

Development and validation of a RPLC-MS/MS method for the quantification of ceramides in human serum of patients with CAD

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Introduction

- Ceramides are key-role compounds that regulates numerous central cellular processes, such as apoptosis, inflammation, etc.
- A plethora of studies have showed that changes on the concentrations of these bioactive lipids in serum are associated with different pathophysiological states, namely obesity, cardiovascular diseases, ovarian cancer, metabolic disorders.
- Four specific molecular ceramides are highly linked to coronary artery disease (CAD), enhancing the prediction of fatal outcomes (Fig. 1).
- Coronary artery disease (CAD) represents the most common form of cardiovascular disease and remains one of the leading causes of mortality and morbidity worldwide, especially in western countries. Greece is not an exception, as 250,000 patients suffering from coronary atherosclerosis.
- Different types of coronary syndrome includes the ST-elevation myocardial infarction (STEMI), the non-STEMI (NSTEMI) ST-segment elevation MI, the unstable angina and stable angina.

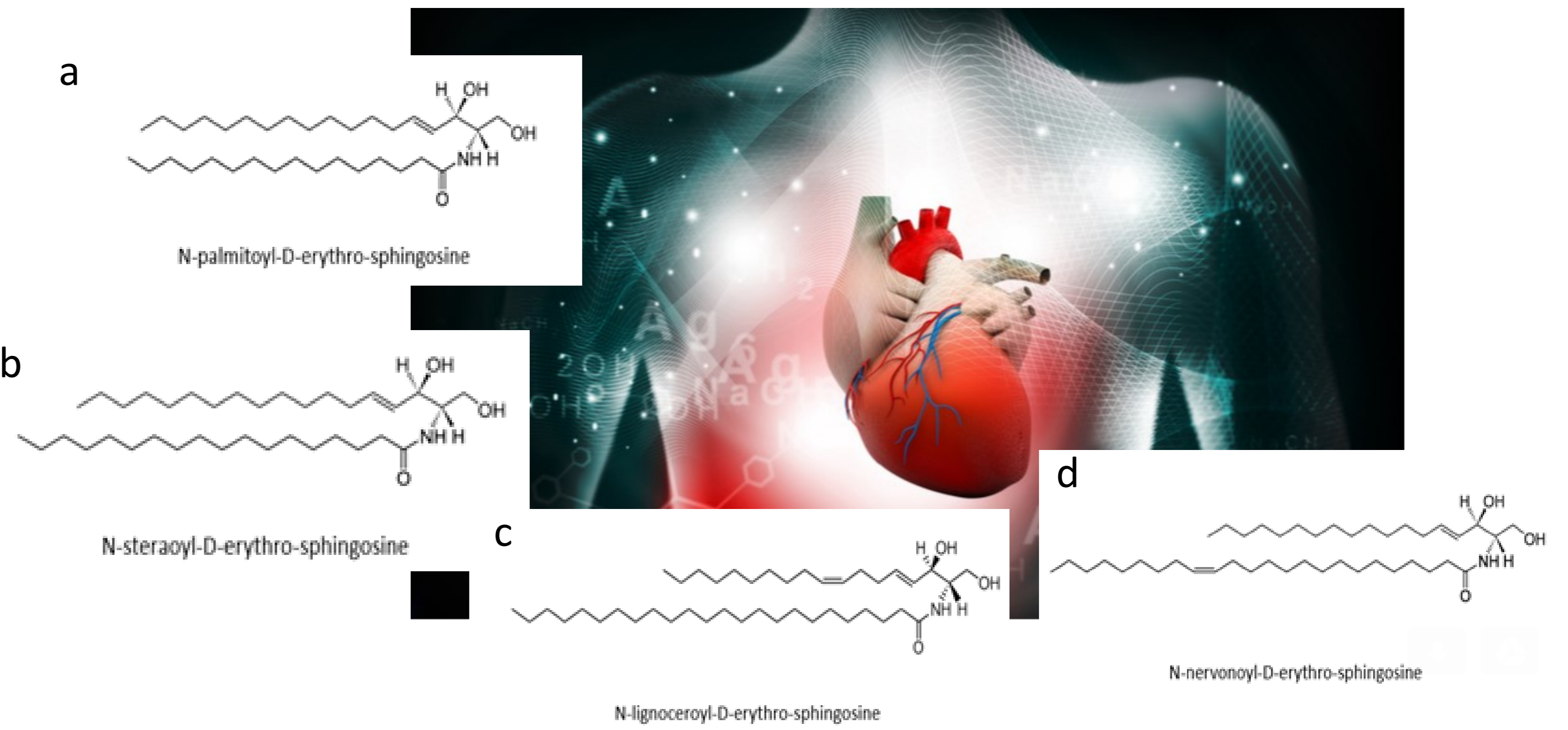


Fig. 1: Molecular structure of C16:0 (a), C18:0 (b), C24:0 (c), C24:1 (d)

Aims

- The aim of the present study was the development and validation of an RPLC-MS/MS method of the accurate quantification of four highly significant ceramides in CAD patient serum samples.
- Patients suffering from CAD were recruited to participate in large-scale study, "CorLipid" (MIS 5030456) aiming the diagnostic utility of serum metabolomics-based biomarkers for determination of the complexity and the severity of coronary artery disease, assessed using SYNTAX score.

