A feasibility study of the determination of B-group vitamins in food by HPLC with Mass Spectrometric detection

LGC/R/2011/181

research • measurement • science • expertise • quality • safety

LGC Setting standards in analytical science

GC11/0005A

A feasibility study of the determination of B-group vitamins in food by HPLC with Mass Spectrometric detection.

A Government Chemist Programme Report

Report number LGC/R/2011/181

Author: Paul Lawrance

(MS evaluation carried out by Sabine Biesenbruch)

Approved by: Peter Colwell

September 2011

A Government Chemist Programme Report

Report number LGC/R/2011/181

Contents **Page**

1. Executive Summary

This was a feasibility study of a High performance Liquid Chromatography (HPLC) method for the simultaneous determination of B-group vitamins using combined extraction and mass spectrometric determination. An HPLC-MS procedure was developed using standards and was applied to samples of infant milk powder.

2. Background

The B-group vitamins are a group of water-soluble vitamins that play important roles in cell metabolism. They have certain characteristics in common, in that they are all water-soluble, they coexist in various foods and they all have roles in human biochemical processes however from a chemical viewpoint, they are a diverse group of substances with different chemical properties.

The B-group vitamins include the following substances:

- Vitamin B1 (thiamine)
- Vitamin B2 (riboflavin)
- Vitamin B3 (niacin or niacinamide)
- Vitamin B5 (pantothenic acid)
- Vitamin B6 (pyridoxine, pyridoxal, or pyridoxamine, or pyridoxine)
- Vitamin B7 (biotin)
- Vitamin B9 (folic acid and folates)
- Vitamin B12 (cyanocobalamin and other cobalamins)

In foods, these compounds exist in the free form, as various derivatives, as salts or may be bound to other food components such as protein or carbohydrate which makes their extraction and subsequent analysis a complex problem.

B-group vitamins can be determined using a number of techniques. These include microbiological assay, ELISA, and HPLC techniques as well as older, chemical or spectrophotometric procedures. HPLC can be used for determination of many of the B-group vitamins but often, these methods are specific to a particular vitamin as the extraction conditions required for one compound may not be appropriate for others. Simultaneous methods have been published for the analysis of B-group vitamins in supplements and fortified foods where the vitamins are present in the simplest form and it is therefore possible to develop combined extraction protocols.

3. Objective

The major B-group vitamins can be determined simultaneously in supplements by HPLC with UV detection (Fig 1) although some modification of the procedures may be required for biotin, vitamin B12 and folic acid.

The vitamins are identified on the basis of retention time however, for referee analysis; it is often beneficial to be able to provide additional proof of identification such as that provided by mass-spectrometry. For this reason, a procedure for the determination of the major B-group vitamins using LC-MS was evaluated.

