

DEVELOPMENT OF HPLC-DAD-MS/MS METHODS FOR THE SIMULTANEOUS DETERMINATION OF SEVERAL FAT- AND WATER-SOLUBLE VITAMINS IN GREEN LEAFY VEGETABLES

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INTRODUCTION

Fat (FSV) and water soluble vitamins (WSV) are **biologically active** compounds necessary for the metabolic and physiological functions.

Vitamin analysis is essential for **quality control** and there is a growing need for rapid and specific methods.

Traditionally, methods for vitamin determination require the analysis of each vitamin individually by using widely differing physical, chemical and biological methods, that normally include very laborious work.

The use of a **single method** to determine several vitamins in food is very challenging due to: the different structures; chemical properties; low stability to light, air and heat; and to the fact that foods are complex matrices.

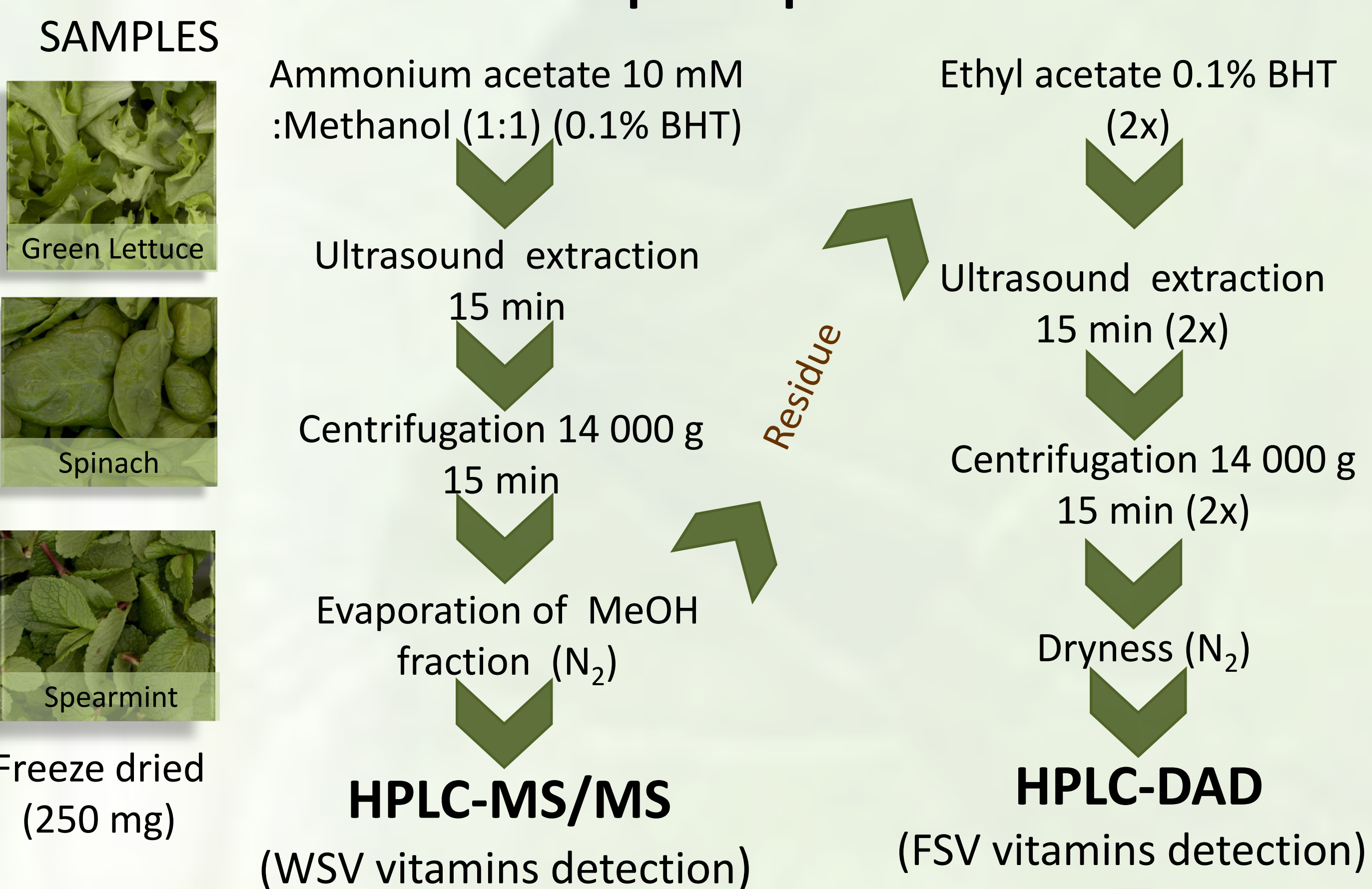
HPLC-DAD is well established for both FSV and WSV measurements, but showed some limitations for certain analytes and also lack of specificity in complex matrices.

