R-Biopharm Rhône

Simultaneous determination of vitamin B12, folic acid and biotin in nutritional products using immunoaffinity clean-up prior to LC-MS/MS or LC-UV analysis

N. Dooley, D. Leeman, E. Marley & C. Milligan
R-Biopharm Rhône, Scotland

Introduction

Vitamins are organic micronutrients necessary for normal metabolic function and are either not synthesised, or not synthesised to a sufficient degree, within the body[1]. Consequently, Vitamins must be obtained from the diet and as such are a key component of nutritional products, often fortified to satisfy complete dietary requirements [2].

Current trends in vitamin analysis are taking a multi-analyte approach, which is consistent with the strategy being implemented by R-Biopharm Rhône, who have developed a multi-vitamin immunoaffinity column to analyse vitamin B12, folic acid and biotin within a single extraction prior to separation and detection by LC-MS/MS or LC-UV.

The vitamins are extracted from the sample and the extract is diluted with buffer, centrifuged, filtered and added to the column reservoir. The top frit is absent from the column allowing the sample to mix directly with the antibody gel where binding takes place between the antibody and the vitamins. The extract is passed through the column before washing which removes any unbound material. The vitamins are released from the antibody following elution before injection onto the LC-MS/MS or LC-UV system.

Column performance is demonstrated through the extraction of vitamins from a commercial Infant Milk Formula an Adult Nutritional drink and the NIST 1849a standard reference material.

LC-MS/MS & LC-UV Method

HPLC Conditions	
Mobile Phase A	10 mM ammonium formate in 0.1 % formic acid
Mobile Phase B	0.1 % formic acid in methanol
Isocratic Conditions	10 mM ammonium formate in 0.1 % formic acid : 0.1 % formic acid in methanol (50 : 50 v/v) for 4.5 minutes
Analytical Column	Gemini C18, 5 µm, 3 mm x 150 mm or equivalent
Flow Rate	0.4 ml per minute
Column Temperature	40 °C

Waters TQD Detector Conditions

Vitamin	Transition		Cone Voltage		Collision Energy	
	Quantifier Ion	Qualifier Ion	Quantifier Ion	Qualifier Ion	Quantifier Ion	Qualifier Ion
Vitamin B12	678.4 > 147.1	678.4 > 359.2	50	50	42	30
Folic Acid	442.0 > 295.1	442.0 > 176.0	32	32	12	46
Biotin	244.9 > 227.0	244.9 > 122.9	32	32	14	28

LC-UV Conditions			
Mobile Phase A	0.0125 % formic acid in water		
Mobile Phase B	0.0125 % formic acid in acetonitrile		
Gradient Conditions	Time	% A	% B
	0	92.5	7.5
	0.1	92.5	7.5
	10.0	80	20
	15.0	80	20
	15.1	92.5	7.5
	25.0	92.5	7.5
UV Detector	Biotin (205 nm), Folic Acid (280 nm), Vitamin B12 (361 nm)		
Analytical Column	Hypersil Gold C18, 3 µm, 4.6 mm x 150 mm or equivalent		
Flow Rate	0.8 ml per minute		
Column Temperature	40 °C		

(1) Vitamin and mineral requirements in Human Nutrition, 2nd Edition. World Health Organisation, Food and Agricultural Organisation of the United Nations. 2004. ISBN: 92 4 154612 3.
(2) Guidelines on food fortification with micronutrients. World Health Organisation, Food and Agricultural Organisation of the United Nations. 2006. ISBN: 92 4 159401 2.

Method

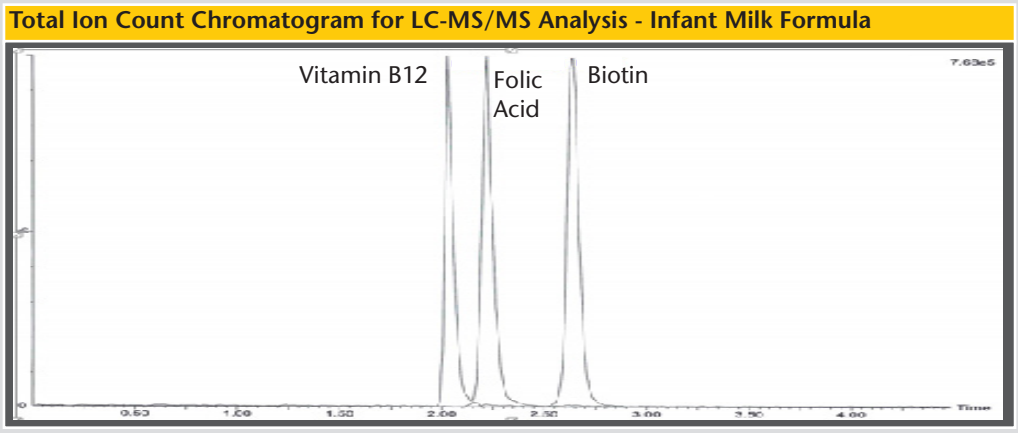
1. Weigh 2 - 10 g of sample (dependent on matrix and concentration).
2. Add 50 ml of 20 mM ammonium acetate buffer.
3. Add 6 ml of 10 % sodium ascorbate and 1 ml of 1 % potassium cyanide.
4. Mix for 10 mins.
5. Incubate in a water bath at 100 °C for 20 mins.
6. Remove sample and allow to cool.
7. Transfer the extract into a 100 ml volumetric flask and fill to the mark with 20 mM ammonium acetate buffer. Invert to mix.
8. Transfer 10 ml of sample into a centrifuge tube and centrifuge at 4,000 rpm for 10 minutes.
9. Filter the sample through a Whatman S&S 597½ filter paper.
10. Drain column and add 7 - 9 ml (depending on matrix) of filtrate to the column and invert by hand to re-suspend the gel.
11. Mix in a rotary shaker for 15 mins. Leave to stand for 5 minutes.
12. Allow filtrate to pass through column by gravity.
13. Wash the column with 18 ml of water.
14. Elute the vitamins using 3 ml of 0.5 % ammonium hydroxide in methanol.
15. Evaporate the eluate to dryness under air at 60 - 70 °C.
16. Reconstitute in 300 µl of 0.1 % formic acid.
17. Inject 10 µl onto LC-MS/MS or 100 µl onto the LC-UV system.

