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Research paper

NIR transmission spectroscopy for rapid determination of lipid and lyoprotector content in liposomal vaccine adjuvant system CAF01

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ABSTRACT

It is of crucial importance to determine the concentration of the different components in the formulation accurately, during production. In this respect, near-infrared (NIR) spectroscopy represents an intriguing alternative that offers rapid, non-invasive and non-destructive sample analysis. This method, combined with multivariate data analysis was successfully applied to quantify the total concentration of lipids in the liposomal CAF01 adjuvant, composed of the cationic surfactant dimethyldioctadecylammonium bromide (DDA) and the immunomodulator α,α′-trehalose 6,6′-dibehenate (TDB). The near-infrared (NIR) detection method was compared to a validated high-performance liquid chromatography (HPLC) method and a differential scanning calorimetry (DSC) analysis, and a blinded study with three different sample concentrations was performed, showing that there was no significant difference in the accuracy of the three methods. However, the NIR and DSC methods were more precise than the HPLC method. Also, with the NIR method it was possible to differentiate between various concentrations of trehalose added as cryo-/lyoprotector. These studies therefore suggest that NIR can be used for real-time process control analysis in the production of CAF01 liposomes.

1. Introduction

CAF01 is a novel adjuvant system composed of the cationic surfactant dimethyldioctadecyl ammonium bromide (DDA) and the immunomodulating glycolipid α,α′-trehalose 6,6′-dibehenate (TDB). Upon dispersion in aqueous solution DDA self-assembles into liposome structures, which are cationic due to the quaternary ammonium head-group of DDA. Incorporation of TDB into the liposomes not only enhances and modulates the immune response towards co-injected antigens in a Th1 direction, but also stabilizes the liposomes. This stabilization is probably due to enhanced hydration of the liposome membrane and sterical hindrance, avoiding reduced charge repulsion and aggregation [1,2]. The addition of trehalose to the formulation has enabled freeze-drying of the formulation, which can be readily redispersed in water [3]. Thereby, one of the big hurdles in developing vaccines intended for the developing world can be overcome, making the formulations less dependent on functioning cold-chains for the distribution of the vaccines.

When bringing liposomes like CAF01 into clinic, it is important to address issues such as how to perform product quality control (QC) during the different stages of production. This consideration is important, especially in light of the recent process analytical technology (PAT) initiative and the guidance documents (Q8–Q10) from the International Conference on Harmonization (ICH) [4] which have been implemented by both European Medicines Agency (EMEA) [5] and the US Food and Drug Administration (FDA) [6]. These guidelines encourage pharmaceutical production sites to implement real-time quality control methods into the manufacturing process. Implementation of a PAT system roughly encompasses (1) identification of critical points in the manufacturing process through risk assessment, (2) identification of suitable analytical techniques and (3) subsequent installation of related sampling devices in the production (e.g. in the form of probes) designed to perform timely measurements of the bulk product and/or manufacturing environment. If unwanted changes are observed during production, measures can be taken, e.g. by adjusting process parameters, to ensure that end product quality is maintained.

Several methods for quantification of lipid content in liposomes have previously been reported. These include chemical techniques

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(e.g. the Bartlett assay [7]), high-performance liquid chromatography (HPLC) [8–10] and enzyme-based assays [11–13]. An alternative method is high sensitivity differential scanning calorimetry (hsDSC) usually used to investigate the nature of phase- and/or structural transitions in lipid membranes and proteins.

All of the above mentioned methods are generally used off-line (i.e. manual sampling with transport to remote laboratory) and, in addition, are resource-intensive with respect to sample preparation and analysis time. Furthermore, HPLC requires extraction of the lipid components, which must be validated as complete and non-selective. Due to the apolar nature of the lipids, organic solvents are needed to ensure proper elution, thus requiring specialized equipment and localization. Furthermore, as lipids lack light absorbing moieties, light detectors like UV and fluorescence detectors are usually not efficient for detecting liposomal components. Hence, other spectroscopic methods like evaporating light scattering detector (ELSD) or mass spectroscopy (MS) could be implemented.