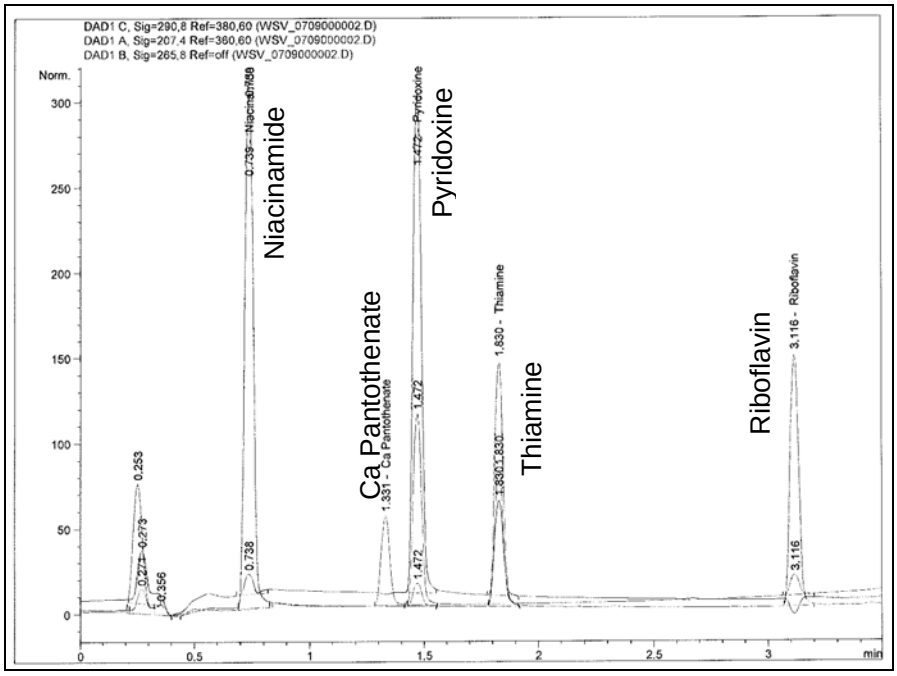

Fig 1: Simultaneous determination of five b-group vitamins by HPLC with UV detection

4. LC-MS evaluation

The conditions used for the determination of the B-group vitamins by HPLC-UV required the use of a phosphate buffer. For LC-MS analyses, volatile buffers are preferred, in order to minimise MS ion suppression and maintain sensitivity. In addition, non-volatile buffers such as phosphate may lead to contamination of the ion source. In addition, shorter HPLC columns are usually used to minimise analytical run times.

Two HPLC systems were evaluated for their ability to separate and determine seven B-group vitamins (B_{12} was not included). The structures of the analytes are shown in Figures 2 - 8 below.

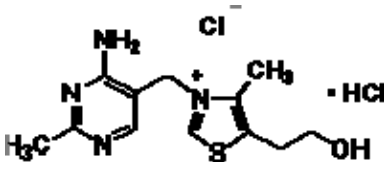

Fig.2: Thiamine HCl

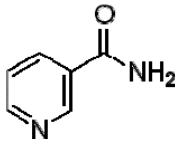

Fig. 4: Nicotinamide

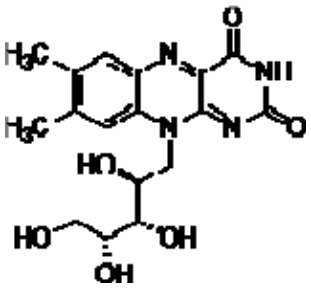

Fig 3: Riboflavin

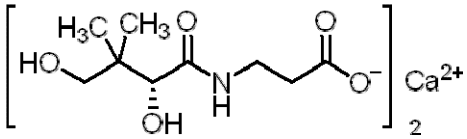

Fig. 5: Calcium pantothenate

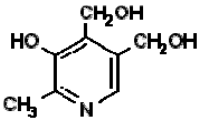

Fig. 6: Pyridoxine

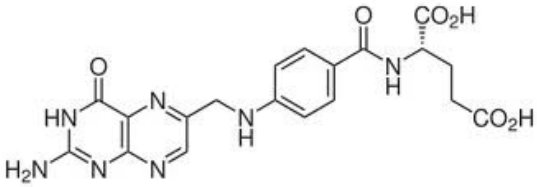

Fig.7: Folic acid

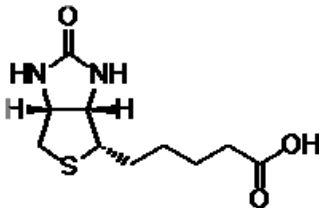

Fig.8: Biotin

4.1 MS Optimisation

Mixed standards containing the B-group vitamins in water were prepared. These contained the target analytes at concentrations of between 1 and 150 ng/mL of each vitamin. These were infused into a solvent flow to tune the mass spectrometer.

The evaluation was carried out using an ABI 4000 QTrap Mass Spectrometer with an Agilent 1100 series Liquid Chromatograph. Various tuning conditions were tested. Although optimum tuning conditions could be established for each vitamin, a compromise had to be reached to provide adequate conditions for all of the vitamins of interest. The following conditions were used:

ESI+ve ion mode	
Ion Source:	Turbo Spray
Curtain Gas (N2):	40
Ion Spray Voltage (V):	4500
Source Temperature (°C):	500
Gas 1 (N2):	40
Gas 2 (N2):	40
Interface Heater:	ON
CAD:	High

Fig 9: ABI QTRAP tuning conditions

Table1: Mass spectrometer conditions for seven B-group vitamins on the ABI 4000 QTrap

