| 1.5 | 60 | 40 | 0 | 0 | 1.0 | | |
| 1.6 | 60 | 40 | 0 | 0 | 0.0 | | |
| 1.7 | 60 | 40 | 0 | 0 | 0.0 | 2 | 1 |
| 5.7 | 60 | 40 | 0 | 0 | 1.0 | | |
| 6.7 | 60 | 40 | 0 | 0 | 1.0 | 2 | 2 |
| 22.7 | 44 | 26 | 30 | 0 | 1.0 | | |
| 23.7 | 60 | 0 | 40 | 0 | 1.0 | | |
| 23.75 | 60 | 0 | 0 | 40 | 1.0 | | |
| 26.7 | 60 | 0 | 0 | 40 | 1.0 | | |
| 27.7 | 60 | 40 | 0 | 0 | 1.0 | | |
| 30.7 | 60 | 40 | 0 | 0 | 1.0 | | |
| 31.0 | 60 | 40 | 0 | 0 | 0.1 | 1 | 1 |

The columns were flushed for 3 min after each injection with DCM/MeOH (60:40). Eluents: (A) DCM; (B) TMP/IPA (98:2); (C) MeOH containing 24 mM NEM and 16.4 mM GAA; (D) MeOH.

and SV program for use with eluents containing NEM/GAA modifiers are shown in Tables 3 and 4. The final system is shown Table 4.

## 3. Results and discussion

### 3.1. Chromatography on guard column

Preliminary experiments were conducted to evaluate the separation of the phospholipids from simple lipids on a guard column. In these experiments the effluent from the guard column was directly monitored with the ELSD. Following the injection of a milk lipid sample onto a PVA-Sil guard column, a relatively non-polar eluent (DCM/TMP 60:40, v/v) removed most of the simple lipids within 3 min as a single peak (Fig. 2). The phospholipids were retained on the guard column under these conditions. They were eluted off the guard column by use of a gradient system comprising DCM, IPA, TMP and MeOH (Table 1). The PL eluted in a total of 5 min, with a total analysis time of 14 min.

Table 4
Gradient and switching valve (SV) program for the pre-concentration and analysis of milk phospholipids on PVA-Sil guard and analytical columns with the NEM and GAA modifiers in both the TMP/IPA (98:2, v/v) and MeOH eluents

| *T* (min) | A (%) | B (%) | C (%) | Flow | SV1 | SV2 |
|---|---|---|---|---|---|---|
| 0.0 | 60 | 40 | 0 | 0.4 | 1 | 1 |
| 0.3 | 60 | 40 | 0 | 1.0 | | |
| 1.5 | 60 | 40 | 0 | 1.0 | | |
| 1.6 | 60 | 40 | 0 | 0.0 | | |
| 1.7 | 60 | 40 | 0 | 0.0 | 2 | 1 |
| 5.0 | 60 | 40 | 0 | 1.0 | | |
| 6.0 | 60 | 40 | 0 | 1.0 | 2 | 2 |
| 16.0 | 55 | 35 | 10 | 1.0 | | |
| 26.0 | 44 | 26 | 30 | 1.0 | | |
| 27.0 | 60 | 40 | 0 | 1.0 | | |
| 30.0 | 60 | 40 | 0 | 1.0 | | |
| 30.1 | 60 | 40 | 0 | 0.2 | 1 | 1 |

Eluents: (A) DCM; (B) TMP/IPA (98:2) containing 48 mM NEM and 32.8 mM GAA; (C) MeOH containing 24 mM NEM and 16.4 mM GAA.

The three main phospholipids (PE, PC and SM) were almost baseline resolved. Separation of PC and SM could not be achieved on two alternative commercial silica guard columns (Waters Resolve Pak and Alltech Econosil SI) that we used. The reasons for this difference may be due to the larger quantity of stationary phase in the PVA-Sil guard column and selectivity differences between the modified and bare silica stationary phases. The PVA-Sil guard cartridge contains approximately two and six times the quantity of stationary phase compared to the Alltech Econosil and Waters Resolve Pak guard cartridges, respectively.

Two unidentified minor components eluted just before PE on the PVA-Sil guard column (Fig. 2). Based on a published chromatogram of milk PL, it is possible that these minor components were glycolipids [13].