Results

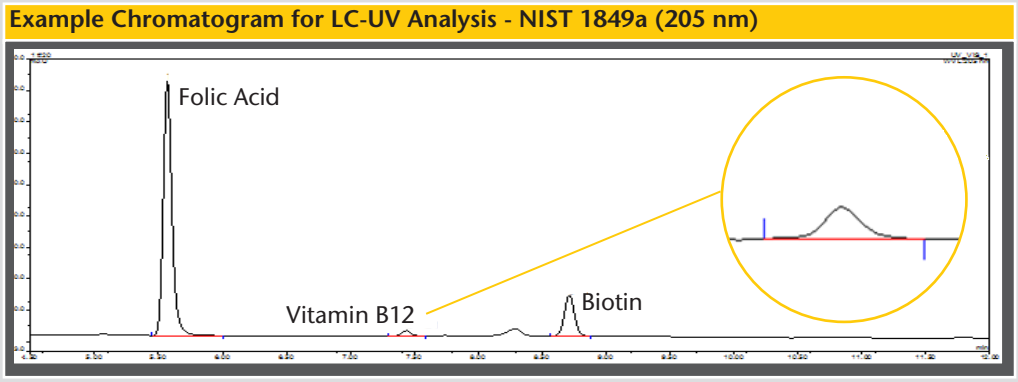
Recoveries Obtained with IAC Clean-Up Relative to the Label Claim (n = 6)

Sample	Vitamin	ng Injected		LC-MS/MS Results			LC-UV Results		
		HPLC	LC-MS/MS	%R	%RSD	LOQ	%R	%RSD	LOQ
NIST 1849a	Vitamin B12	2.25	0.225	92.3	3.2	0.03	86.8	5.1	1.6
	Folic Acid	107.1	10.71	119.9	9.7	0.08	77.5	1.2	1
	Biotin	92.9	9.29	64.1	6.4	0.08	72.7	1.7	4
Infant Formula	Vitamin B12	1.9	0.19	151.3	4.3	0.03	131.8	7.9	1.6
	Folic Acid	130	13	164.2	12.9	0.08	129.2	1.4	1
	Biotin	14	1.4	110.1	4.2	0.08	113.8	5.2	4
Adult Nutritional Drink	Vitamin B12	1.65	0.165	116.8	3.7	0.03	99.7	7.0	1.6
	Folic Acid	120	12	83.1	6.6	0.08	69.8	3.3	1
	Biotin	18	1.8	86.0	5.8	0.08	87.7	6.5	4

Total Ion Count Chromatogram for LC-MS/MS Analysis - Infant Milk Formula



Example Chromatogram for LC-UV Analysis - NIST 1849a (205 nm)



Conclusion

- The new multi-vitamin immunoaffinity column enables the analysis of vitamin B12, folic acid and biotin in nutritional samples simultaneously using a single extraction prior to detection with either LC-MS/MS or LC-UV.
- The need for matrix matched standards is removed since the monoclonal antibodies are highly specific to the selected vitamins and unbound matrix materials are removed during the washing of the immunoaffinity column prior to elution.
- The immunoaffinity column improves the clean-up and pre-concentration of the target vitamins thereby enhancing sensitivity and accuracy of detection.
- Analysis can be performed by either LC-MS/MS or LC-UV depending on instrument availability and/or sample throughput requirements.

R-Biopharm Rhône
Block 10 Todd Campus, West of Scotland Science Park,
Acre Road, Glasgow, Scotland, G20 0XA
Phone: +44 (0) 141 945 2924, Fax: +44 (0) 141 945 2925
www.r-biopharm.com

R-BIOPHARM RHÔNE LTD