Analyte	Precursor Ions [m/z]	Product Ions [m/z]	Declustering potential [V]	Collision cell entrance potential [V]	Collision Energy [eV]	Collision cell exit potential [V]
Thiamine	265	122, 144	70	10	20	11
Riboflavin	377	243, 359	50	10	30	11
Nicotinamide	123	80, 78	30	4	32	5
Pantothenic acid	220	202, 116	30	10	21	11
Pyridoxine	170	152, 134	50	10	23	11
Folic acid	442	295, 313	40	8	20	11
Biotin	245	227, 166	40	10	28, 36	10

Product ion spectra were obtained for each vitamin. An example for riboflavin is shown below:

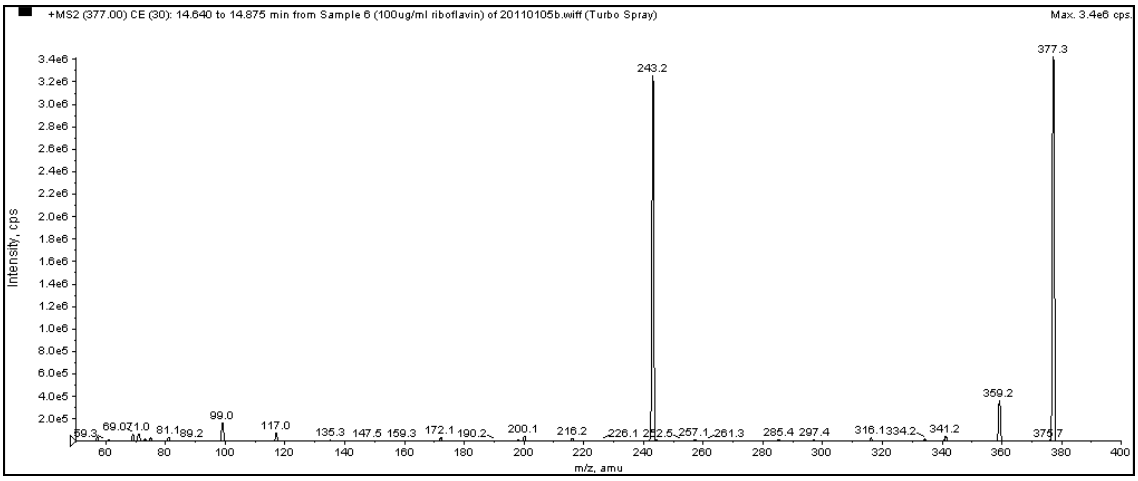

Fig 10: Product Ion spectrum of riboflavin

4.2 Chromatography

It is difficult to separate and resolve the B-group vitamins on C18 columns without the use of modifiers and ion-pair reagents which are severely restricted when using an MS detection system. Two HPLC columns which are designed for the separation of very polar compounds were therefore chosen.

The columns and mobile phases used were as follows:

Option 1

Column: Phenomenex; Kinetex PFP 100 x 2.1mm, 2.6u, 100A.
Mobile phase:
A: 20mM formic acid in water
B: Methanol
Column temperature: 40°C
Sample temperature: 4°C
Injection volume: 1uL

Gradient

Step	Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	200	97	3
1	4.00	200	97	3
2	4.10	200	70	30
3	9.00	200	70	30
4	15.00	200	50	50
5	17.50	200	50	50
6	17.60	250	97	3
7	23.00	250	97	3

Option 2

Column: Hypersil GOLD aQ 150 x 2.1mm, 3u.
Mobile phase
A: 10mM ammonium formate in water, pH 3.47
B: Methanol
Column temperature: 40°C
Sample temperature: 4°C
Injection volume: 1uL

Gradient

Step	Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	200	97	3
1	4.00	200	97	3
2	4.10	200	70	30
3	9.00	200	70	30
4	15.00	200	50	50
5	17.50	200	50	50
6	17.60	250	97	3
7	23.00	250	97	3

Table 2 shows details of the transitions used and the retention times obtained for the two options.