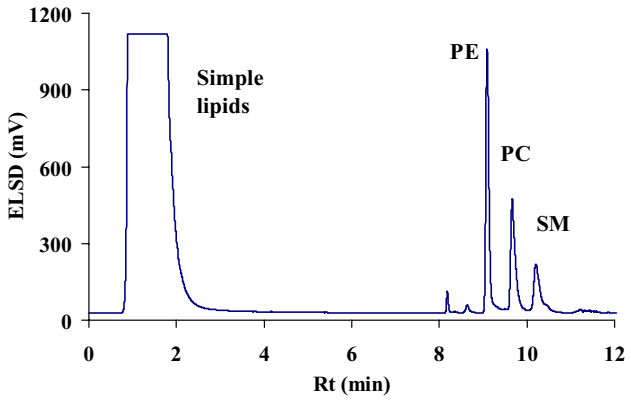


Fig. 2. Separation of milk lipids sample (100 μL of 3.6% (w/v) DCM solution) on a PVA-Sil guard column, with effluent monitored by ELSD. Simple lipids were eluted with DCM/TMP (60:40, v/v) for 4.0 min followed by gradient program shown in Table 1.

While it was possible to separate the major milk PL on a PVA-Sil guard column, PI and PS co-eluted with PE and PC, respectively (results not shown). However, since milk PL contain less than 5% of both PI and PS, their low peak areas would have only a small influence on the quantitation of PE and PC. Therefore, analysis of milk lipids on the guard column is suitable when the concentrations of the major milk PL are only required. This method is simple and rapid. We have, however, not investigated the quantitative aspects of this analysis.

### 3.2. Pre-concentration of phospholipids on guard column and transfer to analytical column

When the measurement of all milk PL, including the minor components PI and PS is required, it is necessary to use an analytical column for separation of the phospholipids. The guard column is used to remove most of the simple lipids and pre-concentrate the PL prior to separation on the analytical column. The retained PL can then be transferred onto the analytical column in either the original or reverse flow direction using a column switching valve (SV). Though it could be expected that the PL would be retained near the injection end of the guard column, the chromatograms obtained from either flow direction were almost identical (results not shown). Hence there was no advantage to be gained by reversing the flow to elute the PL off the guard column.

A single quaternary HPLC pump was used for both the wash-off step and subsequent analysis. This enabled the composition of the wash-off solvent to be easily varied and minimised pressure shocks to the guard column. To avoid damage to the analytical column due to pressure shocks, the pump flow was stopped prior to switching the flow from the guard column (wash-off step) to the analytical column. An alternative configuration has been reported in which a second pump was used in the wash-off step during the pre-concentration of phospholipids from peanut oil [24]. This avoided the need to stop pump flow between injections and allowed the concentrator column to be loaded with the next sample while the previous sample was still being eluted from the analytical column. It was not possible to perform this latter function with our HPLC system.

Under the conditions used in the current study (Tables 2 and 3), approximately 3 min were required to wash off all the simple lipids from the guard column. However, it was found that a reduced wash-off time of 1.5 min was sufficient to remove most simple lipids. This prevented any loss of the early eluting PL (PI and PE) and improved the precision of their measurement.

Separation of the pre-concentrated PL on a PVA-Sil analytical column gave baseline resolution of all the phospholipids, including PI and PS (Fig. 3). However, the analysis time (including column re-equilibration) was more than doubled compared to analysis on a guard column. Moreover, there was at least a four-fold decrease in sensitivity for the PL due to increased chromatographic dispersion.