HPLC-DAD-MS/MS could provide **more sensitivity and specificity** to vitamins determination in food matrices, and permit the simultaneous analysis of multiple vitamins in a single analysis.

OBJECTIVE:

To develop a method that allows the sequential extraction and determination of several free forms of WSV (Vitamins C, B1, B2, B3, B5, B6 and B9) and FSV (vitamins A and E) in green leafy vegetables.

METHODS



HPLC-MS/MS

Accela LC and TSQ Quantum triple quadrupole MS (Thermo)

Analytical column: ACE-100 Å C18 (3µm, 100x2.1 mm)

Gradient elution:
 (A) Ammonium acetate 10 mM (pH 4.5);
 (B) MeOH (0.1% acetic acid)
 (C) MeOH (0.3% acetic acid)

Minute/% (A): 0/90; 3/90; 4/50; 7/50; 10/0; 16/0; 20/90; 30/90.
 Minute/% (B): 0/10; 3/10; 4/0; 7/0; 10/100; 16/100; 20/10; 30/10.

Flow: 0.2 ml/min

MS/MS detection parameters (TABLE 1)

HPLC-DAD

Agilent 1100 HPLC-DAD

Analytical column: YMC C30 (5 µm, 250 × 4.6 mm)

Gradient elution:
 (A) MeOH:H₂O: TEA (90:10:0.1);
 (B) MTBE, MeOH: H₂O:TEA(90:6:4:0.1)

Minute/% (A): 0/93.5;8/93.5; 43/0; 46/93.5; 55/93.5.

Flow: 1 ml/min.

DAD detection :
 295 nm → α-tocopherol
 450 nm → β- carotene

Quantification was done using **vitamins standards**: ascorbic acid (C), thiamine hydrochloride (B1), riboflavin (B2), nicotinamide (B3), D-calcium pantothenate (B5), pyridoxine (B6), folic acid (B9), α-tocopherol (E) and β-carotene(pro- A).

