

Development and Applications of Methods for
Assessing Fat Soluble Micronutrients (FSMs) in Dried
Blood Spots and Plasma by HPLC and
HPLC-MS/MS

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Table of Contents

LIST OF FIGURES	VI
LIST OF TABLES	VIII
ABSTRACT.....	I
DECLARATION	III
ACKNOWLEDGEMENTS	V
ABBREVIATIONS	VII
CHAPTER 1: LITERATURE REVIEW	1
1.1 OVERVIEW OF FAT SOLUBLE MICRONUTRIENTS.....	1
1.1.1 Vitamin A	2
1.1.2 Vitamin E.....	5
1.1.3 Vitamin D	6
1.1.4 Carotenoids	10
1.1.5 Summary	11
1.2 IMPORTANCE OF FSMs FOR FETAL DEVELOPMENT	12
1.2.1 FSM status during pregnancy	12
1.2.1.1 Low-middle income countries.....	13
1.2.1.2 Middle-high income countries.....	14
1.2.1.3 Australia and New Zealand.....	15
1.2.2 Consequences of FSM deficiencies during pregnancy.....	15
1.2.3 Summary	16
1.3 DETERMINATION OF FSMs FROM HUMAN BLOOD SAMPLES	17
1.3.1 Literature selection criteria	17
1.3.2 Sample types: plasma or serum.....	18
1.3.3 Sample types: dried blood spot (DBS).....	19
1.3.4 Extraction processes in plasma/serum	20
1.3.5 Extraction processes used in DBS.....	26
1.3.5.1 Vitamin A in DBS	27
1.3.5.2 Vitamin D in DBS.....	28
1.3.6 HPLC methods for analysis of FSMs.....	32
1.3.7 Summary	37
1.4 THESIS OUTLINE.....	42
CHAPTER 2: ROBUST MEASUREMENT OF VITAMIN A STATUS IN PLASMA AND BLOOD DRIED ON PAPER.....	45
CHAPTER 3: MEASUREMENT OF FAT SOLUBLE MICRONUTRIENTS IN PLASMA BY HPLC.....	53

3.1	CANDIDATE CONTRIBUTION	53
3.2	INTRODUCTION	53
3.3	MATERIALS AND METHODS	56
3.3.1	Chemicals and reagents.....	56
3.3.2	Calibration and internal standard solutions	56
3.3.3	Subjects and sampling.....	58
3.3.4	Sample preparation	58
3.3.5	HPLC analysis	59
3.3.6	Method validation	60
3.3.7	Statistical analysis	62
3.4	RESULTS AND DISCUSSION	63
3.4.1	HPLC chromatography	63
3.4.2	Linearity.....	66
3.4.3	LOD and LOQ	68
3.4.4	Accuracy	70
3.4.5	Precision.....	75
3.4.6	Method validation – clinical application.....	77
3.5	SUMMARY.....	80
CHAPTER 4: MEASUREMENT OF VITAMIN D IN PLASMA BY HPLC-MS/MS		81
4.1	CANDIDATE CONTRIBUTION	81
4.2	INTRODUCTION	81
4.3	MATERIALS AND METHODS	84
4.3.1	Chemicals and reagents.....	84
4.3.2	Subjects and sampling.....	84
4.3.3	Sample extraction.....	85
4.3.4	PTAD Derivatisation	85
4.3.5	HPLC-MS/MS conditions.....	86
4.3.6	Method validation	89
4.3.7	Statistical analysis	91
4.4	RESULTS AND DISCUSSION	92
4.4.1	Optimisation of MS conditions	92
4.4.2	Method validation	98
4.4.3	Clinical application	104
4.5	SUMMARY.....	108
CHAPTER 5: COMPARISON OF HEPARIN AND EDTA ANTICOAGULANTS ON CONCENTRATIONS OF FAT SOLUBLE MICRONUTRIENTS IN HUMAN PLASMA		109