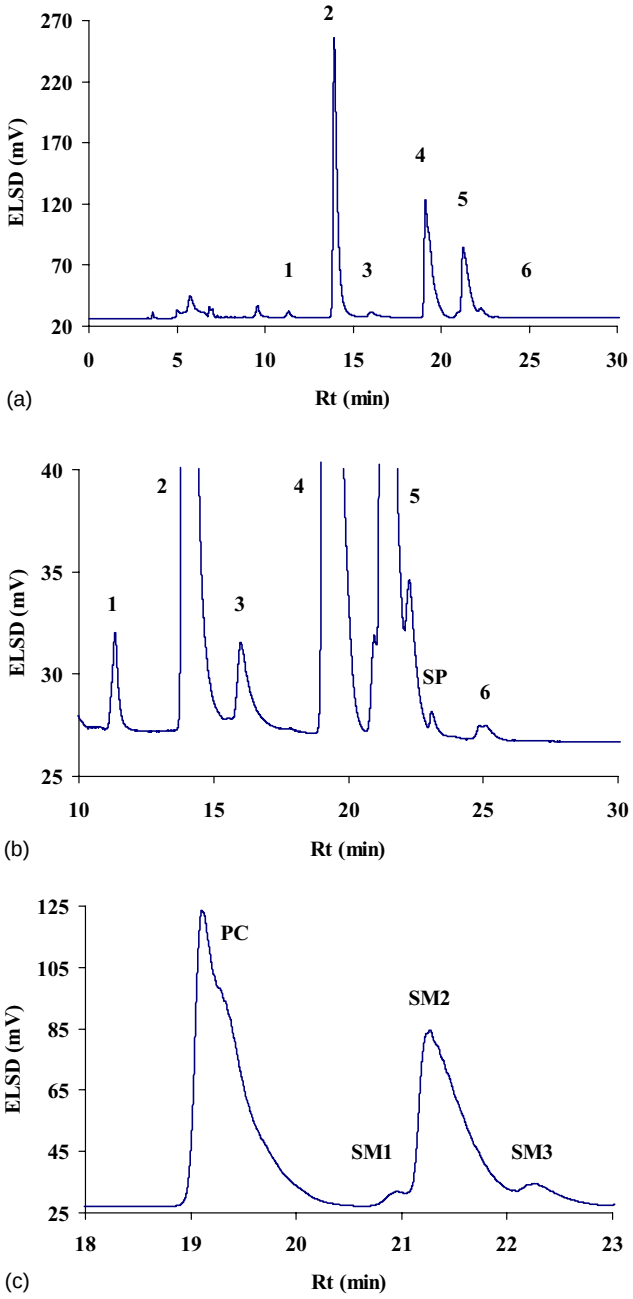


Fig. 3. Separation of milk phospholipids on a PVA-Sil column after on-line removal of simple lipids. Milk lipid sample (300 μL of 3.6% (w/v) DCM solution) injected onto a PVA-Sil guard column. Milk lipid sample spiked with LPC. Peak identities: (1) PI; (2) PE; (3) PS; (4) PC; (5) SM; (6) LPC; (SP) system peak. [PE], [PC] and [SM] were 0.079, 0.073 and 0.084 mg/mL. Eluent composition and gradient and switching valve program are shown in Table 2. (a) Full scale chromatogram. (b) Expanded view showing minor phospholipids. (c) Expanded view of PC and SM peaks. SM1, SM2 and SM3 refer to three SM sub-peaks.

### 3.3. Separation of phospholipids

The stationary phase in PVA-Sil column contains silica that has been coated with polyvinyl alcohol. It affords greater pH stability and allows more rapid column re-equilibration

after the use of very polar solvents, such as water [14]. PVA-Sil columns have been used for the analysis of PL from a variety of sources [14,16,18–20].

Due to the on-line removal of the simple lipids with the guard column, only the separation of the PL was required. It was desirable to incorporate DCM into the initial eluent composition since the milk lipids samples were prepared in this solvent. A binary gradient elution system using DCM and MeOH has been previously reported for the analysis of peanut PL [24]. It has been noted that elution systems employing DCM/MeOH gradients afford sharper PL peaks [25]. However, we failed to achieve complete resolution of the milk PL on a PVA-Sil column with a DCM/MeOH binary system.

In a detailed optimisation study, Deschamps et al. [19] developed a ternary elution system for the separation of most lipid classes on a PVA-Sil column. The three solvents were TMP/IPA (98:2, v/v), DCM and MeOH. Each solvent contained equimolar concentrations (7.2 mM) of triethylamine (TEA) and formic acid (FA). It has been reported that these modifier concentrations enhanced the ELSD detection response [19]. We modified the gradient to suit our requirements and achieved separation of all the milk PL in 20 min, with a total analysis time of 30 min (Fig. 3 and Table 2).

