VALIDATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY FOR THE QUANTIFICATION OF CARDIOLIPIN AND OTHER PHOSPHOLIPIDS IN MITOCHONDRIA

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BACKGROUND

Our previous results suggest that cardiolipin, a specific phospholipid of mitochondria, could be implicated in the hypermetabolism associated to cancer cachexia.

Although several methods exist to quantify phospholipids and especially cardiolipin, none are at the same time sufficiently sensitive, precise, rapid and low-cost.

AIM OF THE STUDY

The aim of the study was to validate the High Performance Thin Layer Chromatography (HPTLC)-densitometry technique, according to the International Conference on Harmonization guidelines, to quantify phospholipids: sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), and especially phosphatidylethanolamine (PE) and cardiolipin (CL). Furthermore, this method was apply to quantify 1/ the enrichments of PE and CL in mitochondria *via* liposomes and 2/ CL content in liver mitochondria from control rats and rats suffering of cancer cachexia.

Structure of cardiolipin
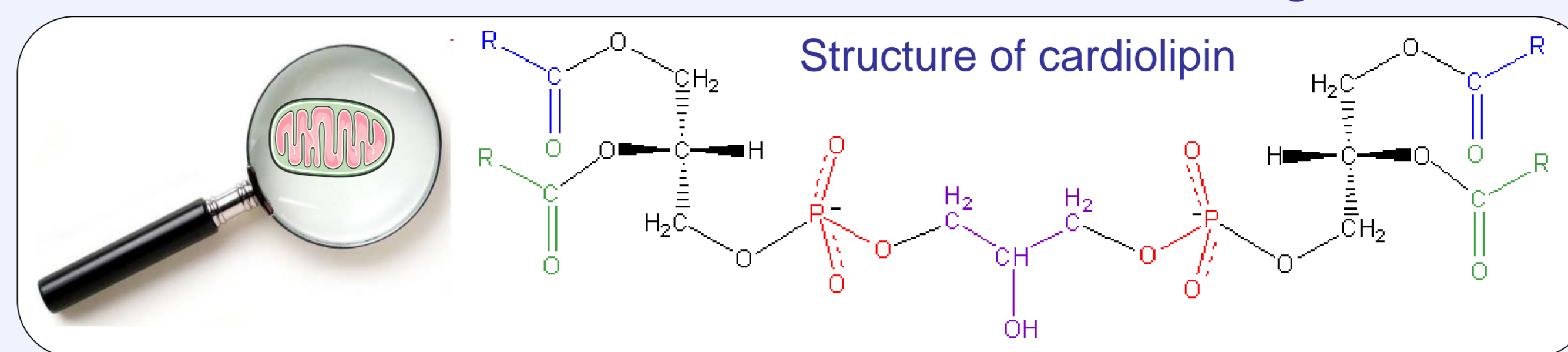
PROTOCOL

Samples were deposited on HPTLC plates using a Camag Linomat V sample applicator. Authentic lipids were deposited as mixtures of phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL) and neutral lipids (NL, a mixture of triglycerides, cholesterol, cholesteryl esters and non-esterified fatty acids). After chromatographic separation, the densitometric image of phospholipid bands was obtained with a Reprostar Camag TLC scanner III. The amount of each phospholipid class was determined with the WinCats 1.4 software. The validation parameters evaluated were: linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter-day and intra-day assay precision, repeatability of measurement, and repeatability for sample application. Validation of the method was performed by statistical analysis using one-way ANOVA (Prism 4 software).

RESULTS

SEPARATION OF DIFFERENT CLASSES OF PHOSPHOLIPIDS

Chromatography optimized for the separation of cardiolipin and phosphatidylethanolamine

