The determination of biotin in food using HPLC

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The determination of biotin in food using HPLC

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Executive Summary

Procedures for the determination of biotin in foods were evaluated in this study. The study was undertaken to underpin the capability of the Government Chemist to perform referee analysis of vitamins by evaluating potential analytical methods.

Existing procedures for the determination of biotin were briefly reviewed.

A method using a commercially available, immunoaffinity cartridge for the selective extraction of biotin from infant formulae was compared with a European Standard HPLC procedure for the determination of biotin in foods. The latter method uses reverse phase HPLC with post-column derivatisation using FITC-labelled avidin to enhance detection sensitivity.

Both methods performed well for the determination of biotin in milk based test samples although further validation of the extraction conditions would be needed for other matrices using the immunoaffinity procedure.

The European Standard procedure has slightly better sensitivity but the immunoaffinity procedure is quicker and easier to perform on a routine basis.

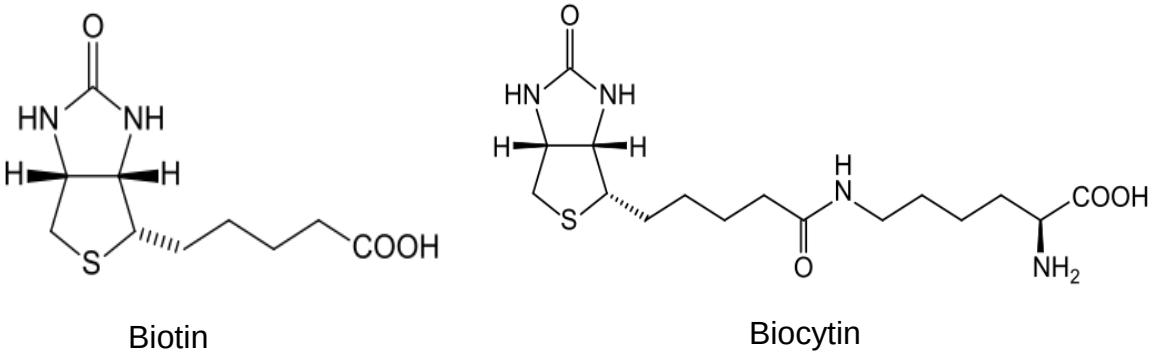
Contents

1 Background

1.1 General properties

Biotin, sometimes called Vitamin H or Vitamin B_7, is cis–hexahydro-2-oxo-1H-thieno [3, 4-d] imidazole-4-pentanoic acid. It has three asymmetric carbon atoms and can exist in eight steroisomeric forms although only D (+) biotin occurs in nature and is biologically active.

In nature, biotin occurs in food in the free form or is bound to the amino acid L-lysine as biocytin, or to other food peptides or proteins.



Biotin Biocytin

Figure 1: Structures of Biotin and Biocytin

Because of the relative complexity of the binding mechanisms in nature, the bioavailability of biotin in different foods varies. For example, the bioavailability varies from 0% in wheat to 100% in corn and is typically around 20 to 30% in most cereals. From an analytical viewpoint, the apparent biotin content of a food will depend upon the extraction procedure used. The relevance of this figure for nutritional use will depend upon how closely this extraction mimics human digestion processes. In practice, analytical procedures that determine the total biotin content of foods are preferred. Bioavailability in humans should be established separately. In order to determine total biotin, the bound forms must be liberated during analysis. This can be accomplished either enzymatically or by strong acid hydrolysis. In some cases, the presence of inactive biotin analogs and metabolites may also need to be considered.

1.2 Physical properties

Biotin is soluble in dilute alkali, sparingly soluble in water and alcohol. It is insoluble in most organic solvents.

Biotin in solution is quite stable at pH 4 to 9 but can be degraded by exposure to UV light. It is relatively stable in food products when heated and it is not easily destroyed in the ordinary processes of cooking but it will leach into cooking water. Processing of food, e.g. canning causes some losses.

Due to a lack of chromophores in the molecular structure, biotin only has weak absorbance in the UV region at 200 – 210 nm, with an absorbance maximum at 204 nm. Direct HPLC

methods using UV detection are therefore only useful for assays of highly concentrated vitamin premixes.

1.3 Dietary sources

Biotin occurs at low concentrations in many foodstuffs. Even the richest sources are low compared to those for other water-soluble vitamins. Foods containing biotin include egg yolk, yeast, liver and kidney, soybeans, nuts and cereals and some vegetables. Biotin exists in the free form to a greater extent in plants than in animal tissues.