We observed that the TEA/FA modifiers enabled resolution of the minor milk phospholipids, PI and PS, from PE and PC, respectively (Fig. 3b). PS eluted as a broad peak, which was in agreement with Deschamps et al. [19]. PI and PE eluted as sharp peaks. Bovine milk SM was characterised by three distinct peaks, SM1, SM2 and SM3 (Fig. 3c). SM2 was the major peak, while SM1 and SM3 had similar peak heights. A detailed study of these three sub-peaks using LC–MS has shown significant differences between the molecular species [26]. By contrast, we observed that bovine brain SM displayed only two peaks under the same chromatographic conditions (results not shown). It is worth noting that in an earlier analysis of milk lipids using a silica column, SM displayed only 2 peaks [13].

We observed milk PC eluted as a broad peak with noticeable sub-peaks (Fig. 3c). Lipid chemists have been aware of a large range of molecular species in bovine milk PC for many years [2,30]. The issue of peak heterogeneity and its ramifications to quantitation are discussed in more detail in later sections. The concentration of lysophosphatidylcholine in most of the milk lipid samples that we analysed was below the detection limit. A milk sample spiked with LPC illustrates that LPC eluted after SM in this separation (Fig. 3).

The addition of the TEA/FA modifiers to DCM resulted in a very large system peak that eluted near the phospholipids. Two sources of HPLC grade DCM, stabilised with either amylene or cyclohexene, produced this system peak, although the greatest effect was noted with the amylene stabilised DCM. The system peak was considerably reduced when the cyclohexene stabilised DCM was distilled immediately prior to use. Washing the amylene stabilised DCM with water and subsequently drying had the greatest effect in reducing the size of the system peak. It is well known that chlorinated solvents are susceptible to degradation by a free radical mechanism that produces HCl and other degradation products [27]. The above observations suggested that the system peak was due to the formation of TEA–Cl. It appears that this ion pair is detected by the ELSD due to its reduced volatility. A similar but smaller system peak was also observed when an alternative pair of modifiers, *N*-ethylmorpholine and glacial acetic acid were added to the DCM. No system peak occurred when a binary gradient was run with DCM/GAA (500:1.0, v/v) and MeOH/NEM (500:1.0, v/v). This provided further confirmation that the system peak was due to an amine–chloride ion pair. To overcome this problem, it was decided to avoid adding modifiers to the DCM solvent in all further work.

When the TEA and FA modifiers were added to the TMP, IPA and MeOH solvents at the concentration (7.2 mM) reported by Deschamps et al. [19], the PS and PC peaks were broad. To compensate for the lack of modifiers in the DCM solvent, the TEA/FA modifier concentrations were doubled in the IPA and MeOH solvents to 14.3 mM. This improved the PS and PC peak shapes, although there was still a noticeable asymmetry of the PC peak (Fig. 3). However, the increased modifier concentrations caused a decrease in the measurement precision for PI and PE compared to PC and SM.

A large injection volume (200–300 μL) of the milk lipid samples was required to enable quantitation of PS, as it was very small peak (Fig. 3b). It is worth noting that a 20 μL injection of a milk lipid sample onto the guard column alone was sufficient to enable quantitation of PE, PC and SM.

Over several days of continuous analysis of milk lipid samples using this method, it was observed that the PS peak shape gradually deteriorated and its retention time changed resulting in its eventual merging with the PC peak. Other peaks were also effected, but to a lesser extent. Due to the problems experienced with PS, it was decided to ignore this minor PL and reduce the sample injection volume to 100 μL. While the reason for this column damage was not certain, it may have been due to the modifiers. However, the column manufacturers (YMC) state that the PVA-Sil column can tolerate a wider pH range (2–9.5) than conventional silica columns. A literature column regeneration scheme employing acetonitrile and 5% ammonium acetate solution partially restored the column [17].

The TEA/FA modifiers were noted to cause variable precision between the PL and gradual deterioration of the PS peak shape. Due to these problems, an alternative pair of modifiers, *N*-ethylmorpholine and glacial acetic acid, was investigated. The p$K_a$ of NEM (7.7) and GAA (4.7) indicate that they are less aggressive on a silica based column than those of TEA (9.8) and FA (3.7) [28,29]. Christie et al. employed 12 mM NEM and 8.2 mM GAA in the final eluent of a ternary gradient [17]. Initially, we employed DCM, TMP/IPA (98:2) and MeOH solvents with the NEM and GAA modifiers (24 and 16.4 mM, respectively) added