5.1	ABSTRACT	112
5.2	INTRODUCTION	114
5.3	MATERIALS AND METHODS	116
5.3.1	Chemicals and reagents.....	116
5.3.2	Subjects and sampling.....	116
5.3.3	Sample preparation	117
5.3.4	HPLC and HPLC-MS/MS analyses	118
5.3.5	Calibration of the analytical method	119
5.3.6	Statistical analysis	120
5.4	RESULTS	122
5.4.1	Comparison of plasma FSMs in blood collected in EDTA and heparin vacutainers	122
5.4.2	Effect of delayed blood fractionation on FSM levels.....	123
5.5	DISCUSSION	125
5.6	ACKNOWLEDGEMENT	130
5.7	CONFLICTS OF INTERESTS	130
CHAPTER 6: CLINICAL APPLICATION – MEASURING FAT SOLUBLE		
MICRONUTRIENTS FROM ORIP SIDE STUDY		
		136
6.1	CANDIDATE CONTRIBUTION	136
6.2	INTRODUCTION	136
6.3	MATERIALS AND METHODS	139
6.3.1	Chemicals and reagents.....	139
6.3.2	Subjects and sample collection	139
6.3.3	Sample preparation	141
6.3.4	Quality control	141
6.3.5	Statistical analysis	141
6.4	RESULTS	143
6.4.1	Basic demographic characteristics of participants	143
6.4.2	Concentrations of vitamin A (retinol) in plasma at both the first and second trimesters of pregnancy	143
6.4.3	Plasma concentrations of vitamin E (α -tocopherol, γ -tocopherol and δ -tocopherol) at both the first and second trimesters of pregnancy	145
6.4.4	Concentrations of carotenoids (lutein + zeaxanthin, lycopene and β -carotene) in plasma during the first and second trimester of pregnancy.....	149
6.4.5	Concentrations of 25OHD in plasma during first and second trimester of pregnancy	154
6.4.6	Seasonal changes in plasma 25OHD.....	159
6.5	DISCUSSION	162
6.6	SUMMARY.....	167

CHAPTER 7: GENERAL DISCUSSION.....	168
FUTURE DIRECTIONS	174
BIBLIOGRAPHY.....	176
APPENDIX 1.....	191
APPENDIX 2.....	192
APPENDIX 3.....	193