2 Review of methods

Methods for the determination of biotin have been developed for foods, feeds, pharmaceutical preparations and for biological fluids such as blood and urine however, their sensitivity and specificity varies.[1-3]

Methods can be divided into four categories:- biological, microbiological, physiochemical and binding methods although some hybrid methods have been used.

2.1 Biological methods

Bioassays, using rats or chickens were used to determine the bioavailable fraction of biotin in food or feed by observing growth factors in response to controlled biotin intake. These assays have largely been replaced by more sophisticated techniques

2.2 Chemical methods

Chemical methods based on spectrophotometry or colorimetry have been available since the 1960's although because of their lack of sensitivity, their use has been mainly restricted to products with high concentrations of biotin such as pharmaceutical preparations. Polarography and TLC methods have also been used. A GC method was reported in the 1970's and HPLC methods have been used since the 1980's, primarily for use with multivitamin formulations.

HPLC is able to separate biotin from its analogs (e.g. dethiobiotin, biotin sulfoxides, and biotin sulfone) but is hampered by the poor, non-specific absorption in the UV range, requiring derivatisation techniques to enhance sensitivity, especially for the low concentrations found in food. Recent techniques using the strong binding properties of fluorescently-labelled avidin to biotin have been used to enhance sensitivity of detection. One such method has been published as an EN Standard and is evaluated later in this report.

2.3 Binding methods

Binding methods for biotin have been in use for many years. These are based on the same principles as immunoassay except that they utilise a specific binding protein instead of an antibody. Almost all of these use avidin or streptoavidin as the binding protein because of their inherent affinity for biotin. Various labelling ligands, reagents and sample extraction protocols have been described.

It should be noted that although such assays are specific in measuring biotin from other chemical components, they will also detect other biotin analogs and metabolites such as biocytin, and biotin-4-amidobenozoic acid. This may or may not be desirable depending upon the application.

In the past, these kits relied on radioactive labelling and were restricted to research use for blood and serum samples only. However modern kits are available with more user-friendly labelling mechanisms and include sample extraction conditions for other matrices such as foods. Typically, conditions for milk powders and cereal products may be given. The kits are sensitive but are relatively expensive and may require additional validation for the foodstuff being anlaysed.

2.4 Microbiological assays

Microbiological assays have been used for the determination of biotin in food for many years and are still in use today. They are sensitive and specific but are relatively complex to perform, may be prone to contamination and have a relatively high associated analytical uncertainty compared to chromatographic techniques. They remain however, the "classical" procedure for the determination of total biotin in foods.

Microbiological assays rely on the use of specific microorganisms such as *Lactobacillus plantarum* which require B-group vitamins for growth. The growth of these organisms under appropriate conditions is proportional to the biotin content of the sample extracts tested.

Microbiological assay kits are now available from suppliers such as R-Biopharm ("VitaFast®"). These kits remove the need to prepare and maintain the assay organisms, media etc although careful use is still required to avoid contamination and variability. Selection of extraction procedures is also key to ensuring that meaningful values are obtained. For the determination of total biotin in most foods enzymatic treatment or digestion with strong acid is required to release bound forms of biotin.

In the VitaFast® kit instructions there are a range of extraction conditions given for the extraction of various sample matrices including milk products, cereals, flour, baby food, multivitamin juices, fitness drinks, capsules, pills and vitamin mixes, yeast and yeast products.

Relatively simple, aqueous extraction is described for the determination of added biotin in tablets and similar matrices. Digestion with takadiastase is described for the determination of biotin in more complex food matrices such as cereals. It remains for the user to select and validate the most suitable extraction conditions for their particular application.

3 Evaluation of selected HPLC methods

The analysis of biotin in foods is hampered by the lack of sensitive and specific detection mechanisms for underivatised biotin. For the detection of biotin at naturally occurring concentrations, it is necessary to remove potentially interfering components and to enhance the detection sensitivity. UV detection can be used for added biotin at relatively high concentration, but for lower concentrations, derivatisation is required. A number of mechanisms have been proposed to produce fluorescent adducts[4.] One such procedure, using fluorogenically-labelled avidin has been published as a CEN standard[5].