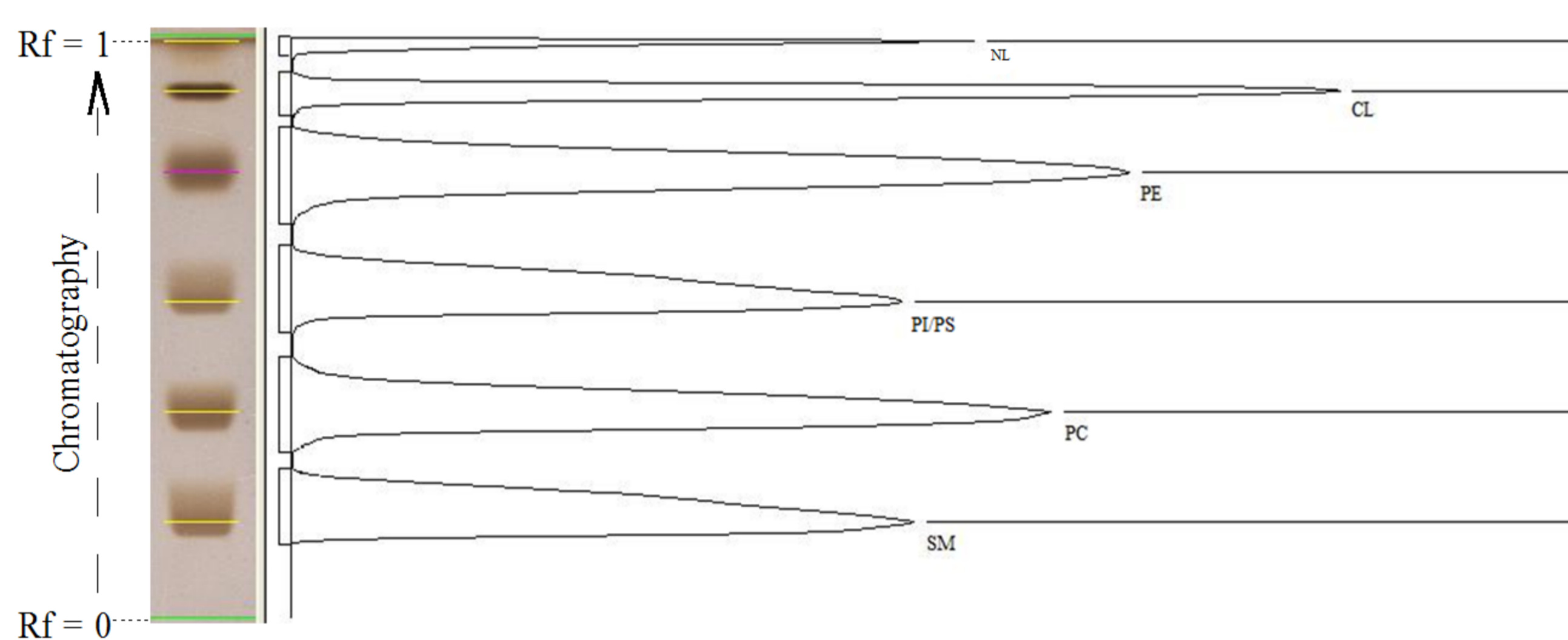**Chromatography optimized for the separation of phosphatidylserine and phosphatidylinositol**

