



Comprehensive quantitation of water-soluble vitamins in infant formula

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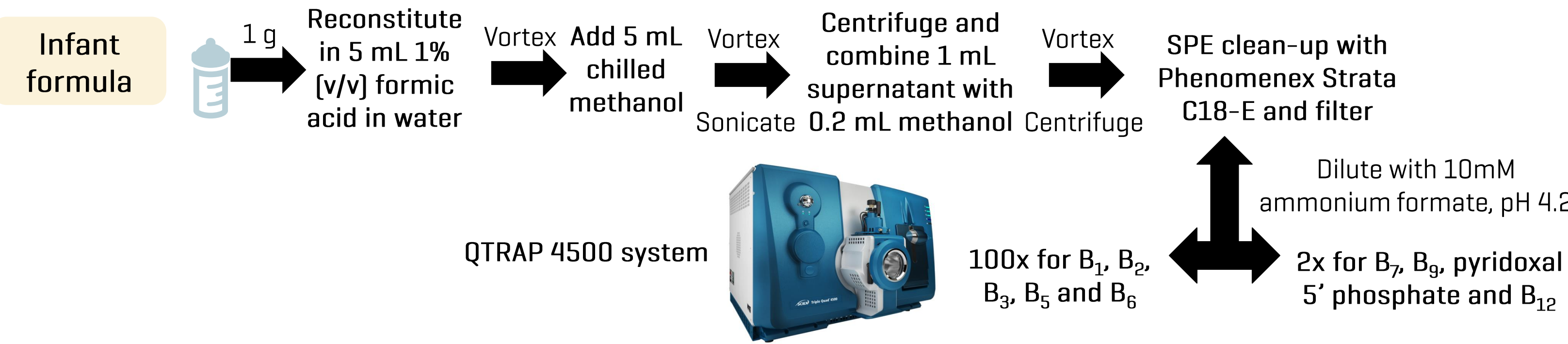
INTRODUCTION

Water-soluble vitamins (WSVs) are essential nutrients that support healthy growth and development during infancy, which include vitamin C and the B complex group comprised of thiamine [B₁], riboflavin [B₂], niacin [B₃], pantothenic acid [B₅], pyridoxine and related forms [B₆], biotin [B₇], folate/folic acid [B₉] and cobalamin [B₁₂]. As they are highly water-soluble, WSVs are readily excreted from the body, necessitating recommended dietary allowances (RDAs) to ensure adequate dietary intake across age groups, especially for infants.¹ As an alternative to human breastmilk, infant formula is fortified with water- and fat-soluble vitamins, minerals and other functional ingredients to ensure compliance with nutritional requirements. Infant formula manufacturers typically over-fortify vitamins to offset processing- and storage-related losses and degradation and to ensure nutritional adequacy during the labeled shelf life.² As such, infant formula is highly regulated^{2,3} and requires accurate vitamin verification in the finished product.

Quantitation of WSVs in infant formula is challenging due to their structural diversity, wide range of polarities, multiple vitamers and susceptibility to degradation. These challenges are exacerbated by the compositional complexity of infant formula, often leading to significant matrix effects. Variability in sensitivities among WSVs across detection techniques often requires separate methods to meet the different quantitation criteria specified in the AOAC standard method performance requirements (SMPRs). These are individually specified for each B-vitamin by the Stakeholder Program on Infant Formula and Adult Nutrition [SPIFAN]. This work describes the simultaneous analysis of 13 WSVs in infant formula using a combined sample preparation and LC-MS/MS method developed on the QTRAP 4500 system.