In this project, two analytical procedures were evaluated:

a) the determination of added biotin in infant formula using UV detection after sample purification using immunoaffinity media

b) the determination of biotin in foods using EN 15607: 2009.

3.1 Determination of biotin in foods by HPLC with UV detection after concentration using an immunoaffinity cartridge (IAC)

Biotin can be determined selectively by HPLC with UV detection but the sensitivity is poor. Without a means of selective extraction, such methods can only be used for products containing very high levels of biotin such as vitamin premixes.

However, an immunoaffinity cartridge for the selective extraction of biotin is available from R-Biopharm (Biotin EASI-EXTRACT®). This cartridge contains an immobilised antibody, specific to biotin which selectively retains biotin from food extracts and can therefore be used to concentrate the biotin and remove interferences so that it can be analysed by HPLC.

The procedure involves extraction of the sample using a pancreatin digestion, dilution with buffer and centrifugation. The supernatant is then passed through the immunoaffinity cartridge. Biotin present in the sample binds to the column, unbound material is washed off and the retained biotin is collected by eluting with methanol. The eluate is concentrated by evaporation if necessary and analysed by HPLC

The kit instructions refer, in the introduction, to determination of biotin from food and feed but in fact the procedure is only described for infant formula, ready-to-use infant formula, infant cereal and liquid milk. The sensitivity of the procedure is dependent on the end determination which for a typical HPLC with UV detection will be reasonably close to the detection limit. Biotin does not possess a chromophore in its structure, which means that it is only possibly to use a non-specific detection wavelength at around 204nm. Since many compounds absorb light at this wavelength, it is not usually possible to determine biotin in foods without selective extraction from other matrix components.

The instructions suggest that a sample weight of 1 - 5g should be extracted into 100mL and that a total of 0.3 - 0.4 μg of biotin should be loaded onto the cartridge in a volume of 1 - 30mL. If it is assumed that 5g of sample is extracted into 100mL and that 30ml of extract is loaded onto the immunoaffinity cartridge, an initial biotin content of 10μg/100g would be required to produce an injected solution containing 0.15μg/mL (assuming 100% recovery) which is close the bottom of the calibration curve. Although this would be suitable for some natural products, there are many foods which contain less than this amount. It would also be necessary to verify that the extraction described was applicable to other matrices of interest.

The procedure was tested for the described matrix, i.e. infant formula powder containing added biotin. The kit instructions were followed as described below:

3.1.1 Extraction

5g of sample was stirred for 15 min on a magnetic stirrer in 0.1M phosphate buffer containing 4g of pancreatin. It was then incubated for 2 hours at 37°C. The extract was heated for 30 min at 100°C, cooled and diluted to volume before being centrifuged and filtered. An aliquot of the cleared extract was then passed through the IAC cartridge, washed with buffer, and the biotin was eluted with methanol. The methanol was evaporated to dryness and the residue dissolved in mobile phase.

3.1.2 HPLC

Standards (0.05 – 0.5 µg/mL) and samples were injected onto an HPLC system using the following conditions:

Column: ACE 3 AQ (Phenomenex); 3µm, 150 x 3mm
Column temp: 30°C
Mobile phase: 0.025%TFA / Acetonitrile (85/15)
Flow rate 0.35 mL/min (isocratic with 40% wash step in each run)
Detection: UV @ 210nm
Injection volume: 100 µL
Standards: External. Calibration range 0.05 – 0.5µg/mL in mobile phase

The calibration line and example chromatograms are shown in Figures 2 & 3 below:

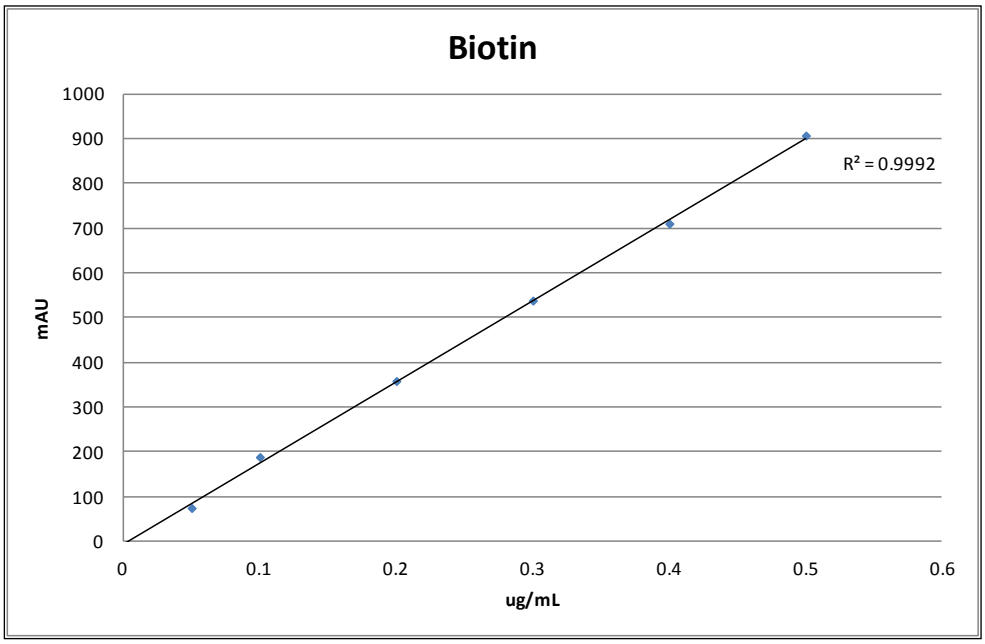
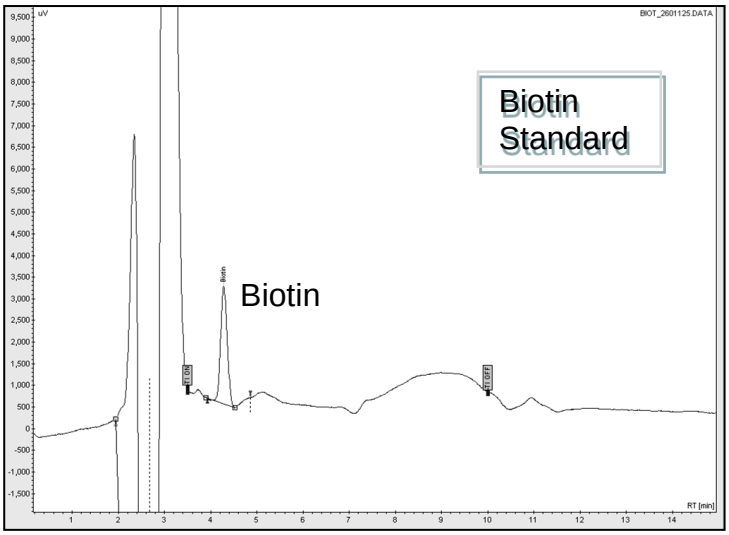


Figure 2: Calibration line for Biotin EASI_EXTRACT method



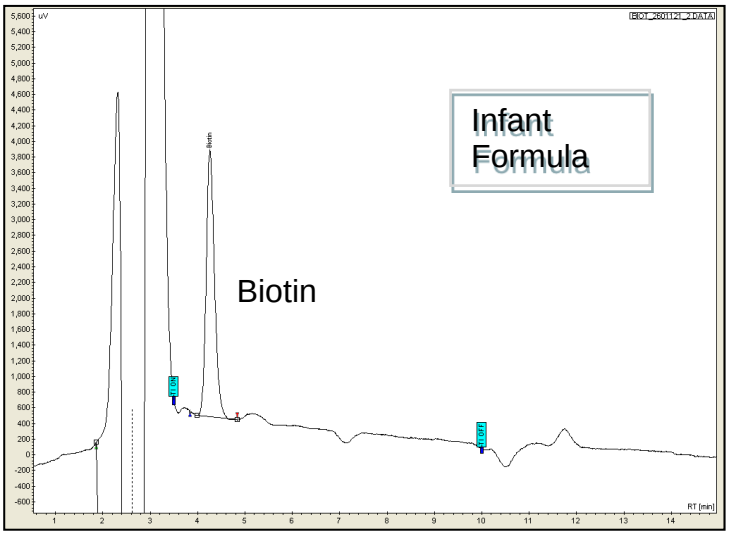


Figure 3: Chromatograms for a standard and an infant formula using the EASI-EXTRACT procedure

The procedure worked well for the infant formula samples and for this sample type, the kit provides a rapid and convenient means of selectively extracting and concentrating biotin from the sample so that HPLC can be used without derivatisation.