List of Figures

FIGURE 1.1. STRUCTURAL FORMULA OF FSMs [17].	4
FIGURE 1.2. FLOW CHART OF A TYPICAL FSM EXTRACTION PROCEDURE FROM BLOOD/PLASMA.....	23
FIGURE 1.3. SCHEMATIC OF PTAD (4-PHENYL-1,2,4-TRIAZOLE-3,5-DIONE) DERIVATISATION OF 25OHD3 IN ETHYL ACETATE. PICTURE ADAPTED FROM [175].....	29
FIGURE 1.4. SCHEMATIC OF A MULTIPLE REACTION MONITORING (MRM) IN A TRIPLE QUADRUPOLE MASS SPECTROMETER. PICTURE ADAPTED FROM [184].....	34
FIGURE 3.1. ILLUSTRATION OF MPB PERCENTAGE RAMP IN A HPLC GRADIENT SYSTEM FOR FSM ANALYSIS.	60
FIGURE 3.2. HPLC CHROMATOGRAMS OF 7 FSMs IN 20 μ L INJECTION OF STANDARD 3 (A) AND 100 μ L OF HUMAN PLASMA (B).....	65
FIGURE 3.3. LINEARITY OF PEAK AREAS OF 8 FSMs AND 1 INTERNAL STANDARD VERSUS THEIR PREPARED CONCENTRATIONS AT DILUTION 2 – DILUTION 8 USING THE HPLC SYSTEM.....	67
FIGURE 3.4. THE LINEARITY REGRESSION PLOTS OF ANALYTE CONCENTRATIONS MEASURE WITH SPIKED STANDARDS INTO 100 μ L OF PLASMA (Y-AXIS) AGAINST THE CONCENTRATIONS SPIKED (X-AXIS).....	72
FIGURE 3.5. CONCENTRATIONS OF 8 FSMs IN PLASMA FROM 22 HEALTHY SUBJECTS.	79
FIGURE 4.1. COLLISION INDUCED DISSOCIATION (CID) SPECTRA OF (A) 25OHD3, INTERNAL STANDARD (B) D6-25OHD3 AND (C) 25OHD2. CHEMICAL STRUCTURES WERE ADAPTED FROM [175].....	95
FIGURE 4.2. REPRESENTATIVE MRM EXTRACTED ION CHROMATOGRAMS OF 25OHD ALL AT CONCENTRATIONS 25 NMOL/L WITH (A, B, AND C) AND WITHOUT (D, E, AND F) PTAD DERIVATISATION PROCEDURE USING A KINETEX PFP COLUMN.	97
FIGURE 4.2. LINEAR RESPONSES OF STANDARDS (25OHD3 AND 25OHD2) WITHOUT (A AND B) AND WITH (C AND D) PTAD DERIVATISATION AT BOTH NORMAL RANGES AND LOWER CONCENTRATION RANGES. ...	100
FIGURE 4.3. LINEAR RESPONSES OF 25OHD STANDARDS (25OHD3 AND 25OHD2) IN PLASMA MATRICES POST PTAD DERIVATISATION.	101
FIGURE 4.4. CONCENTRATIONS OF 25OHD3 (A), 25OHD2 (B) AND TOTAL 25OHD (C) IN PLASMA FROM HEALTHY SUBJECTS MEASURED BY HPLC-MS/MS.	107
FIGURE 5.1. BOX AND WHISKER PLOTS OF PLASMA FSM CONCENTRATIONS WHEN BLOOD SAMPLES WERE COLLECTED IN EDTA (EDTA) AND HEPARIN (HEP) VACUTAINERS.	134
FIGURE 5.2. BLAND-ALTMAN PLOTS COMPARING METHODOLOGICAL DIFFERENCES BETWEEN LEVELS FOUND IN EDTA AND HEPARIN VACUTAINERS.	135
FIGURE 6.1. BOX AND WHISKER PLOT OF PLASMA RETINOL CONCENTRATIONS IN THE FIRST TRIMESTER (ENROL, ENROLMENT) AND SECOND TRIMESTER (22WK) OF GESTATION (N = 103 AT BOTH TIME POINTS).	144
FIGURE 6.2. BOX AND WHISKER PLOT OF VITAMIN E (A, A-TOCOPHEROL, B, Γ -TOCOPHEROL, C, Δ -TOCOPHEROL, AND D, TOTAL TOCOPHEROLS) CONCENTRATIONS IN PLASMA IN THE FIRST TRIMESTER (ENROL, ENROLMENT) AND SECOND TRIMESTER (22WK) OF PREGNANCY.	148

FIGURE 6.3. BOX AND WHISKER PLOT OF CAROTENOID (<i>A</i> , LUTEIN + ZEAXANTHIN, <i>B</i> , LYCOPENE, <i>C</i> , B-CAROTENE, AND <i>D</i> , TOTAL CAROTENOIDS) CONCENTRATIONS IN PLASMA IN THE FIRST TRIMESTER (ENROL , ENROLMENT) AND SECOND TRIMESTER (22WK) OF PREGNANCY.....	152
FIGURE 6.4. CORRELATION OF B-CAROTENE AND RETINOL STATUS IN MATCHED PLASMA SAMPLES FROM PREGNANT WOMEN AT EARLY TO MIDDLE GESTATIONAL STAGES (N = 206).....	153
FIGURE 6.5. BOX AND WHISKER PLOT OF 25OHD (<i>A</i> , 25OHD3, <i>B</i> , 25OHD2, AND <i>C</i> , TOTAL 25OHD) CONCENTRATIONS IN PLASMA IN THE FIRST TRIMESTER (ENROL , ENROLMENT) AND SECOND TRIMESTER (22WK) OF PREGNANCY.	158
FIGURE 6.6. PLASMA 25OHD CONCENTRATIONS (<i>A</i>) AND THE NUMBER OF SAMPLES (<i>B</i>) PLOTTED AGAINST MONTH OF COLLECTION IN PREGNANT WOMEN DURING BOTH THE FIRST AND SECOND TRIMESTER.....	160
FIGURE 6.7. GEOMETRIC MEANS OF PLASMA 25OHD CONCENTRATIONS IN SAMPLES COLLECTED IN WINTER AND SUMMER.	161