MATERIALS AND METHODS

Sample preparation



LC-MS/MS instrumental conditions

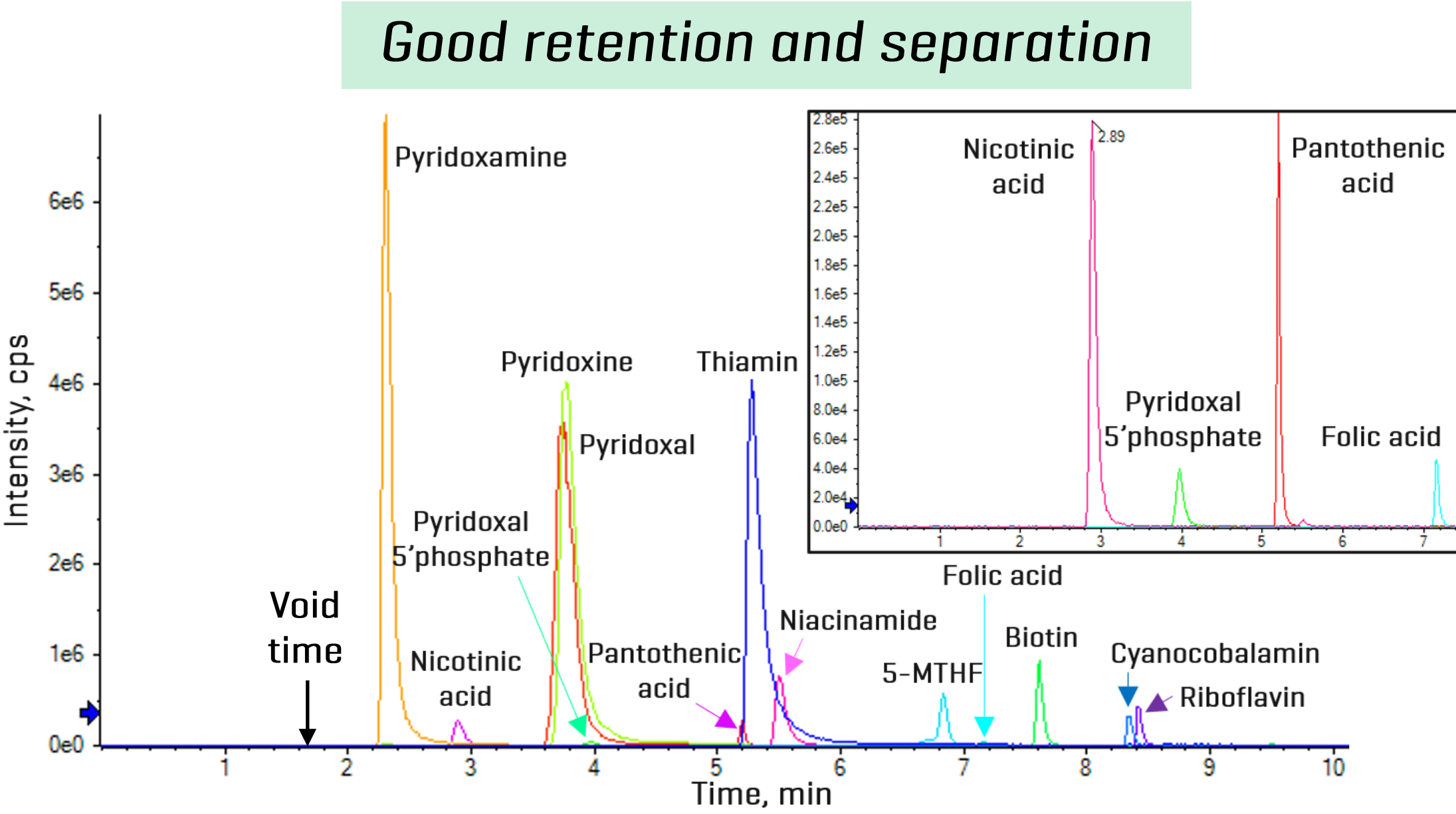
Chromatography

Time [min]	Flow [mL/min]	%A	%B
0	0.45	99	1
2.0	0.45	99	1
3.0	0.45	85	15
6.0	0.45	65	35
8.0	0.45	5	95
10.0	0.45	5	95
10.1	0.45	99	1
14.0	0.45	99	1

MPA: Water with 10mM ammonium formate, pH 4.2
MPB: Methanol
Column: Phenomenex Luna Omega Polar C18 (150 x 3 mm, 3 µm)
Injection volume: 5 µL

Source parameters

Parameter	Value
Polarity	+
Ion source gas 1	75 psi
Ion source gas 2	55 psi
Curtain gas	40 psi
Source temperature	550°C
Ion spray voltage	5000 V
CAD gas	9 psi