Figure 1: Representative HPTLC-densitometric profile of authentic phospholipid standards after optimized separation of cardiolipin and phosphatidylethanolamine.
Eluent: chloroform-acetone-acetic acid- methanol-water 6:8:2:2:1 (v/v) (Arvier. M. *et al.* Am J Physiol Endocrinol Metab 2007, [293]: 1320–1324)
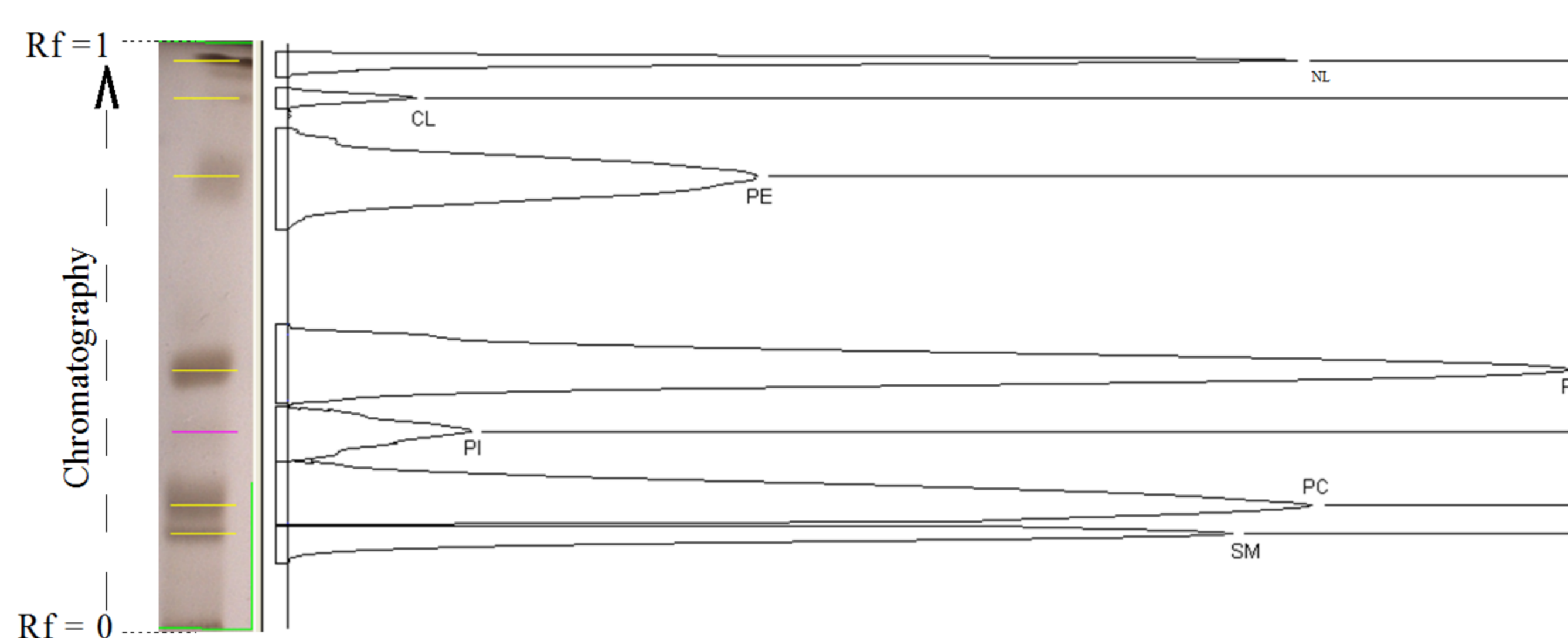Developer: 0.18% copper sulfate in orthophosphoric acid-distilled water (40:460, v/v)