The enzyme-based assays are commercially available kits, which only require a spectrophotometer. The main drawback is that they can only detect cholesterol and cholesterol esters, choline-containing phospholipids or non-esterified fatty acids [13]. This excludes the quantification of a long range of lipids including DDA and TDB. DSC enables accurate determination of the concentration-dependent change in enthalpy (ΔH) of the liposomes when going from a lower temperature gel-phase to a higher temperature fluid-phase. The applicability of this method, however, is highly dependent on the composition of the liposomes, since a well-defined gel–liquid phase transition is a prerequisite. Furthermore, DSC is time consuming due to the low scanning speed. Finally, all the above mentioned methods are destructive and thus not suitable for real-time analysis of CAF01 liposomes and trehalose.

Near-infrared (NIR) spectroscopy presents an intriguing alternative, requiring no sample preparation while offering rapid (seconds rather than minutes), non-invasive and non-destructive sample analysis. Furthermore, NIR spectroscopy allows for measurements directly through transparent sample containers, e.g. glass and plastic. This enables monitoring of large numbers of samples in the production line, without affecting the throughput of the production. For these reasons, the technique has gained wide acceptance within the pharmaceutical industry, either as part of off-line quality control or as a PAT tool [14]. NIR spectroscopy measures overtones and combinations of fundamental vibrations from the mid-IR region: –OH, –NH, –SH and –CH. Anharmonicity of the atomic vibrations gives rise to lower absorption (compared to mid-IR) and, as a result, the bands related to particular functional groups of the analyte in question are often broad and overlapping [15]. Hence, data analysis of more than a single wavelength is often needed to adequately describe the physicochemical features of the sample found in the spectra. For this purpose, the multivariate modeling method principal component analysis (PCA) may be used [16]. PCA reduces the number of original (and highly co-linear) variables into so-called principal components (PCs), best describing the systematic variance found in the spectra. These new variables (i.e. the PCs) contain physicochemical information on the sample. PCA therefore facilitates interpretation of NIR spectroscopy data, while modified versions of the algorithm, e.g. partial least squares (PLS) regression, are often used for building quantitative calibration models [17].

NIR spectroscopy has been used extensively for quantification of fatty acids and proteins in the food industry (e.g. [18–20]). Few efforts, however, have been done to thoroughly scrutinize the use of NIR transmission spectroscopy and multivariate data analysis for detection of lipids in liposomes. The work presented in this paper is therefore a feasibility study, in which the objective was to evaluate the applicability of NIR transmission spectroscopy for the quantification of CAF01 liposome content in liquid formulations. The developed model will be compared to results obtained by established HPLC and DSC methods and the results from the three methods will be evaluated using HPLC as the reference method. Furthermore, the possibility to qualitatively determine trehalose content in liposome formulations by NIR transmission spectroscopy is investigated. It is expected that implementation of NIR methods will provide faster – yet reliable – analyses, compared to existing HPLC and DSC methods. Hopefully, this should also help set the scene for future use of in-line NIR spectroscopy methods within the field of liposomology.

2. Materials and methods

2.1. Materials

Dimethyldioctadecylammonium (DDA) bromide and α,α′-trehalose 6,6′-dibehenate (TDB) were obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the compounds was >99% by HPLC. Methanol (extra pure) and chloroform (extra pure) were purchased from Merck (Darmstadt, Germany). Tris base (99%) was obtained from Sigma–Aldrich (St. Louis, MO). Trehalose dihydrate (≥99%) was obtained from Sigma–Aldrich (Brøndby, Denmark). Purified water of Milli-Q quality was used to prepare all buffers.

2.2. Liposome preparation

The adjuvant was prepared by the thin film method as described previously [1]. For the quantitative analysis, weighed amounts of DDA and TDB were dissolved in chloroform/methanol (9:1 v/v), and the organic solvent was removed using a gentle stream of N_2, forming a thin lipid film on the bottom of the test vial. The lipid film was dried overnight under vacuum to remove trace amounts of the organic solvent. The lipid films were hydrated to their final concentration (Table 1) in 10 mM Tris-buffer (pH 7.4) containing 0–100 mg/ml trehalose (Table 1) by heating for 20 min at 10 °C above the main phase transition temperature of DDA ($T_m \approx 47$ °C) [21].