Table 2: Table of B-group vitamins transitions and retention times for the two LC options

Analyte	Transition [m/z]	Retention Time option 1	Retention Time option 2
Thiamine	265>122	3.3	2.6
Riboflavin	377>243	13.7	14.7
Nicotinamide	123>80	1.6	5.1
Pantothenic acid	220>202	4.6	9.6
Pyridoxine	170>152	2.7	3.7
Folic acid	442>295	13.8	12.7
Biotin	245>227	12.6	13.8

Example chromatograms for both columns are shown in figures 11 and 12 below:

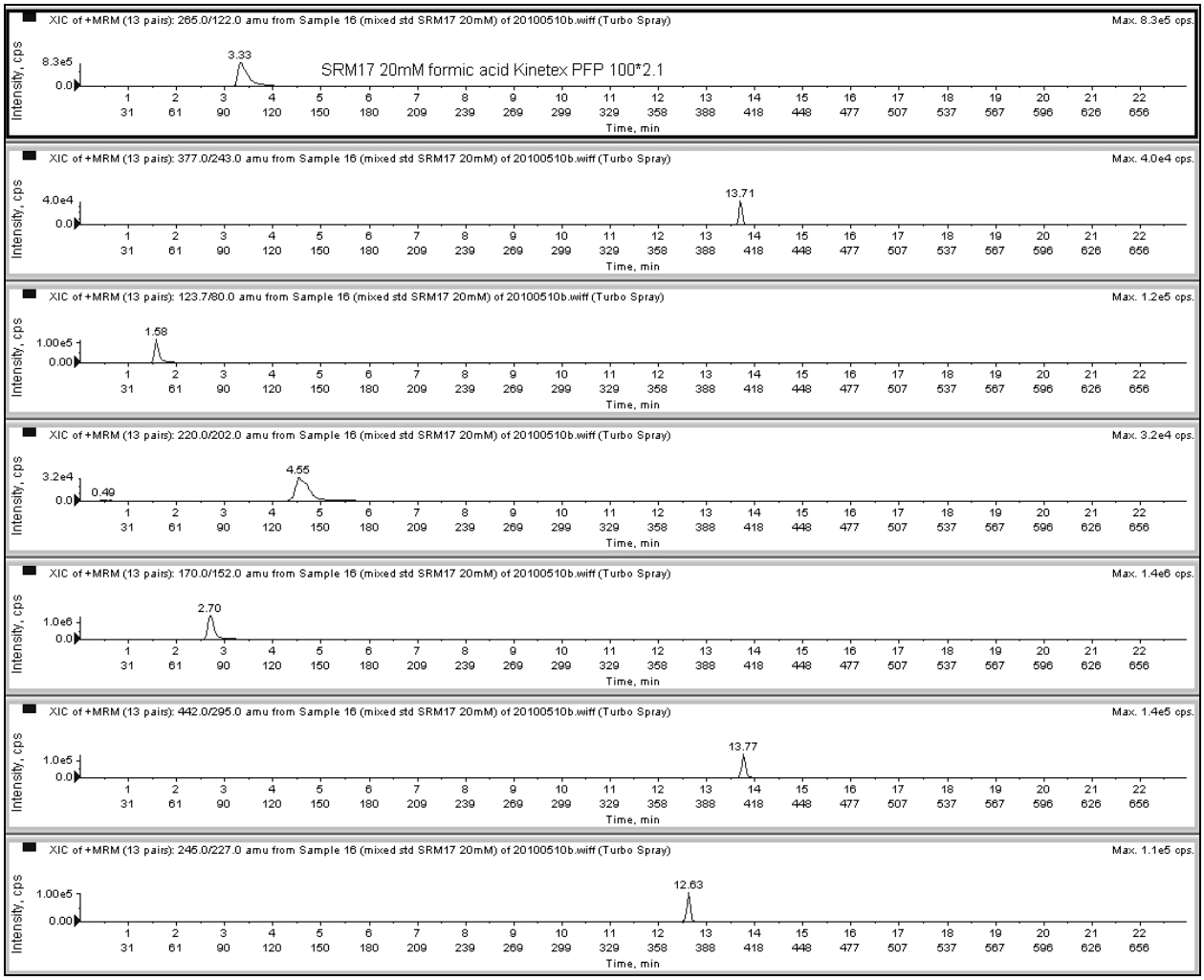

Fig. 11: Separation of a solution containing seven B-group vitamins on Phenomenex Kinetex PFP