MRM parameters

Vitamin	Compound	MRM	DP [V]	CE [V]	CXP [V]
B ₁	Thiamin 1	265 > 122	30	25	8
	Thiamin 2	265 > 144	30	20	10
B ₂	Riboflavin 1	376.9 > 243.1	50	35	12
	Riboflavin 2	376.9 > 198	50	50	6
B ₃	Nicotinic acid 1	123.9 > 78	55	30	8
	Nicotinic acid 2	123.9 > 80	55	30	8
	Niacinamide 1	122.9 > 80	55	30	8
	Niacinamide 2	122.9 > 78	55	30	8
B ₅	Pantothenic acid 1	220 > 90	35	20	6
	Pantothenic acid 2	220 > 98	35	30	10
	Pyridoxal 1	167.9 > 150.1	35	20	10
	Pyridoxal 2	167.9 > 94.1	35	30	5
B ₆	Pyridoxal 3	167.9 > 122	35	30	10
	Pyridoxamine 1	169 > 152	25	20	10
	Pyridoxamine 2	169 > 134	25	30	10
	Pyridoxine 1	170 > 152	30	20	10
B ₇	Pyridoxine 2	170 > 134	30	30	10
	Pyridoxal 5'phosphate 1	247.9 > 150.1	85	25	10
	Pyridoxal 5'phosphate 2	247.9 > 122.1	85	30	8
	Pyridoxal 5'phosphate 3	247.9 > 94.1	85	40	6
B ₉	Biotin 1	244.9 > 227.1	55	20	12
	Biotin 2	244.9 > 97	55	40	6
	Folic acid 1	442 > 295.1	65	20	10
	Folic acid 2	442 > 176	65	55	12
B ₁₂	Folic acid 3	442 > 120.1	65	50	8
	5-MTHF 1	460 > 313.1	80	30	12
	5-MTHF 2	460 > 180.1	80	50	12
	Cyanocobalamin 1	678.5 > 147.1	110	85	10
	Cyanocobalamin 2	678.5 > 359.1	110	35	16

REFERENCES

1. National Research Council (US) Subcommittee on the Tenth Edition of the Recommended Dietary Allowances. Recommended Dietary Allowances: 10th Edition. Washington (DC): National Academies Press [US]; 1989. 8, Water-Soluble Vitamins.
2. Food and Agriculture Organization of the United Nations. World Health Organization. Standard for infant formula and formulas for special medical purposes intended for infants. Codex Alimentarius International Food Standards. CXS 72-1981.
3. Food Safety and Standards [Foods for Infant Nutrition] Regulations, 2020. Version-II [04.01.2024].
4. PP, H. et al. Comprehensive quantitation of water-soluble vitamins in infant formula. SCIEX technical note. MKT-37592-A.