2.3. High-performance liquid chromatography analysis

Standard curves for DDA and TDB were prepared according to the protocol for the QC-validated method at Statens Serum Institut [22] analyzing 1, 2, 3 and 4 mg DDA and 0.2, 0.4, 0.6 and 0.8 mg TDB. Briefly a Vydac 218TP52 reversed-phase C_{18} column (250 × 2.1 mm, 5 μm particles) was used on a Dionex Summit HPLC with a Shimadzu ELSD-LT evaporative light scattering detector. Twenty-five microliters of sample was injected and eluted in 68/32% (v/v) of eluent A (0.1 M ammonium acetate in methanol) and B (chloroform) for 6 min. This gradient was followed by a 10 min plateau at 10/90% eluents A and B, before going back to the initial eluent mixture for 9 min. All runs were performed at 25 °C at a flow rate of 0.400 ml/min. ELSD detection was done at

Table 1
Overview of the liposome preparations containing DDA, CAF01 and CAF01 + trehalose

Formulation	Content		
	DDA (mg/ml)	TDB (mg/ml)	Trehalose (mg/ml)
DDA	0.50–3.50	–	–
CAF01 (DDA + TDB fixed ratio = 5:1)	0.50–3.50	0.10–0.70	–
CAF01 + trehalose	0.625	0.125	0–100
	1.25	0.25	0–100
	2.50	0.50	0–100

DDA and CAF01 were prepared in concentration intervals of 0.50 mg/ml DDA.

55 °C in 350 kPa N_2-flow. Three repetitive injections of each sample were used for data analysis. Standard curves were obtained from an average peak area of three analyses against the absolute amount of weighed lipid in chloroform:methanol (9:1 v/v) injected. Prior to the analysis, the unknown samples were freeze-dried and dissolved in chloroform:methanol (9:1 v/v) to an estimated concentration of 2.0 mg/ml DDA and 0.4 mg/ml TDB ($n = 6$). The HPLC results were used for reference since this method was QC-validated for quantification.

2.4. Differential scanning calorimetry

The thermotropic phase behavior of the liposomes in suspension was determined using *hs*DSC. Thermograms were obtained as described previously [1] using a MicroCal VP-DSC MicroCalorimeter (MicroCal LLC, Northamton, USA), scanning at a rate of 30 °C/h from 25 to 60 °C [1]. *VPViewer 2000* and *Origin® 7 scientific plotting* software (OriginLab, Northampton, USA) were used for data analysis. One scan on each sample ($n = 3$) was used for data analysis. Standard curves were obtained by plotting an average peak area of three analyses against the absolute amount of the liposome preparations shown in Table 1.

2.5. Near-infrared spectroscopy

NIR spectra of the liposome formulations were obtained in the transmission mode using a Fourier-Transform NIR spectrometer equipped with an InGaAs detector and a quartz halogen lamp (Nicolet Antaris Near-IR Analyzer, Thermo Fisher Scientific, Waltham, MA). A total of 32 scans per spectrum were acquired with a resolution of 8 cm^{-1}. Prior to analysis in this study, an adequate amount of sample was transferred to a 300 μl glass vial with an inner and outer diameter of 3.6 and 5.5 mm, respectively. Samples were measured in random order through the side of the glass vial. For each sample triplicate measurements were included in the randomization scheme.

2.6. NIR data analysis

2.6.1. Spectral processing

All spectra were preprocessed by standard normal variate (SNV) using *SIMCA-P+ 11.5* (Umetrics, Umeå, Sweden). This was done to remove unwanted intensity features of the spectra as a result of varying path length between samples [15].

2.6.2. Multivariate modeling

PCA is a bi-linear modeling method that approximates the original data table, *X*, (with objects/samples and variables/wavenumbers organized as rows and columns, respectively) through decomposition into a set of mutually orthogonal components termed principal components (PCs) and a residual matrix, *E* [16]. The first PC corresponds to the direction of maximum explained variance with each successive PC accounting for as much of the remaining variance as possible. All *X*-variables were centered prior to analysis.

Quantification of the total liposome concentration was done using partial least squares (PLS) regression on preprocessed NIR data. PLS is used for the purpose of predicting a particular (and user-selected) sample property. Lipid concentration, based on weighed amount at seven levels ranging from 0.6 to 3.0 mg/ml, was used as input for *y*. Ultimately, a simple one component PLS model, explaining 94.2% and 99.6% variance of *X* and *y*, respectively, was used for predicting new samples.