Method Development

- Instrument: Alliance HT Waters 2790 (Milford, Massachusetts, USA) equipped with a SCIEX API 3200 TM (Warrington, Cheshire, UK)
- Column: ReproShell ODS-3 (50 mm x 2 mm, 2.7 μm).
- Mobile Phase: A: H₂O + 10 mM CH₃COONa (pH=5), B: MeOH: IPA 1:1 v/v + 10 mM CH₃COONa + 0.1 % FA.
- IS: Deuterated Ceramide LIPIDOMIX® Mass Spec Standard solution (1:1 dichloromethane:methanol), (Avanti Polar Lipids)

- Both liquid-liquid (LLE) and supported liquid (SLE) extraction were applied examining the efficacy of the sample preparation process.
- Different extraction solvents for LLE included MTBE, CHCl₃: MeOH, 1:1 v/v, CHCl₃: MeOH, 1:1 v/v + NaCl and for SLE included EA, MTBE, CHCl₃: IPA 1:1 v/v were tested.
- SLE with EA were selected based on the accuracy, precision, extraction recovery, process efficiency and matrix effect.

Table 1. Gradient System

Time	A%	B%	Flow Rate (mL/min)
0	20	80	0.6
1.5	0	100	0.6
3.0	0	100	0.6
3.01	20	80	0.6
5.0	20	80	0.6

Table 2a & 2b: MS parameters

	MW	Parent Ion	Daughter Ions	DP (volts)	EP (volts)	CEP (volts)	CE (volts)	CXP (volts)
C16:0	537.512	538.470	264.300*	55.0	4.5	24.0	31.0	4.0
			520.500				21.0	6.0
C18:0	565.543	566.460	264.300*	51.0	5.0	30.0	33.0	4.0
			548.500				19.0	8.0
C24:0	649.637	650.532	264.400*	41.0	10.0	36.0	39.0	4.0
			632.600				27.0	6.0
C24:1	647.622	648.482	264.300*	61.0	4.0	46.0	41.0	4.0
			630.500				24.0	8.0

* Quantifier Ion

2b.	
Curtain Gas (CUR)	20.0
Collision Gas (CAD)	5
IonSpray Voltage	5500 V
Temperature (TEM)	450.0 °C
Ion Source Gas 1 (GS1)	55.0
Ion Source Gas 2 (GS2)	35.0

Sample Preparation

- Serum dilution with H₂O, 1:1 v/v
- Sample loading into cartridge & vacuum application (2-5 sec)
- Gradual elution gravity -assisted for 5 min
- Addition of 2 mL of EA & elution as previous.
- Vacuum application
- Evaporation to dryness under N₂ stream
- Reconstitution in IPA: MeOH 1:1 v/v