List of Tables

TABLE 1.1. COMPARISON OF SELECTED FSM EXTRACTION METHODS FROM PLASMA/SERUM SAMPLE. ^A	24
TABLE 1.2. COMPARISON OF FSM EXTRACTION FROM DRIED BLOOD SPOTS (DBS) IN SELECTED METHODS. ^A .	31
TABLE 1.3. SUMMARY OF HPLC ANALYSIS OF FSMs IN HUMAN PLASMA/SERUM SAMPLES IN SELECTED METHODS. ^A	38
TABLE 3.1. SERIAL DILUTION OF A STANDARD SOLUTION AT 12 DIFFERENT LEVELS.	57
TABLE 3.2. LODs AND LOQs OF FSMs BY HPLC AND POPULATION REFERENCE VALUES FROM PLASMA ($\mu\text{MOL/L}$).....	69
TABLE 3.3. RECOVERIES OF FSMs FROM HUMAN PLASMA SPIKED WITH 4 LEVELS OF STANDARD SOLUTIONS.	73
TABLE 3.4. DIRECT COMPARISON OF VITAMIN E AND CAROTENOID CONCENTRATIONS FROM NIST [206] AND MY METHOD.	74
TABLE 3.5. INTRA-DAY AND INTER-DAY COEFFICIENT OF VARIATIONS (CVs) OF VITAMIN E AND CAROTENOIDS IN QC SAMPLES	76
TABLE 4.1. COMPOUND SPECIFIC PARAMETERS FOR 25OHD IN MULTIPLE REACTION MONITORING (MRM) MODE.	88
TABLE 4.2. RECOVERIES OF 25OHD ₃ FROM HUMAN PLASMA SPIKED WITH 4 LEVELS OF STANDARD SOLUTIONS.	102
TABLE 4.3. DIRECT COMPARISON OF 25OHD ₃ CONCENTRATIONS FROM NIST [206] AND OUR METHOD.....	103
TABLE 4.4. INTRA-DAY AND INTER-DAY COEFFICIENT OF VARIATIONS (CVs) OF 25OHD IN QC SAMPLES....	103
TABLE 5.1. CORRELATIONS OF PLASMA FSM CONCENTRATIONS BETWEEN BLOOD SAMPLES COLLECTED EDTA AND HEPARIN AS ANTICOAGULANTS ^A	131
TABLE 5.2. CONCENTRATIONS OF FSMs MEASURED USING EDTA AND HEPARIN BLOOD COLLECTING VACUTAINERS FOR TWO TIMES PERIODS.	132
TABLE 6.1. CONCENTRATIONS OF VITAMIN E HOMOLOGUES IN WOMEN AT BOTH THE FIRST AND SECOND TRIMESTERS ($\mu\text{MOL/L}$) ^A	147
TABLE 6.2. CONCENTRATIONS OF CAROTENOIDS IN PLASMA IN WOMEN AT BOTH THE FIRST AND SECOND TRIMESTERS ($\mu\text{MOL/L}$) ^A	150
TABLE 6.3. CONCENTRATIONS OF 25OHD IN PLASMA AT BOTH THE FIRST AND SECOND TRIMESTERS (NMOL/L) ^A	155
TABLE 6.4. PREVALENCE OF VITAMIN D DEFICIENCY AND INSUFFICIENCY BASED ON THE LEVEL OF 25OHD IN PLASMA DURING FIRST AND SECOND TRIMESTER ^A	156

Abstract

Epidemiological studies have shown that deficiencies in vitamin A and vitamin E are very high in developing countries, whereas vitamin D deficiency is a global health concern. Deficiencies in fat soluble vitamins have been associated with negative health consequences, and are most severe when they occur during pregnancy, where they may lead to complications in both the mother and the offspring. In order to better understand the status of populations and associations with diseases, the establishment of analytical methods for determining fat soluble micronutrient (FSM) concentrations is essential. Therefore, the central aim of this thesis was to develop simple and reliable assays for FSMs in human blood and plasma, which would be suitable for application in large-scale clinical studies.