PCA and PLS modeling was performed using *SIMCA-P+ 11.5* (Umetrics, Umeå, Sweden) and the results were visualized using *Origin® 7 scientific plotting* software (OriginLab, Northampton, USA).

3. Results and discussion

3.1. Lipid quantification using HPLC

Each lipid starting material was analyzed individually by HPLC. Dry lipid powder was dissolved in organic solvent and injected directly for sample analysis. Superimposed chromatograms of CAF01 are presented in Fig. 1A. Linearity between the injected concentrations and the elution peak areas was obtained in the analyzed concentration range for DDA (Fig. 1B) and TDB (Fig. 1C). The correlation coefficients and standard deviations for both DDA and TDB indicate that the method is suitable for quantification.

This HPLC method is easy to operate and the ability to measure the concentration of DDA and TDB separately supports its use. However, the necessity for removal of water from the samples before analysis makes it a time consuming and expensive procedure. Another argument against this method is that the chlorinated eluent resulted in problems with air bubbles in the system as well as leakage problems, despite strong precautions were taken, with solvent resistant hardware and comprehensive cleaning procedures, to secure a tight system. Additionally, the use of chloroform should be avoided if possible, due to the adverse effects of chlorinated solvents on health and environment.

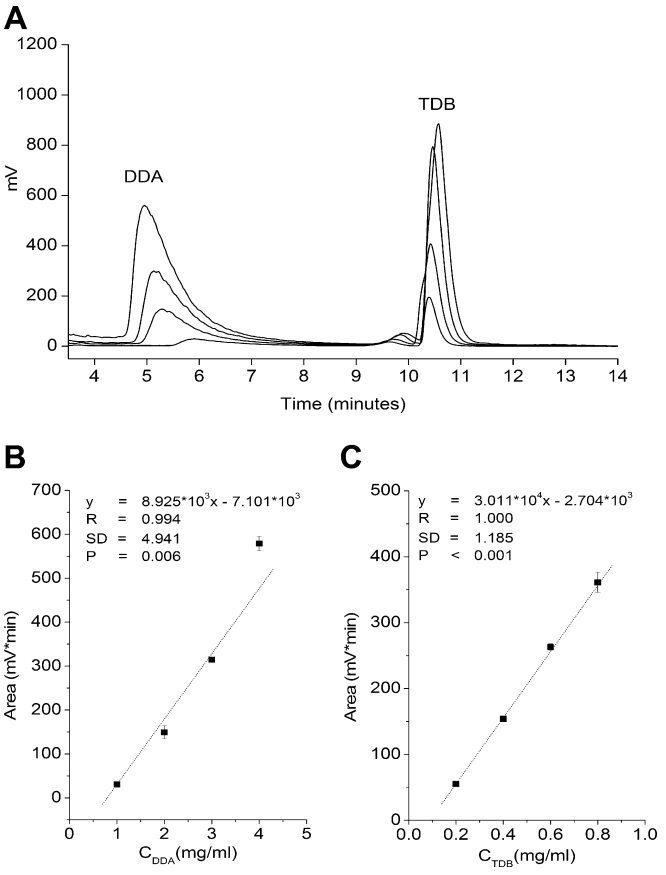

Fig. 1. Superimposed chromatograms (A) and standard curves of DDA (B) and TDB (C) analyzed by rp-HPLC and ELSD detection. The chromatograms (A) illustrate an injected quantity of 1.2, 2.4, 3.6 and 4.8 mg/ml CAF01, respectively. In the standard curves (B and C) the peak area of each lipid is plotted against the absolute amount of injected lipid. Slopes, intercepts and correlation coefficients were determined from linear regression analysis. Each point is the mean of three determinations ± standard deviation.

3.2. Lipid quantification using DSC

The thermotropic phase behavior of CAF01 vesicles (DDA to TDB ratio of 5:1 w/w) was determined at various concentrations according to Table 1. The thermograms obtained are shown in Fig. 2A (average of triplicates) and are in accordance with previously reported data [1,3]. There is a small decrease in T_m of the main transition peak when increasing the lipid concentration from 0.6 mg/ml (T_m = 42.74 ± 0.01 °C) to 3.0 mg/ml (T_m = 42.20 ± 0.07 °C). With the increased concentration of lipids, there might be an increased possibility for a uniform distribution of DDA and TDB causing a similar change in the phase transition pattern, which could explain the small decrease in T_m.