Figure 2: Representative HPTLC-densitometric profile of authentic phospholipid standards after optimized separation of phosphatidylserine and phosphatidylinositol.
Eluent: chloroform-methanol-petroleum ether-acetic acid (40:20:30:10, v/v) with 1.8% boric acid (Skipski *et al.* Biochem Journal, 1964, [90], 374-378)
Developer: 0.18% copper sulfate in orthophosphoric acid-distilled water (40:460, v/v)
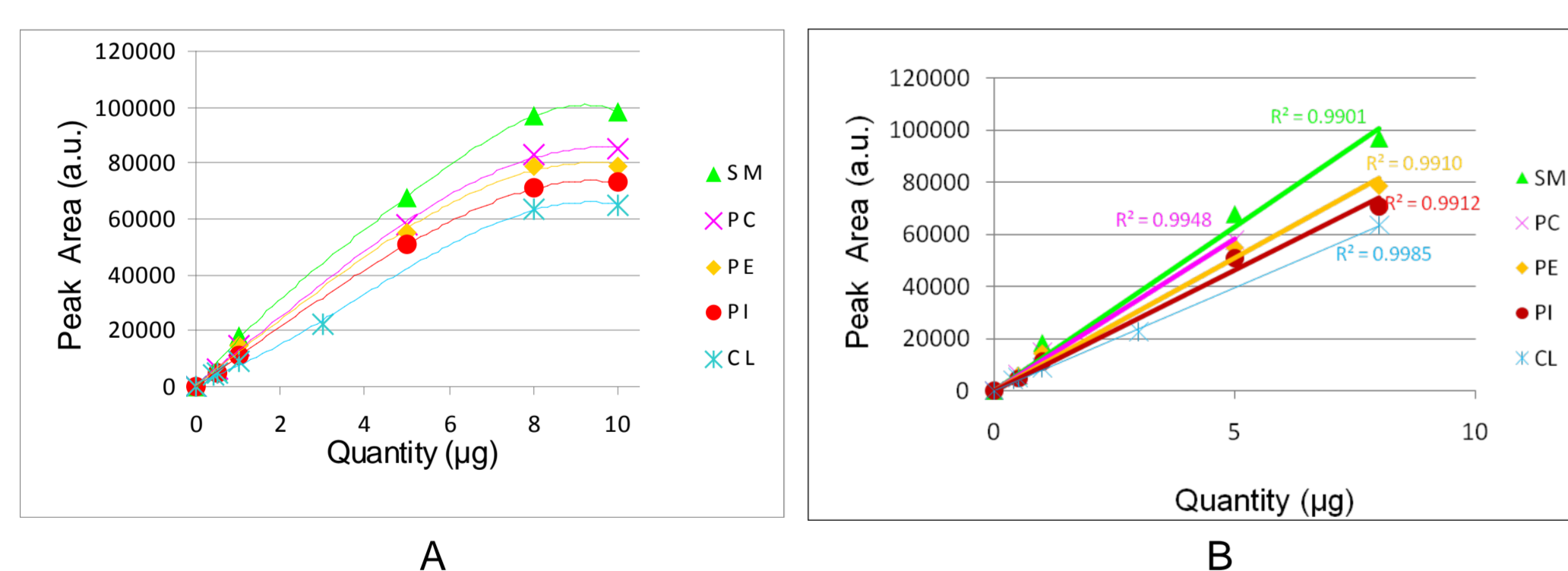
METHOD VALIDATION

Linearity

Figure 3: (A). Concentration range of linearity for the quantification of five phospholipid classes. Integrated peak area (arbitrary units of band density) were plotted against the amount of phospholipids spotted on the plates. Detection of phospholipids was linear up to 8 μg. (B). Linear regression coefficients are indicated for each compound.

Validation results

Classes	SM	PC	PI	PE	CL
Cochran C	0.8350	0.8947	0.6413	0.7543	0.8232
Fischer test	3.4903	3.2389	3.8853	3.2389	4.0662
Significance threshold	0.0045	0.0288	0.0061	0.0020	0.0469
LOD (ng/spot)	38.96	22.61	148.37	67.18	7.46
LOQ (ng/spot)	89.56	46.94	347.13	173.61	18.04
Validation	accepted	accepted	accepted	accepted	accepted

Table 1 : Results of the statistical analyses used to validate the chromatographic separation of cardiolipin and other phospholipids. The method validated is described in the legend of figure 1. Experiments were repeated 10 times.
Cochran test was obtained after an ANOVA analysis to see the variance homogeneity. The values found were compared with the Cochran table.
The Fischer test was used to test the homogeneity of the data.
The significance threshold must be below 0.05 to validate the method. Moreover, the variation coefficient of repeatability (CVr) and reproducibility (CVR) were calculated.
The linearity range was 0.4 to 8 μg/spot. All statistical tests were significant to validate the method for all phospholipid classes.

APPLICATIONS

Determination of the enrichment of rat liver mitochondria in CL and PE *via* liposomes