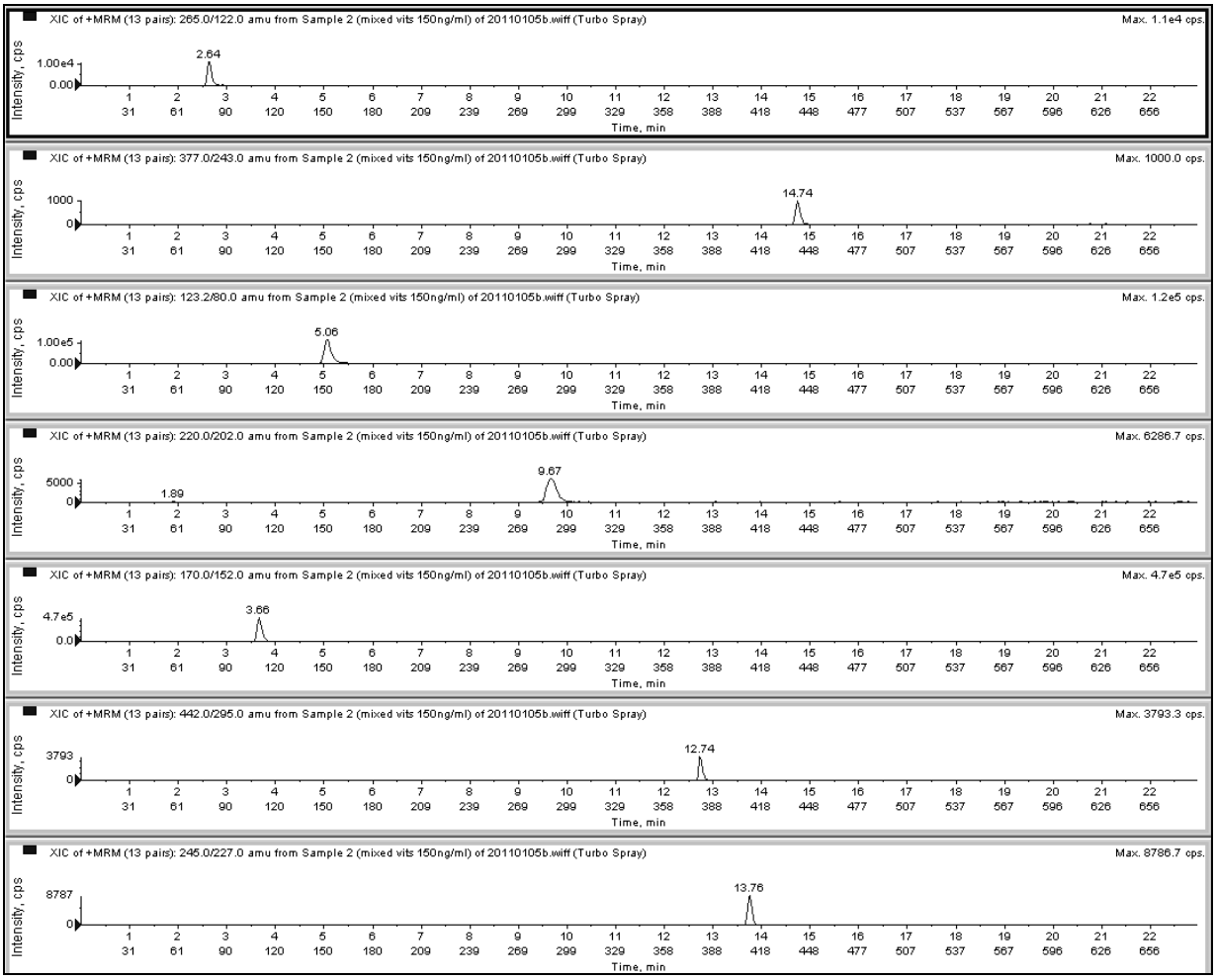

Fig.12: Separation of a solution containing seven B-group vitamins on Hypersil GOLD aQ