RESULTS

Table 1: MS/MS detection parameters for the WSV analyzed

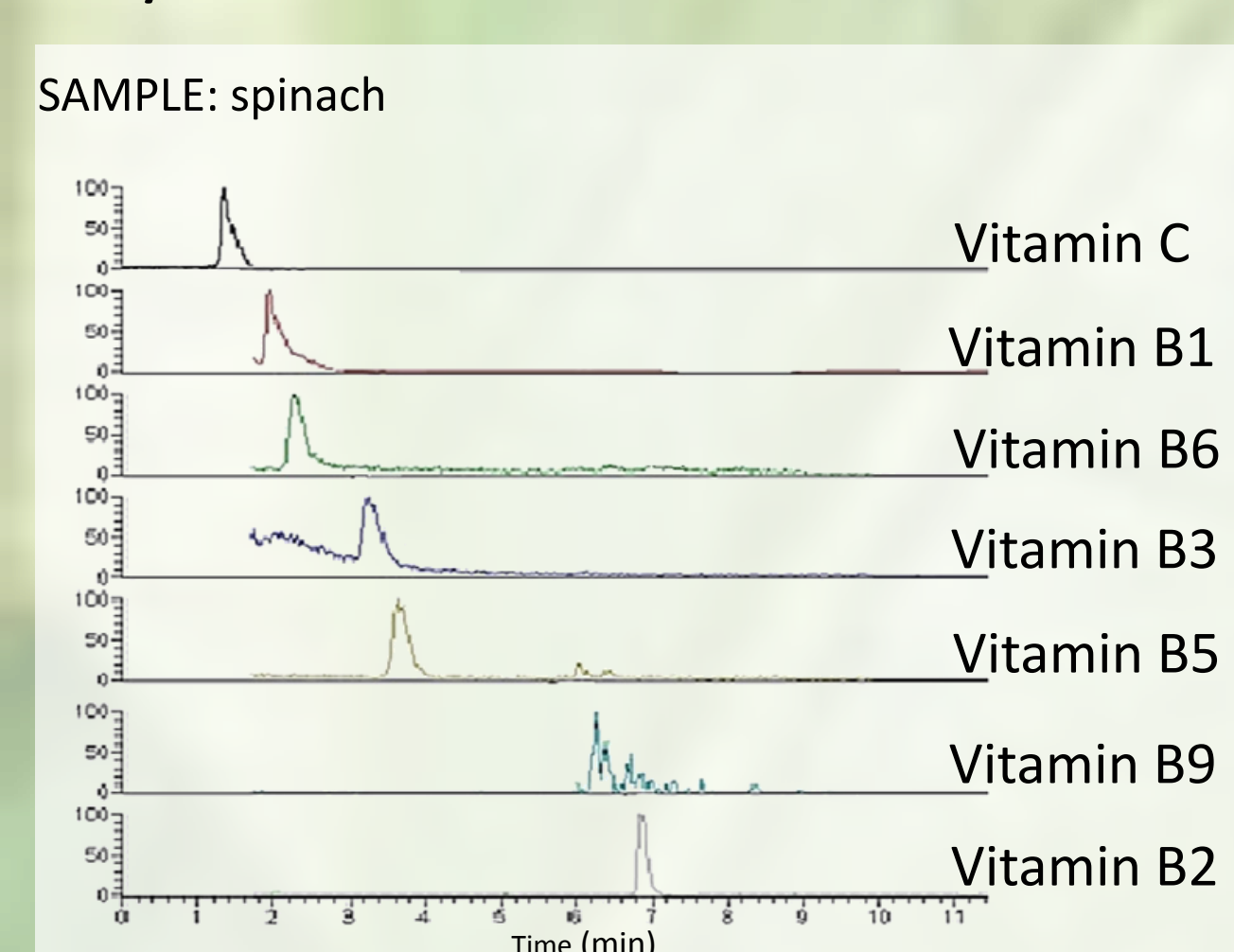
Vitamin	Precursor ion (m/z)	SRM transitions		Tube lens offset (V)
		Quantifier ion (m/z) (Collision energy, V)	Qualifier ion (m/z) (Collision energy, V)	
Ascorbic Acid (C)	174.9 [M-H] ⁻	115.2 (14)	87.3 (18)	76
Thiamine (B1)	265.1 [M+H] ⁺	122.1 (10)	144.1 (16)	48
Pyridoxine (B6)	169.9 [M+H] ⁺	152.1 (11)	134.1 (19)	55
Nicotinamide (B3)	123.0 [M+H] ⁺	80.3 (16)	78.3 (24)	67
Pantothenic Acid (B5)	220.0 [M+H] ⁺	202.1 (12)	184.1 (12)	60
Folic Acid (B9)	442.0 [M+H] ⁺	294.9 (13)	176.0 (34)	69
Riboflavin (B2)	377.1 [M+H] ⁺	243.0 (23)	147.1 (37)	93