As illustrated in Fig. 2B, a linear relationship was obtained between CAF01 concentration and the ΔH in the analyzed concentration range (0.5–3.0 mg/ml). At the analytical condition described, the correlation coefficient (r) and the standard deviation (SD) for the linear regression indicated that the correlation was suitable for quantification.

Importantly, the DSC quantifies the structural interactions between the molecules in the formulation, and thereby the amount of liposome-formations rather than the amount of lipid components as the HPLC method. However, as illustrated previously [1,2] the phase transition pattern changed with varying concentrations of TDB. This could form basis for a validation model, which also addresses the proportion between DDA and TDB. In addition, there is no need for preparation of the samples before DSC analysis. This, and the practically non-existing influence from the surroundings on the analysis equipment, might prolong the time between standard measurements.

The method protocol used here is time consuming, and even though a method optimization could reduce the analysis time substantially and auto-sampling would make unattended sample handling possible, it will still be more time consuming than HPLC.

3.3. Lipid quantification using NIR

As expected, strong absorption bands were observed in the NIR spectra. To minimize the interference from water, only the spectral regions 6340–5500 cm^{-1} and 10,000–7500 cm^{-1}, related to 1st and 2nd overtone vibrations of C–H, respectively, were used for analysis (Fig. 3). Preprocessed NIR spectral data on liposome formulations of DDA and CAF01 were decomposed into three principal components (PCs) explaining cumulative 99.9% variance. As apparent from Fig. 4A, PC1 (96.7%) describes an increase in lipid concentration, turning from negative to positive PC1-score values. In contrast, PC2 (2.7%) describes intrasample variation, i.e. minor differences between glass vials, as indicated by the relatively large PC2-score standard deviations of triplicate measurements. Discrimination between DDA and TDB is possible in PC3 (0.5%), however, only at concentrations above 2 mg/ml. Since PC3 describes a small proportion of the total variance, differentiating between DDA and TDB by NIR spectroscopy is not possible in the current study.

To investigate the possibility of lipid quantification, PC1-scores for the CAF01 samples were plotted against total lipid concentrations. A linear relationship was found between spectral information (as retained in PC1) and total lipid concentration, with an excellent linear correlation (Fig. 4C). These findings served as basis for developing the final one component PLS model described in Section 2.6.2. This illustrates that it is possible to build a multivariate regression model based on NIR data for the quantification of the lipid content of liposomes.

In comparison to HPLC and DSC, NIR spectroscopy is a fast and easy operational method that requires little manual intervention upon implementation in a pharmaceutical process. It is, however, relevant to underscore the importance of model maintenance. Multivariate models, in particular, may loose their validity over