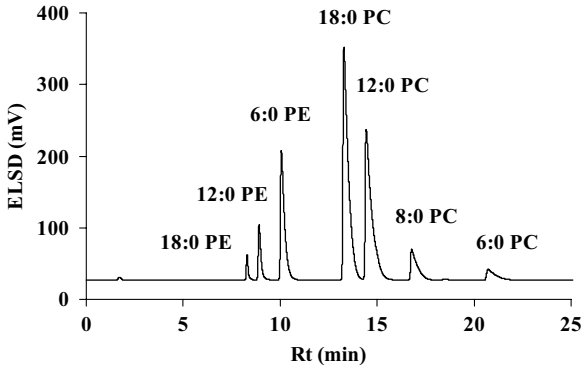


Fig. 4. Mixture of synthetic diacyl PE and PC standards separated on a PVA-Sil analytical column. 100 μL injection. Concentrations (mg/mL): 6:0 PE (0.206); 12:0 PE (0.082); 18:0 PE (0.078); 6:0 PC (0.013); 8:0 PC (0.013); 12:0 PC (0.090); 18:0 PC (0.348).

only to the MeOH solvent. The column was flushed with DCM/MeOH (60:40) for 3 min after each injection to prevent any build-up of modifiers on the column (Table 3). Under these conditions, PI was eluted after PE, which was the reverse elution order compared to that obtained with the TEA/FA modifiers.

Since there was a gradual increase in tailing of the PE peak, it was desirable to elute PI before PE to prevent interference in the quantitation of PI. It was found that the addition of the NEM/GAA modifiers to both the TMP/IPA (98:2) and MeOH solvents resulted in elution of PI before PE. The NEM and GAA concentrations in the TMP/IPA (98:2, v/v) solvent were 48 and 32.8 mM, respectively (Table 4). This further demonstrates that elution order of PI and PE can be manipulated by changes to the modifier concentration. A very gradual decrease in column performance was observed with this method during the analysis of approximately 200 milk lipids samples.

### 3.4. Effect of acyl length on PE and PC elution

The milk PC eluted as a broader peak with sub-peaks evident within the PC peak (Fig. 3c), while the corresponding PE peak displayed a more symmetrical peak shape. The PC peak shape reflected partial separation of molecular species containing different acyl groups. The effect of acyl chain length on the retention of PE and PC standards with various saturated acyl groupings was investigated by analysing three synthetic PE standards (di-6:0, di-12:0 and di-18:0) and four synthetic PC standards (di-6:0, di-8:0, di-12:0 and di-18:0). The seven PL standards were all resolved (Fig. 4). Within each PL class, the component containing the shortest acyl chain eluted last, reflecting its greater polarity. It was also observed that the PC standards had a larger range of retention times compared to the PE standards. This is in agreement with a recent study in which it was shown that the capacity factors of saturated diacyl PC varied 3–10 times as much as the capacity factors of the equivalent diacyl PE [31].

It has been noted that the use ionic modifiers should mostly negate this effect [15]. While we observed an improvement in PC peak shape when the concentration of the TEA/FA modifiers was doubled, there was still a noticeable peak asymmetry for PC. This effect may, in part, be due to the higher resolution afforded by a PVA-Sil column compared to bare silica columns.

### 3.5. Calibration and detection limits

Given that each milk PL class is composed of a complex mixture of molecular species, calibrations for the analysis of milk PL should be prepared from standards obtained from bovine milk. However, only bovine milk SM was commercially available as a pure standard. Consequently, synthetic di-18:0 PE and di-18:0 PC were used for calibration of these two PL, while the PI calibration standard was obtained from bovine liver.

While bovine milk PE eluted as a sharp peak, bovine milk PC eluted as a broad peak with sub-peaks evident under some conditions, for the reasons discussed earlier. This could lead to a potential error due to the inherent non-linear response displayed by ELS detectors. There have been no reported efforts to determine the level of this error. However, it is evident that the ELSD response is approximately linear over a narrow range [14,16].

Since it was possible to obtain bovine milk sphingomyelin as a pure standard, the above calibration issues for PC were not relevant to SM. However, lower values for sphingomyelin determined by NP-HPLC/ELSD compared to other methods has been previously noted [32]. This may, in part, be due to variation between different sources of sphingomyelin as calibration standards.