Figure 4 : Cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) phosphatidylcholine (PC) and sphyngomyelin (SM).
Spots 1 to 3 represent curve range of authentic phospholipids.
Spots 4 to 9 represent samples enriched with PE at 2 differents dilutions for each sample, and the spot 10 is the control sample associated. Spots 11-12 are samples enriched with CL and spots 13-14, its control sample.
Eluent: chloroform-acetone-acetic acid- methanol-water 6:8:2:2:1 (v/v). Developer: 0.18% copper sulfate in orthophosphoric acid-distilled water (40:460, v/v).
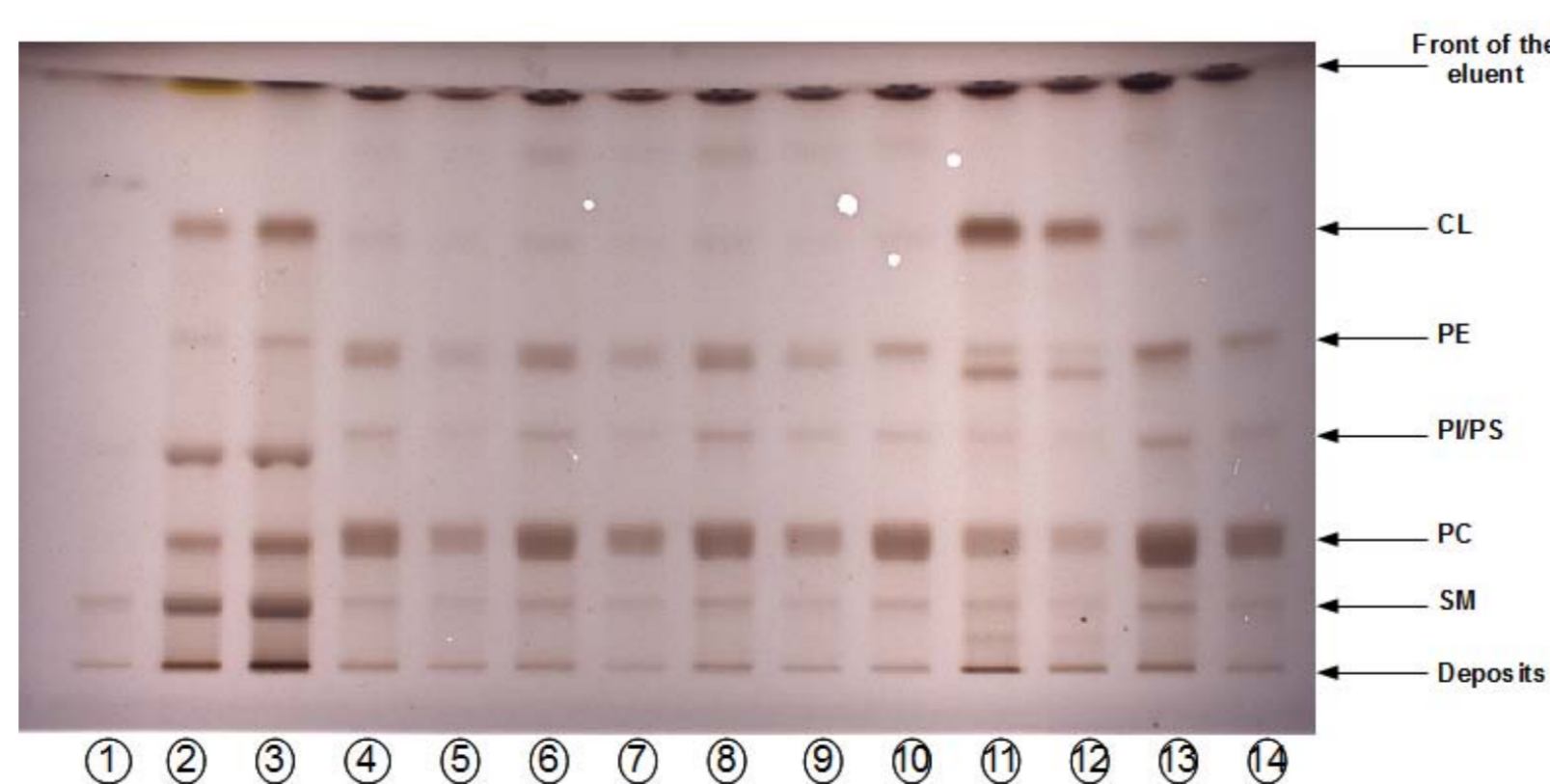
Quantification of CL by HPTLC-densitometry and comparison to probe NAO