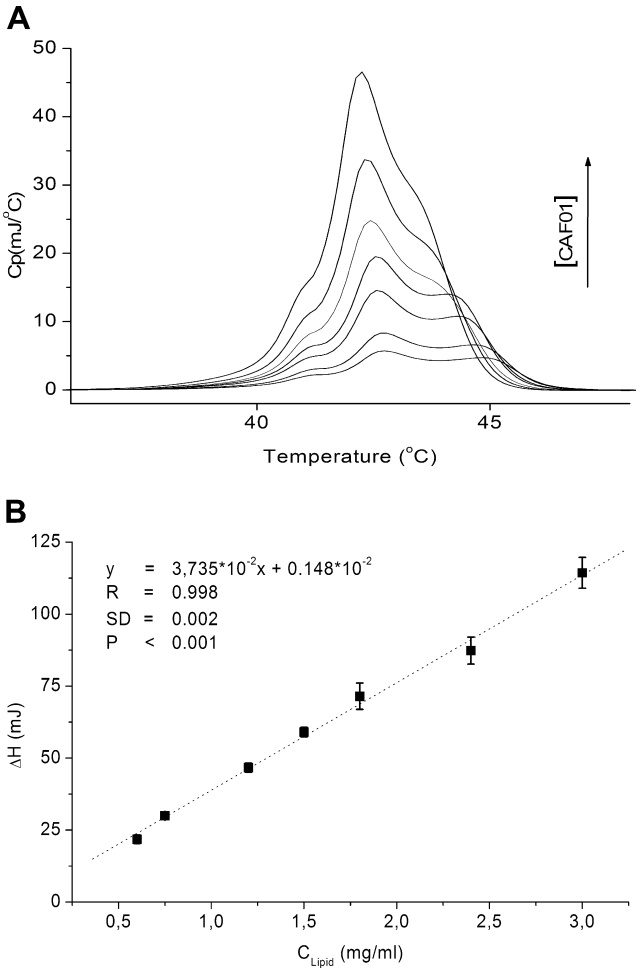

Fig. 2. Thermograms (A) and standard curve (B) of freshly prepared liposome samples ranging from 0.6 to 3.0 mg/ml CAF01. In the standard curve (B) the peak area (ΔH) of each liposome formulation was plotted against the absolute amount of CAF01. Slopes, intercepts and correlation coefficients were determined from linear regression analysis. Each point is the mean of three determinations ± standard deviation.

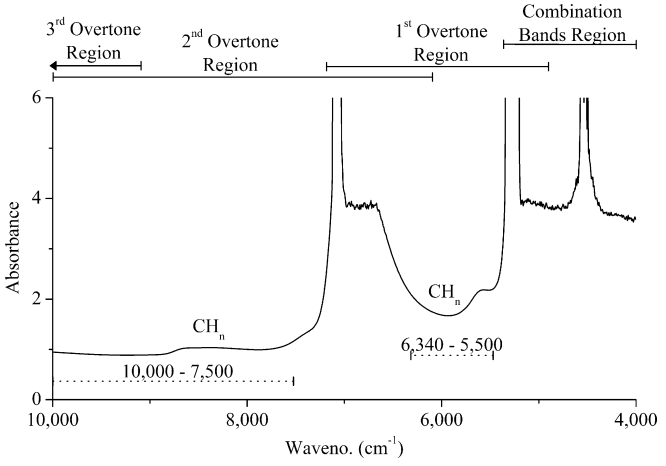

Fig. 3. Raw NIR spectrum of 4.2 mg/ml CAF01 sample. To minimize the interference from water, only the spectral regions 10,000–7500 cm^{-1} and 6350–5500 cm^{-1} were used for analysis.

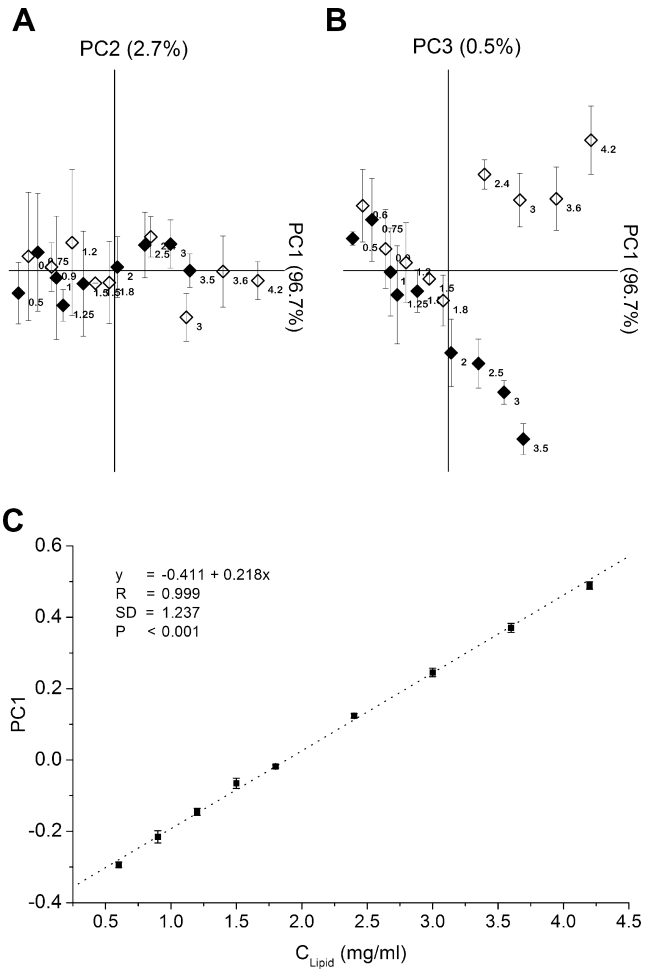

Fig. 4. The position of sample-NIR spectra in the (A) PC1/PC2 and (B) PC1/PC3 subspace for various concentrations of DDA (♦) and CAF01 (◇). (C) PC1-scores plotted against the total liposome concentration. Slopes, intercepts and correlation coefficients were determined from linear regression analysis. Error bars indicate the scores standard deviation of triplicate measurements in the particular PC direction. The numbers next to the symbols constitute the lipid concentrations.