The process of analytical method development in this thesis included: i) establishing and validating a dried blood spot (DBS) test for the measurement of retinol (biomarker for vitamin A) concentrations; ii) an assay to simultaneously determine all FSM (retinol, α -tocopherol, γ -tocopherol, β -tocopherol, 25OHD3, 25OHD2, lutein, zeaxanthin, lycopene and β -carotene) concentrations using a minimal volume of plasma; and iii) to validate these methods through the measurement of concentrations of FSMs in a sub-set of plasma samples in an ongoing clinical study (n = 103 women at two time points, one of which is first and the other the second trimester). This enabled me to evaluate the impact different anti-coagulants on FSM concentrations, and to obtain pilot data on the concentrations of FSMs in pregnant women in South Australia, and how they change across gestation.

The DBS method that I established for measuring retinol showed good reliability and robustness when extracted using acidic conditions. Additionally, using this method, retinol in DBS was shown to be structurally stable at room temperature for 10 weeks, when stored

in the dark and with desiccants. The measurement of all FSMs in plasma also showed reliable and sensitive results including high precision for all analytes (coefficient of variation < 13%), high accuracy when compared to standard reference materials (82% - 112%), and excellent linearity in the plasma matrix ($R^2 \geq 0.99$).

The evaluation of the impact of anti-coagulant type on FSM levels showed significant differences in concentrations for retinol, lycopene and β -carotene between EDTA and heparin-treated plasma samples. Additionally, FSM concentrations decreased by up to 20% for some analytes when they were stored at room temperature for > 20 h before processing.

The assessment of FSMs in the group of pregnant women in South Australia indicated vitamin A, E and carotenoid sufficiency, as well as low prevalence of vitamin D insufficiency (< 10%). Significant changes in concentrations of vitamin D, E and carotenoids between the first trimester and second trimester during pregnancy were also identified.

In conclusion, this thesis reports the successful development of methods for measuring vitamin A in DBS and FSMs in plasma using HPLC, which are reliable, accurate and robust. The DBS method will be a valuable tool for clinical studies and studies in remote areas where sample collection and transportation are difficult.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Publications

Y. Huang, P.R. Clements, R.A. Gibson, Robust measurement of vitamin A status in plasma and blood dried on paper, *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)*, 102–103 (2015) 31-36.

Y. Huang, J. Carragher, D. Cozzolino, Measurement of Fructose, Glucose, Maltose and Sucrose in Barley Malt Using Attenuated Total Reflectance Mid-infrared Spectroscopy, *Food Anal. Methods*, (2015) 1-7.

Abstract:

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Abbreviations

1,25(OH) ₂ D	1,25-dihydroxyvitamin D
25OHD ₃	25-hydroxycholecalciferol; or 25-hydroxyvitamin D ₃
25OHD ₂	25-Hydroxyergocalciferol; or 25-Hydroxyvitamin D ₂
ANOVA	analysis of variance
BHT	butylated hydroxytoluene
CI	confidence interval
DAD	diode array detector
DAPTAD	4-(4'-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione
DBS	dried blood spot
DEQAS	vitamin D External Quality Assessment Scheme
DHA	docosahexaenoic acid
EDTA	ethylene diamine tetraacetic acid
FLD	fluorescence detector
FSM	fat soluble micronutrient, including vitamin A, D, E and carotenoids
HPLC	high performance liquid chromatography
HPLC-MS/MS	high performance liquid chromatography coupled with tandem mass spectrometry
IoM	Institute of Medicine, US
IQR	interquartile range
IS	internal standards
LoA	limits of agreement (in Bland-Altman tests)
LOD	limit of detection
LOQ	limit of quantification

MTBE	methyl <i>tert</i> -butyl ether
MPA	mobile phase A
MPB	mobile phase B
MRM	multiple reaction monitoring
MS	mass spectrometry
<i>m/z</i>	mass to charge ratio
NHMRC	National Health and Medical Research Council of Australia
NIST	National Institute of Standards and Technology
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
QC	quality control
RAE	retinol active equivalents
RBP	retinol binding protein
RDI	recommended dietary intakes
SD	standard deviation
S/N	signal to noise ratio
SPE	solid phase extraction
SRM	standard reference material
THF	tetrahydrofuran
UPLC	ultra performance liquid chromatography
UV	ultra violet
WHO	World Health Organisation