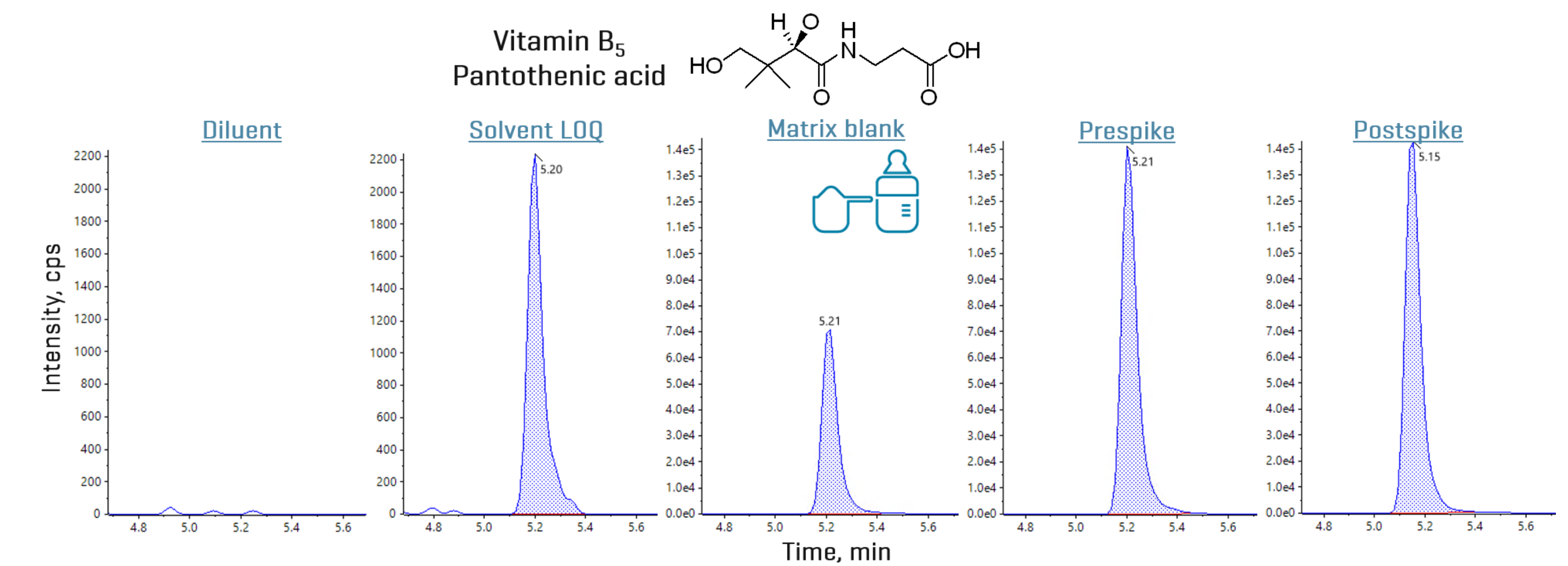
SENSITIVITY PERFORMANCE IN SOLVENT AND MATRIX

Compound	Solvent			Matrix	
	LOQ [ng/mL]	Mean accuracy [%CV]	r ²	LOQ [µg/100 g] _{calc}	LOQ [µg/100 g] _{SMPR}
Thiamine [B ₁]	1.0	118 [1.0]	0.997	1.33	20
Riboflavin [B ₂]	2.5	118 [4.2]	0.997	3.33	30
Nicotinic acid [B ₃]	2.5	78.9 [14]	0.996	3.33	200
Niacinamide [B ₃]	2.5	78.9 [6.0]	0.996	3.33	200
Pantothenic acid [B ₅]	2.5	70.3 [15]	0.997	3.33	50
Pyridoxal [B ₆]	0.5	81.4 [5.2]	0.997	0.67	10
Pyridoxamine [B ₆]	0.1	112 [8.5]	0.999	0.13	10
Pyridoxine [B ₆]	0.1	102 [8.1]	0.998	0.13	10
Pyridoxal 5' phosphate [B ₆]	10	101 [11.1]	0.999	0.27	10
Biotin [B ₇]	1.0	109 [13]	0.999	0.03	0.1
Folic acid [B ₉]	10	101 [4.2]	0.996	0.27	0.5
5-MTHF [B ₉]	50	99.5 [1.5]	0.999	1.33	0.5
Cyanocobalamin [B ₁₂]	2.5	112 [4.2]	0.999	0.07	0.01