Both columns gave sufficient resolution of the vitamins in an acceptable run time. The Hypersil Gold aQ column was used for the remaining work. Calibration lines were obtained for each vitamin using the standards and conditions described above. Fig 13 shows the calibration line obtained for nicotinamide

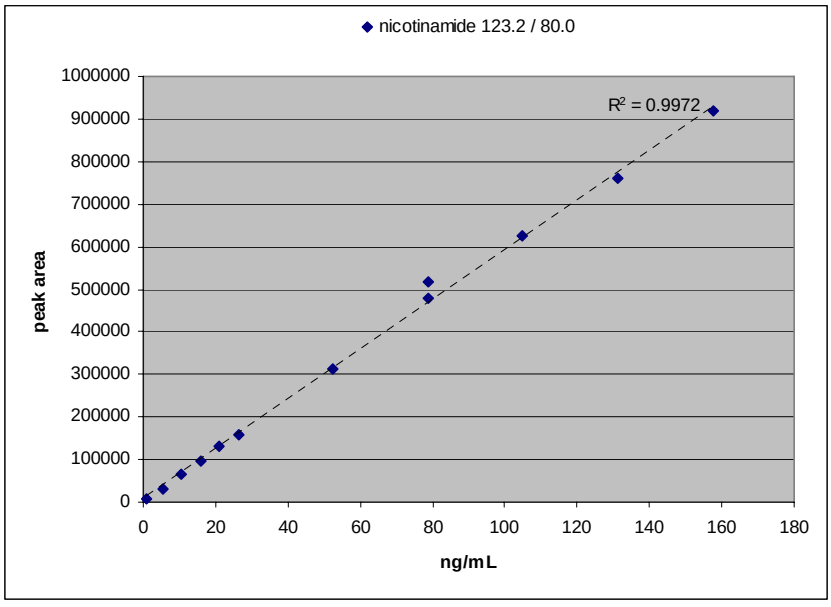

Fig 13: Calibration line for nicotinamide on Hypersil Gold aQ column

The calibration lines for all of the vitamins were linear with the exception of thiamine which showed some non-linearity. Further work is required to investigate the cause of this non-linearity.

5. Analysis of Infant milk formula

Two samples of infant milk formula were extracted with water at 50°C. The samples were shaken for 1 minute and ultrasonicated for 5 minutes. The extracts were then adjusted to pH 4.5 with acetic acid and centrifuged for 5 minutes at 4000 rpm. The supernatant was adjusted to ~pH 7 with 1M sodium hydroxide and filtered through a 0.45µm syringe filter for HPLC.

The vitamins were analysed using the conditions described for method 2, above. Chromatograms for one of the samples are shown below:

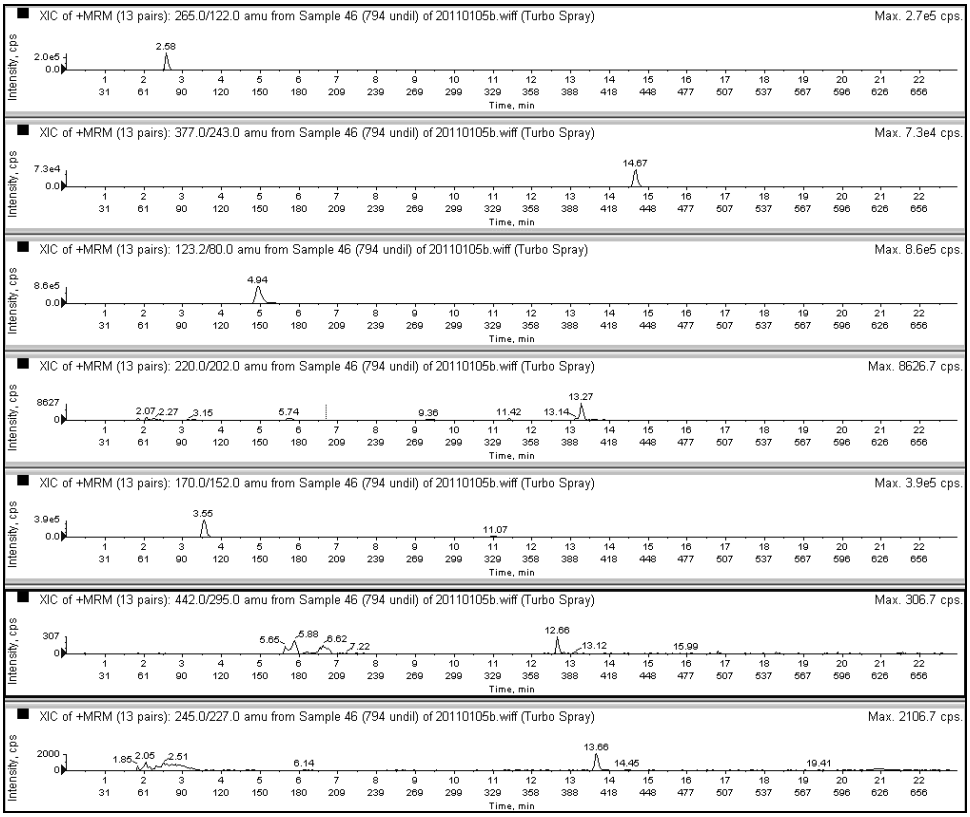

Fig.14: Chromatograms of an infant formula extract on Hypersil GOLD aQ

The vitamins were detected in both sample extracts.There was no evidence of matrix interferences with the exception of a small interference with the calcium pantothenate peak at ~ 13.3 minutes.

Due to variability in the responses obtained however, it was difficult to quantify the amounts of each vitamin present from an external standard. Isotopically labelled standards would ideally be required to allow full quantitation of vitamins in sample extracts. This is a limitation as the commercial availability of such standards is poor at the current time.

6. Conclusion

An LC-MS procedure that was suitable for the separation and qualitative analysis of seven B-group vitamins was developed however further work is required to develop a fully quantitative procedure. Such work would include a review of the availability of isotopically–labelled standards and the development of internal standard procedures for quantitation. Matrix studies with different sample types would also be needed.

It should also be noted that the current procedure is only suitable for the determination of the forms of vitamins studied and therefore is suitable for the determination of fortified products only. In addition, it is likely that much of the sample pretreatment and extraction that is required for HPLC with UV detection would still be required when using MS. There would therefore be little advantage in using MS detection in relation to time / cost of analysis, however the ability to provide better proof of identity would be beneficial.

7. References

[1] A. Gentilli, F. Caretti, G. D'Ascenzo, S Marchese, D. Perret, D. Di Corcia, L. M. Rocca: "Simultaneous determination of water soluble vitamins in selected food matrices by liquid chromatography./electrospray ionisation tandem mass spectrometry". Rapid Commun. Mass Spectrom. (2008) 22 2029-2043.

[2] R. J. Goldschmidt, W. R. Wolf: "Simultaneous determination of water-soluble vitamins in SRM 1849 Infant/Adult Nutritional Formula powder by liquid chromatography-isotope dilution mass spectrometry". Anal. Bioanal. Chem (2010) 397 471-481.

[3] Hong Zhang, Si Chen, Wenjuan Liao, Yiping Ren: "Fast simultaneous determination of multiple water-soluble vitamins and vitamin-like compounds in infant formula by UPLC-MS/MS". J Food, Agriculture & Environment (2009) 7 (2) 88-93.

[4] Pei Chen, M Ozcan, W R Wolf: "Contents of selected B vitamins in NIST SRM 3280 multivitamin/multielement tablets by liquid chromatography isotope dilution mass spectrometry". Anal Bioanal Chem (2007) 389:343-347

[5] Baiyi Lu, Yinping Ren, Baifen Huang, Wenqun Liao, Zengxuan Cai, Xiaowei Tie: "Simultaneous determination of four water soluble vitamins in fortified infant foods by ultra-performance liquid chromatography coupled with triple quadruple mass spectrometry". J Chromatographic Science (2008) 46 225-232

[6] A A Kandutsch, C A Baumann: "Factors affecting the stability of thiamine in a typical laboratory diet". J. Nutr. February 1, 1953 vol. 49 no. 2 209-219