Table 2: Method evaluation parameters

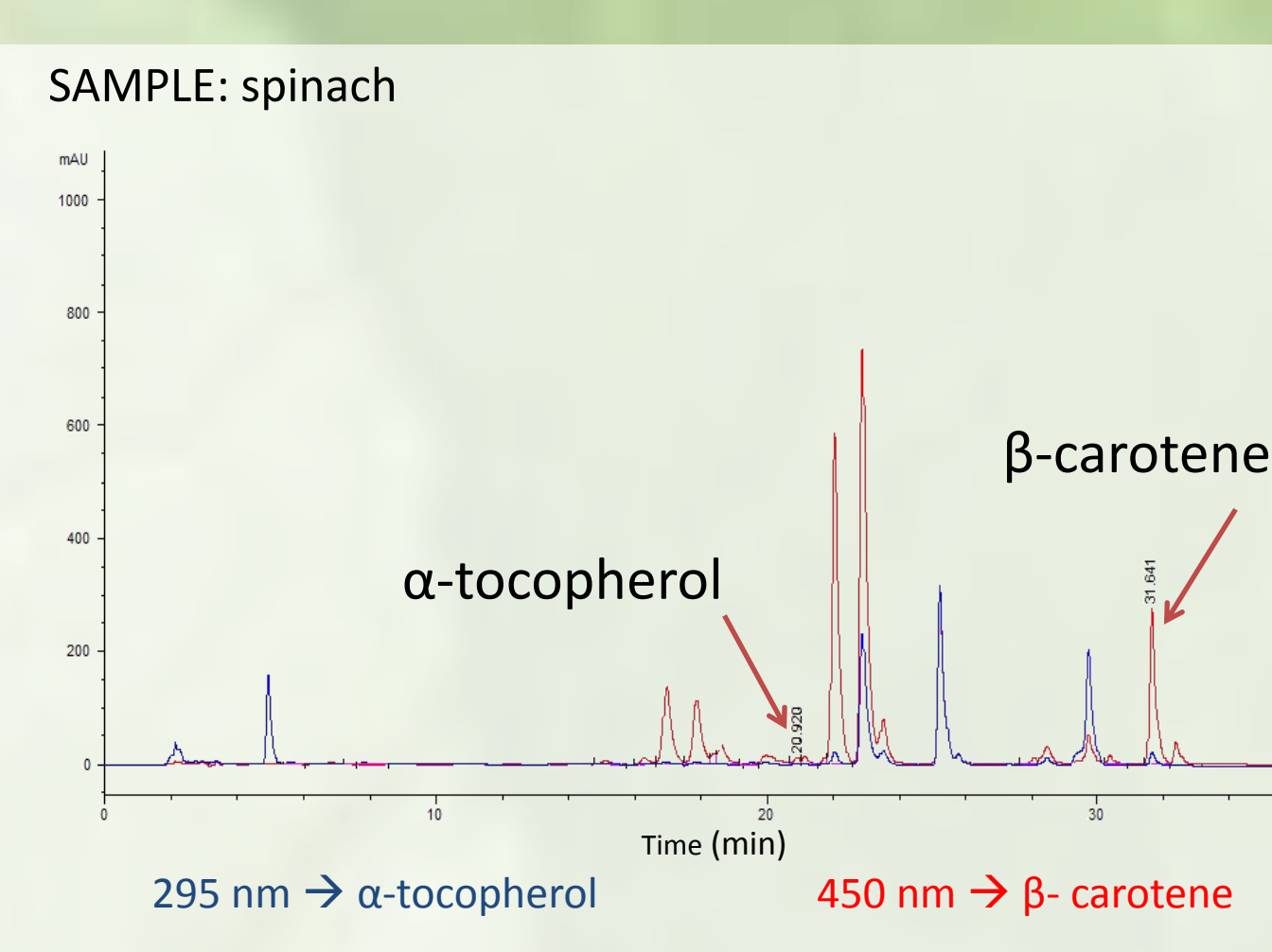
Vitamin	Repeatability (CV %)				Linearity		Mean Recovery					
	Detection	LOD (ng/ml)	LOQ (ng/ml)	Intra day	Inter day	Range tested (µg/ml)	R ²	added µg/ml	%* RSD			
Ascorbic Acid	MS/MS	42.28	128.13	5.6	2.0	6.3	2.0	0.07 - 5.28	0.992	3.30	99.6	5.8
Thiamine	MS/MS	0.79	2.41	3.1	0.7	3.6	0.7	0.0015 - 0.96	0.993	0.60	91.7	4.3
Riboflavin	MS/MS	0.07	0.20	2.5	0.2	4.3	0.2	0.0009 - 1.5	0.996	0.75	92.1	5.9
Nicotinamide	MS/MS	4.35	13.17	2.3	0.5	9.8	0.5	0.004 - 2.80	0.995	1.75	88.1	4.8
Pantothenic Acid	MS/MS	7.67	23.25	0.9	0.6	9.1	0.6	0.02 - 1.67	0.998	0.83	91.2	7.5
Pyridoxine	MS/MS	0.18	0.56	1.7	0.6	9.1	0.6	0.0008 - 0.53	0.997	0.67	90.0	2.7
Folic Acid	MS/MS	0.63	1.90	7.2	0.0	8.4	0.0	0.0004 - 0.53	0.995	0.33	110.1	13.9
α-tocopherol	UV	170	520	0.73	0.12	2.09	0.54	6.25 - 100	0.999	50	87.5	4.9
β-carotene	UV	70.0	200	0.36	0.07	0.34	0.23	6.25 - 100	0.999	50	108.1	13.5