Since the concentrations of individual PL in bovine milk lipids were typically confined to a narrow range, it was possible to establish calibration curves that were approximately linear over the sample range. This would reduce the error associated with the heterogeneity of bovine milk PC. It has been noted that variations in the lipid and mobile phase composition will affect the calibration curves [14]. Under the conditions used in our investigations, it was observed that the NEM/GAA modifiers produced a more linear response than the TEA/FA modifiers. The calibration equations, correlation coefficient and concentration range for each PL with the TEA/FA and NEM/GAA modifiers are shown in Table 5.

The detection limits for PE, PC and SM eluted from a PVA-Sil guard column were 0.08, 0.10 and 0.12 μg, respectively. The detection limits for PI, PE, PC and SM eluted from the coupled system were 0.7, 0.6, 1.6 and 3.0 μg, respectively. The detection limits were determined as the injection mass corresponding to six times the S/N ratio of individual PL standards analysed in triplicate, in a manner similar to that previously reported [16].

By comparison, Nordback et al. [16] reported a detection limit of 0.61 μg for PC, with separation on a 100 mm × 3.0 mm i.d. PVA-Sil analytical column. The detection limits

Table 5
Calibration equation coefficients, correlation coefficients and concentration ranges for phospholipid calibrations

| PL | $ax^2$ | $bx$ | $c$ | $R^2$ | Range (μg/mL) |
|---|---|---|---|---|---|
| (A) TMP/IPA (98:2) and MeOH solvents containing 14.4 mM TEA and FA | | | | | |
| PI | 323718 | 4009 | −46.4 | 0.9999 | 12–51 |
| PE | 71234 | 41426 | −1832 | 0.9993 | 56–154 |
| PC | 81257 | 20103 | −604 | 0.9992 | 63–254 |
| SM | 42493 | 3774 | −142 | 0.9969 | 61–249 |
| (B) TMP/IPA (98:2, v/v) containing 48 mM NEM and 32.8 mM GAA, and MeOH containing 24 mM NEM and 16.4 mM GAA | | | | | |
| PL | $ax$ | | $c$ | $R^2$ | Range (μg/mL) |
| PE | 25549 | | −527 | 0.9994 | 41–105 |
| PC | 21487 | | −378 | 0.9969 | 37–96 |
| SM | 12579 | | −253 | 0.9906 | 37–94 |

Injection volume: 100 μL.

Table 6
Comparison of phospholipid assays with literature values

| | This work | Christie et al. [13] | Bitman and Wood [2] |
|---|---|---|---|
| PE (%) | 38.6 ± 1.7 | 39.8 | 23.6–36.4 |
| PC (%) | 32.2 ± 1.3 | 32.8 | 28.9–40.9 |
| SM (%) | 29.2 ± 1.7 | 27.4 | 32.2–38.6 |
| Total PL (mg/10 mL milk) | 2.42 ± 0.26 | 2.28 | 1.10–3.08 |

This work: average of 21 Victorian milk samples with variation between samples. Christie et al. [13]: NP-HPLC/ELSD analysis. Bitman and Wood [2]: data obtained with TLC/densitometry [2]. Variation over lactation (days 3–180). The literature values for PI and PS were ignored in this comparison. The PL (%) literature values were re-calculated for PE, PC, and SM only.

that we observed are higher from the analytical column due to increased chromatographic dispersion. However, because the guard column allows pre-concentration of the PL, an analytical column in combination with a guard column and column switching valves can still be used for the analysis of samples with low concentrations of PL.

### 3.6. Comparison with literature values

We analysed 21 samples of fresh milk from different dairy farms in Victoria for PL composition using the above method. The relative percentages of PE, PC and SM for these milks were very similar to those reported by Christie et al. who also used NP-HPLC/ELSD [13] (Table 6). Our results were also within the range of values determined in a separate study that examined the PL composition of cows' milk over the lactation period (days 3–180) using TLC/densitometry (Table 6) [2].

## 4. Conclusions

The utility of analysis on a PVA-Sil guard column for the rapid determination of the major milk phospholipids has been demonstrated. The use of on-line removal of simple lipids and simultaneous pre-concentration of phospholipids has been shown to be useful for the analysis of milk lipids. The use of a narrow dynamic range enabled linear calibration responses of the ELSD for the major milk phospholipids (PE, PC and SM). The retention time of PI was significantly influenced by the type and concentration of the modifiers. The TEA/FA modifiers and not the NEM/GAA modifiers caused considerable deterioration of the PVA-Sil column over an extended time.

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