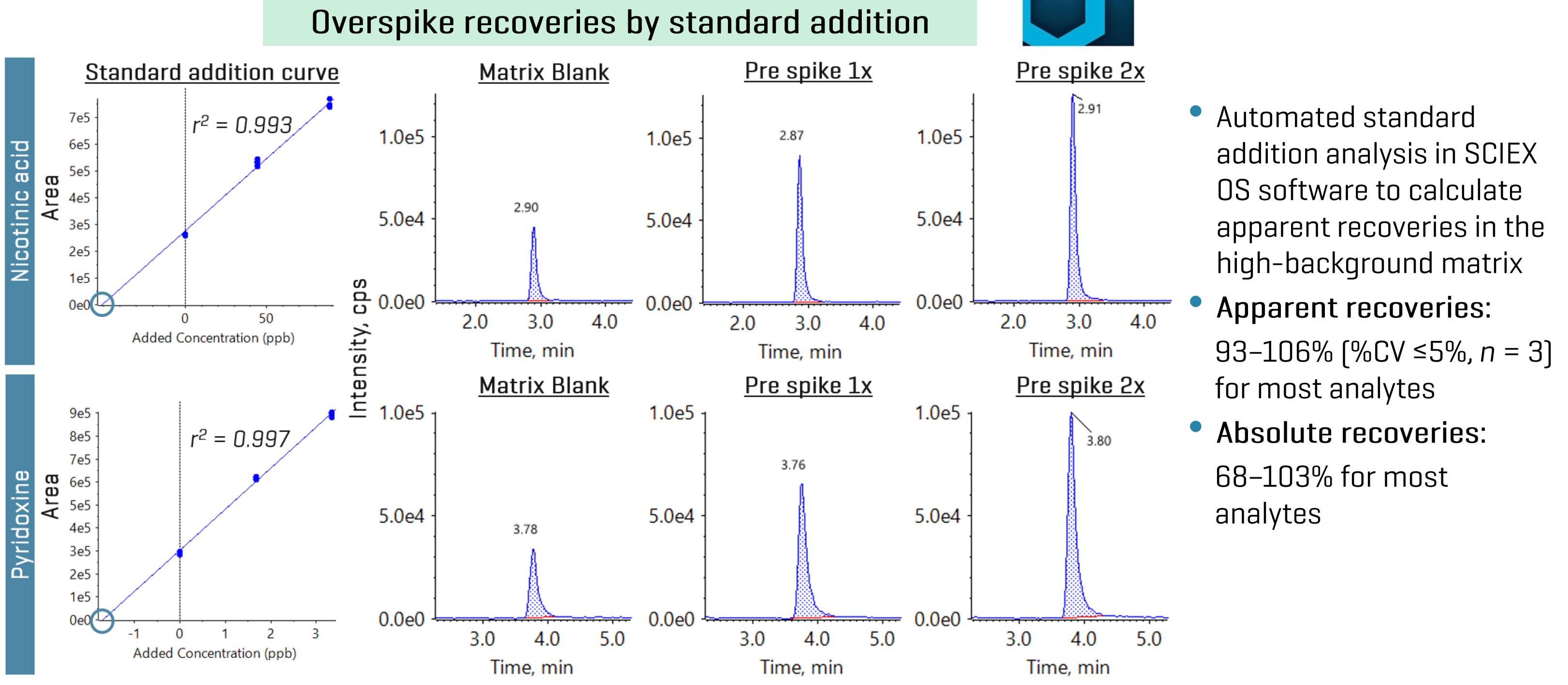
$$LOQ(\mu\text{g}/100\text{ g reconstituted}) = \frac{LOQ(\mu\text{g}/\text{g powder}) \times 25\text{ g powder}}{(25\text{ g powder} + 200\text{ g water})} \times 100$$

Solvent LOQ

- Linearity of r² ≥ 0.996 and linear dynamic range of 2–3 orders of magnitude for most analytes
- In-vial solvent LOQs 0.1–2.5 ng/mL for most of the vitamins, except for pyridoxal 5' phosphate, folic acid and 5-MTHF
- LOQ based on accuracy (±30%), %CV <20% (n = 3), S/N ≥10, ion ratios (±30%)



QUANTITATIVE PERFORMANCE IN MATRIX SPIKES



METHOD APPLICABILITY IN COMMERCIAL INFANT FORMULA AND NIST SRM 1869

Commercial infant formula				NIST SRM 1869			
Compound	Label [µg/100 g]	Measured [%CV] [µg/100 g]	FSSAI requirement [µg/100 g]	Compound	Reference [mg/kg]	Measured [%CV] [mg/kg]	Accuracy [%]
Thiamine	200	425 [10.7]	200–517	Thiamine	13.36 ± 0.32	16.2 [3.9]	121
Riboflavin	400	1218 [7.6]	400–2000	Riboflavin	13.6 ± 1.5	15.9 [6.7]	117
Niacin equivalent	3800	6114 [3.1]	3800–9900	Nicotinamide	98.4 ± 2.2	121.5 [2.9]	124
Pantothenic acid	3300	7335 [3.8]	2000–10000	Pantothenic acid	64.9 ± 6.6	83.7 [7.0]	129
Pyridoxine	110	204 [1.4]	100–400	Pyridoxine	13.09 ± 0.32	14.9 [2.6]	114
Biotin	7.5	45 [4.7]	7.5–50	Biotin	1.89 ± 0.24	2.3 [1.1]	121
Dietary folate equivalent	15	18 [12.7]	15–56.9				
Cyanocobalamin	0.25	nd	0.25–4.0				

- Measured concentrations in commercial sample fell within allowed ranges specified by FSSAI³
- Method trueness within ±30% of NIST SRM 1869 values

TRADEMARKS/LICENSING

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