* mean of five extractions

MS/MS CROMATOGRAMS

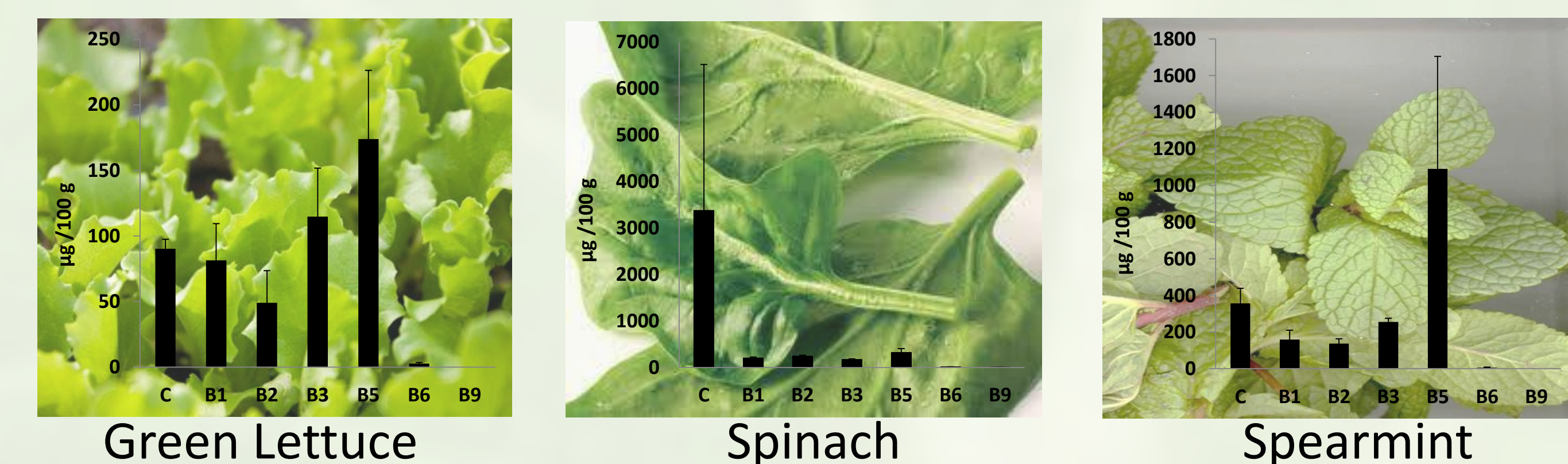


UV CROMATOGRAMS



All WSV and FSV analyzed were perfectly separated in LC columns and identified in the green leaf extracts

Water soluble Vitamins



Fat soluble Vitamins

	Green lettuce		Spinach		Spearmint	
	mean	SD	mean	SD	mean	SD
β-carotene	4527,29	1432,08	12187,55	1563,35	9356,87	1278,74
α-tocopherol	N.D		3462,26	490,52	1425,24	261,570

CONCLUSION

The sequential extraction and the detection methods employed permitted the identification and quantification of several free forms of fat- and water soluble vitamins in green leafy extracts.

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