time, resulting in erroneous predictions. This is often due to unforeseen changes in raw materials, instrument and/or manufacturing environment, thus creating new sources of variation not included in the initial calibration model. If not corrected for, the robustness of the NIR spectroscopy model may be impaired. Several approaches to deal with these issues exist [23]. As this study is stating proof-of-concept rather than actual implementation of a NIR spectroscopy method, this topic will not be described further.

3.4. Comparative feasibility study on the determination of lipid concentration in CAF01 liposomes

Blinded samples with three different concentrations of CAF01 were prepared in six replicates, and the concentrations were determined using the three methods described above (Fig. 5).

Accuracy calculation was performed to determine the closeness between the HPLC values, which due to the status as a validated method make up the true concentration values, and the experimental values of the DSC and NIR spectroscopy. The accuracy of the methods was investigated by one-way ANOVA statistics ($F_{0.05}$) between DSC or NIR spectroscopy replicate measurements of each concentration and the "true" HPLC concentration using the single factor, fixed effect model testing the hypothesis H_0: $\mu_{HPLC} = \mu_{DSC} = \mu_{NIR}$. This hypothesis was accepted for all three batch concentrations, indicating that both DSC and NIR spectroscopy obtained similar results as the HPLC method (Table 2) confirming the results indicated in Fig. 5.

Precision studies were performed to determine the ability of the values to be consistently reproduced within the methods. The precision was estimated by comparing the variances of the HPLC, DSC and NIR spectroscopy replicate measurements at each concentration. Submitting absolute deviation of the observations from the median to ANOVA statistics ($F_{0.05}$) (modified Levene test [24]) rejected the hypothesis H_{method}: $\sigma^2_{HPLC} = \sigma^2_{DSC} = \sigma^2_{NIR}$ but accepted the hypothesis $H_{conc.}$: $\sigma^2_{High} = \sigma^2_{Medium} = \sigma^2_{Low}$ (Table 2) suggesting that there is a difference between the precision of the methods but not between the precisions at different concentrations. Tukey's test showed that $\sigma^2_{HPLC} > \sigma^2_{DSC} \approx \sigma^2_{NIR}$ indicating that both DSC and NIR were more precise than HPLC. This essentially confirms the conclusions drawn from visual examination of Fig. 5. Thus it seems that the HPLC method is associated with problems related to high loading of lipids on the column, which could be circumvented by diluting the sample further before analysis. However, since this was a blinded study, no attempts were done to improve the results.

3.5. Further prospects of NIR: Presence of trehalose in CAF01 formulations

Trehalose can be used as a cryo-/lyoprotector of CAF01 liposomes during freeze-drying [2,3], and would therefore be added to the formulation together with a buffer during production. A successful outcome of the freeze-drying process is highly dependent on the concentration of trehalose [3], making it crucial to be able to determine this concentration in the liquid formulation before freeze-drying. The position of sample-spectra in the PC1/PC2 subspace at three different concentrations of CAF01 containing increasing concentrations of trehalose is shown in Fig. 6 Clearly, with the NIR method it is possible to detect differences in the CAF01 concentration in PC1 (59%) even at high concentrations of trehalose. Furthermore, variance related to the trehalose concen-

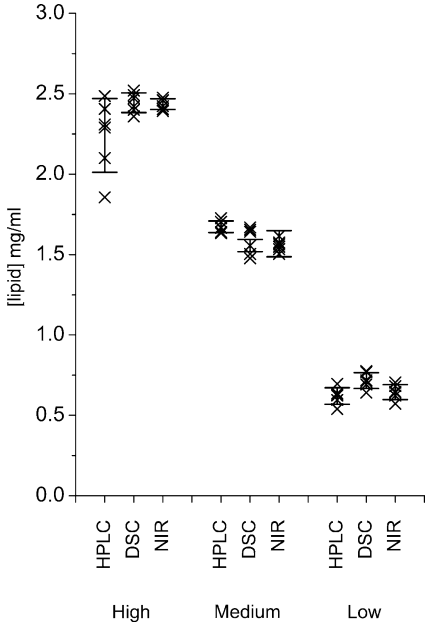

Fig. 5. Plot of predicted lipid concentration versus method for the three different blinded CAF01 preparations denoted *high*, *medium* and *low*. Error bars indicate the standard error of mean ($n = 6$). Visual inspection indicates that the three methods produce similar results, the HPLC method, however, with significantly higher standard deviations than the DSC and NIR methods. This is consistent for all three concentrations.