Figure 5 : HPTLC-densitometry was compared to the NAO (10N-nonyl acridine orange) technique to quantify cardiolipin in liver mitochondria from control and cancer rats. Our results showed that data were very similar as change between cancer and control was the same whatever the used technique (1.55 vs 1.53 for HPTLC-densitometry and NAO, respectively, by calculating their ratio cancer/control).
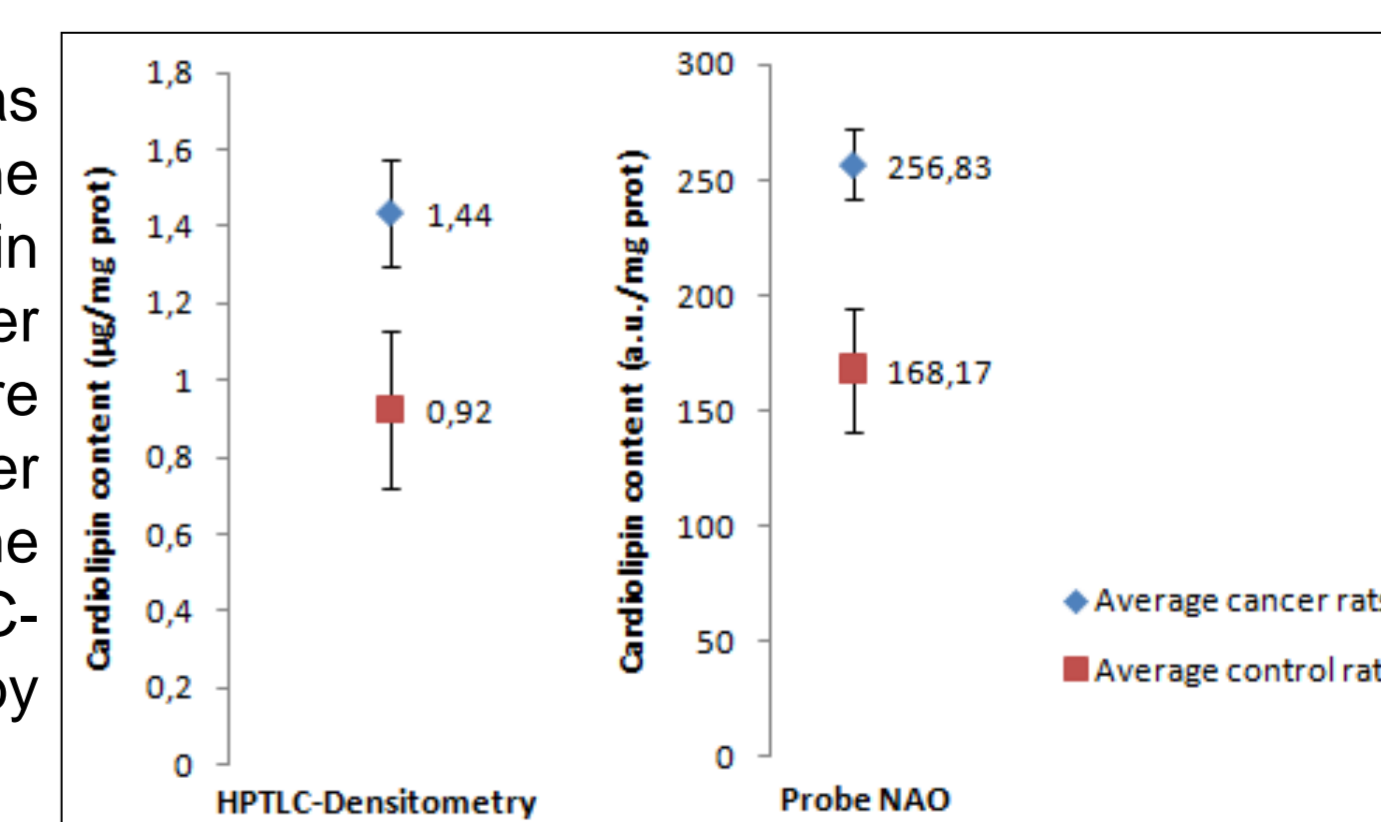
CONCLUSIONS

The HPTLC-densitometry method is a new, selective, precise, quantitative and low-cost method for the determination of cardiolipin and other phospholipids. Quantification is linear up to 8 μg. It will allow the determination of cardiolipin level, as well as other phospholipid classes in mitochondria in diverse pathologies where a default of phospholipids metabolism in present. For instance, our results have shown that CL content in liver mitochondria is increased by cancer cachexia.