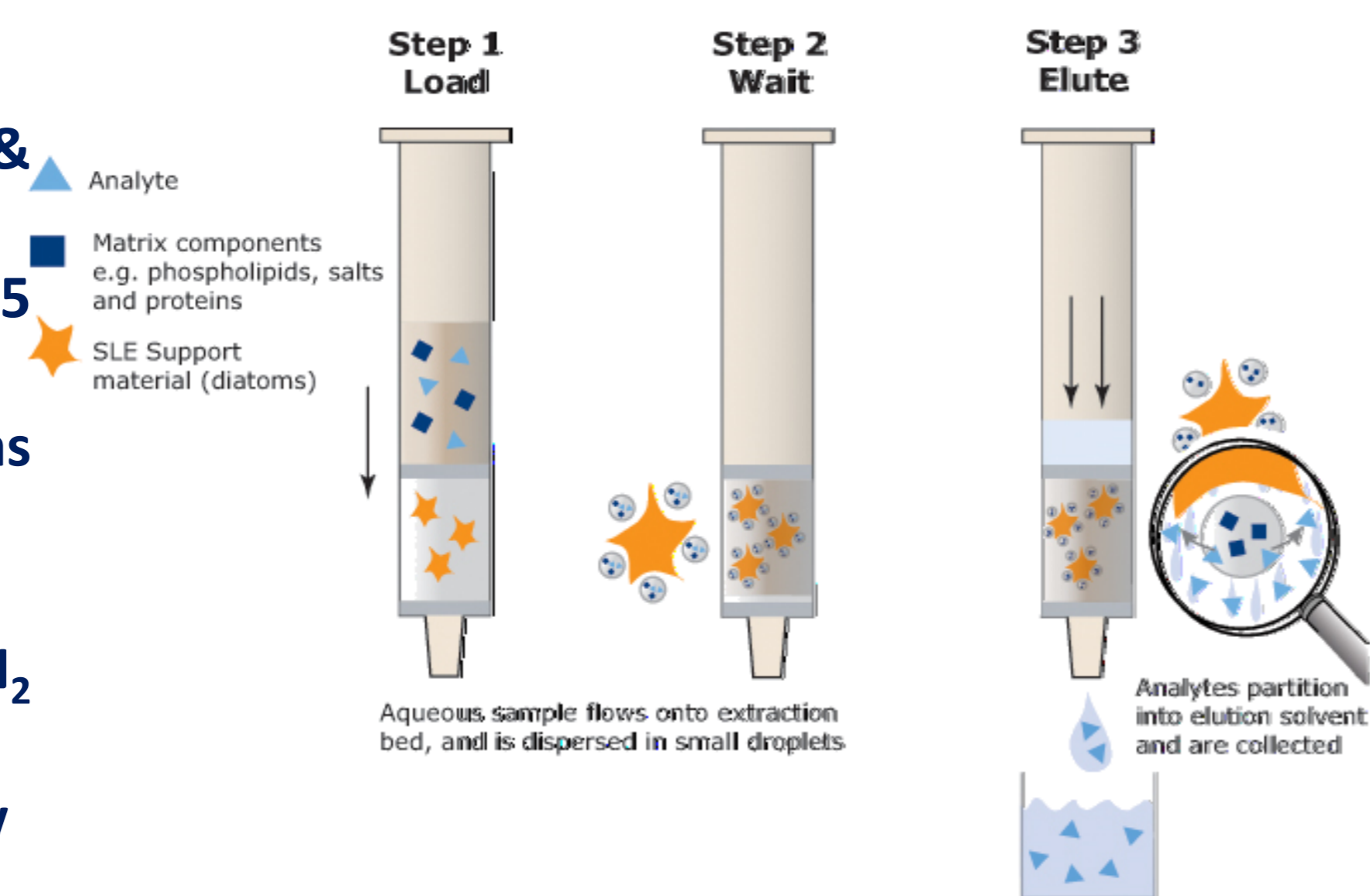


Fig. 2: Graphical illustration of SLE experimental steps.

Application

- Preliminary results were obtained applying the newly developed method. Twenty serum samples from 4 groups ACS (STEMI, NSTEMI, unstable angina) and stable angina were analyzed.
- The integration of the chromatographic peaks was achieved using Analyst Software. The data handling were performed using Graph Pad Prism 7.0 where ANOVA and FDR correction were processed.
- Univariate statistical analysis demonstrated significant differentiation of Ratio C18:0/C24:0 between patient serum samples with STEMI and Unstable angina ($p=0.047$), as well as STEMI and Stable angina ($p=0.025$).

Results

- The evaluation of the analytical parameters were performed in 3 different concentration levels (LQC, MQC, HQC) of spiked human serum (standard addition method) followed by SLE.
- Bovine Serum Albumin solution was tested as surrogate matrix of human serum, demonstrating satisfying results.
- LOD and LOQ were calculated as 3 ng/mL & 10 ng/mL, respectively.

Table 3: Analytical figures of merit

Analyte	Nominal Conc. ng/mL	Calculated conc. ng/mL	%RSD	Accuracy (% RE)	Extraction recovery (%)	Matrix effect
C16:0	250	207.9	1.4	83.1	84.6	105.2
	500	528.1	1.1	105.6	120.5	
	1000	996.5	4.0	99.6	106.4	
C18:0	250	230.4	1.2	92.2	92.1	98.5
	500	503.6	0.4	100.7	115.2	
	1000	1003.1	2.7	100.3	96.3	
C24:0	1500	1290.0	3.0	86.0	88.03	102.5
	3000	3541.6	0.5	118.1	113.7	
	6000	5825.4	3.6	97.1	108.7	
C24:1	1500	1329.9	0.5	88.6	87.3	106.3
	3000	3243.1	0	108.1	115.9	
	6000	5958.7	2.5	99.3	107.2	

Table 4: Mean concentration of the analyzed samples.

	Group	Mean concentration (n=20) ng/mL						
		C16:0	C18:0	C24:0	C24:1	Ratio C16:0/C24:0	Ratio C18:0/C24:0	Ratio C24:1/C24:0
Acute Coronary Syndrome	STEMI	559.8	153.2	2288.0	1488.2	0.28	0.07	0.66
	NSTEMI	337.4	69.4	2092.0	990.0	0.18	0.04	0.55
	Unstable angina	356.2	77.0	2334.0	1075.8	0.16	0.04	0.50
	Stable angina	437.0	68.6	2584.0	1276.2	0.18	0.03	0.56

Conclusion

- The developed and validated method was proven to be accurate, precise, sensitive and free of interferences.
- Suitable for application in clinical studies and routine analyses.
- Comparisons between STEMI - Unstable angina, as well as between STEMI - Stable angina were found to be significant.

Acknowledgments

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