Table 2
Analysis of variance (ANOVA) results on precision and accuracy of the quantification of CAF01 in the comparative feasibility study using HPLC, DSC and NIR as quantification methods

Sample		Method			Accuracy ANOVA ($F_{0.05}$ = 2.81)	
		HPLC	DSC	NIR	F_0	Significant
A		2.241 ± 0.229	2.444 ± 0.062	2.436 ± 0.034	1.44	No
B		1.673 ± 0.036	1.557 ± 0.083	1.568 ± 0.081	2.75	No
C		0.620 ± 0.051	0.716 ± 0.049	0.645 ± 0.046	2.16	No
Precision (method) ANOVA ($F_{0.05}$ = 3.21)	F_0	11.19				
	Significant	Yes				
	Tukey ($q_{0.05}$)	$\sigma^2_{HPLC} > \sigma^2_{DSC} = \sigma^2_{NIR}$				
Precision (conc.) ANOVA ($F_{0.05}$ = 3.21)	F_0	2.19				
	Significant	No				

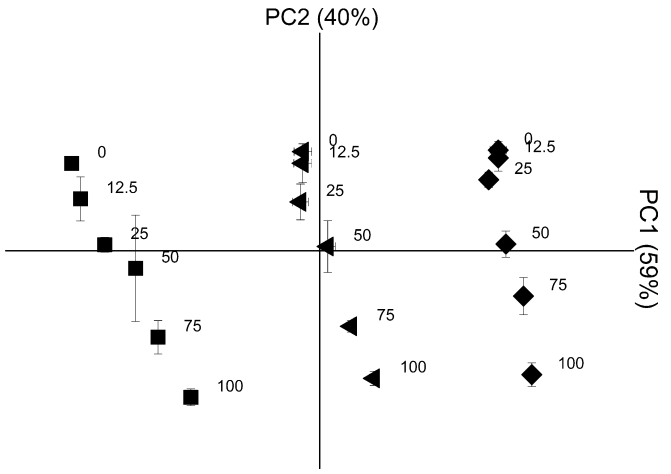

Fig. 6. The position of sample-NIR spectra in the PC1/PC2 subspace for 0.75 mg/ml (■), 1.5 mg/ml (◄) and 3.0 mg/ml (♦) CAF01 with varying concentrations of trehalose (mg/ml). Error bars indicate the scores standard deviation of triplicate measurements in the particular PC direction. The numbers next to the symbols constitute the trehalose concentration.

tration was primarily described in PC2 (40%). These results suggest that it is possible to obtain information not only on total lipid content, but also trehalose content from a single NIR measurement. This is not possible with either the HPLC method or the DSC method. Since trehalose is a constituent of the formulation from the beginning of the formulation process, and since the success of the freeze-drying process is very much dependent on the trehalose, the ability to determine the concentration of this sugar without interfering with the lipid detection is of great importance.

4. Conclusion

This study is the first to document that NIR spectroscopy can be applied to determine the lipid content of liposomes (CAF01). The precision and accuracy of the constructed NIR model were at least as good as those of the HPLC and DSC methods described. Furthermore, use of multivariate analysis on NIR data enabled capturing variance related to changes in trehalose content. This suggests that a multivariate NIR model can provide information on not only CAF01 liposomes but also trehalose content from a single NIR spectrum.

HPLC and DSC are both analytical methods which require batch processing and testing on collected batch samples to evaluate quality. This procedure is time consuming and can result in considerably delayed batch releases with the resulting expenses. The implementation of in- or at-line quality control would therefore improve the efficiencies of both the manufacturing and regulatory processes. By implementing NIR as a quality control method it is possible to incorporate continuous real-time analysis as recommended within the PAT framework. Undoubtedly, more research on the use of NIR within the field of liposomology is to come.

Acknowledgments

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