Table 1: Results for Biotin in infant formula samples

Sample	Analytical result µg/100g	Label declaration µg/100g
Proprietary Infant Formula powder 1	21.51	20
Proprietary Infant Formula powder 2	22.01	20
Proprietary Infant Formula powder 3	24.36	20
Proprietary Infant Formula powder 4	21.65	20

The cartridges have a maximum capacity of approximately 0.4µg of biotin and it is necessary to ensure that this is not exceeded; however, this is not normally a problem as biotin concentrations tend to be low. For samples containing very low levels of biotin, it would be necessary to use a higher sample weight, lower extract volume and/or a higher cartridge loading volume and these aspects have not been evaluated. The suitability of the described extraction conditions for other matrices would need to be proven and, further validation would be needed under these circumstances.

3.2 BS EN 15607: 2009 - Foodstuffs. Determination of d-biotin by LC

A European Standard method for the analysis of biotin in foods (BS EN 15607:2009) was published in June 2009. This procedure uses an enzymatic extraction followed by derivatisation of the extracted biotin using fluorescently-labelled avidin to enhance the HPLC detection sensitivity. The method can also be used to determine biocytin in food.

The method is presented as a generic method for biotin in food and the original validation study[6] was carried out using breakfast cereal powder, infant milk powder, fortified orange juice, freeze dried, green peas with ham and freeze-dried chicken soup. The biotin content of the samples ranged between 16 – 200 µg/100g. Performance data for the ten participating laboratories, showed repeatability relative standard deviations (RSD_r) between 2 – 11% and reproducibility relative standard deviations (RSD_R) between 14 - 30%. Horrat values were between 1.0 and 1.4.

The method was evaluated using samples of infant formula, milk powder and standard reference materials.

3.2.1 Preparation of reagents and stock standards

Biotin is poorly soluble in water and can take 4-5 hours to dissolve if prepared as described in the CEN procedure. However, biotin is much more soluble in even mildly alkaline solution and dissolution can be rapidly achieved by addition of a few drops of 1M sodium hydroxide to a solution of biotin in water. Biotin standards prepared in this way and subsequently diluted with water were stable for approximately one week if stored in a fridge but further work may be required if longer storage times are envisaged.

Avidin fluorescein isothiocyanate reagent (Avidin - FITC) has limited stability and needs to be prepared just before use. The reagent is expensive and is usually purchased in 5mg or 10mg

ampoules. The published method specifies that the stock solution should be prepared by dissolving 2.5 mg of the reagent in 50 mL of 0.1M phosphate buffer solution at pH 7.0. At this level, losses can occur during weighing as the freeze dried form available from the commercial supplier is light and sticks to the vial electrostatically. An easier option is to dissolve the entire contents of a 5mg vial in 100 ml of buffer although it has to be used within 2 weeks when stored at 4-8°C.

The diluted avidin-FTIC reagent must be used within 8 hours and should be protected from light. The cost of this reagent and the poor stability once prepared is a limitation when using this method, especially if the method is only used occasionally or for small numbers of samples.

3.2.2 Extraction of biotin

Biotin has traditionally been extracted using strong mineral acids but the time and temperature required varies depending on the source of the biotin (animal or plant). Longer extraction times are required for animal products but these extended extraction times can cause degradation of biotin from plant sources. Enzymatic hydrolysis will release the bound forms from both types of food without degradation and is preferred for this reason.

Biotin is water-soluble and most stable at pH 4 – 9. In the EN Standard, samples are extracted using overnight enzymatic hydrolysis with citrate buffer at 37°C and pH 5.7. Bound forms are released by the use of papain and for starchy samples, takadiastase is also added. The extracted biotin (& biocytin if present) is quantified by HPLC with external standardisation using a post-column derivatisation with avidin-FTIC and fluorescence detection.

It is important to shake the samples intermittently or continuously to ensure that the sample remains dispersed during hydrolysis. The sample extracts are cooled to room temperature and made up to 50 mL with buffer in a volumetric flask. The extracts are filtered using both filter paper and a syringe filter (0.45µm) prior to HPLC.

3.2.3 HPLC using Post Column Derivatisation

The HPLC system was set up as described in the published procedure except that a Luna C18 (Phenomenex) HPLC column was used. The CEN method indicates a Lichospher C18 column as an example but allows for the use of alternative columns with similar characteristics. The derivatisation reagent was delivered by means of a second HPLC pump, a T-piece and a knitted, mixing coil. The T-piece and coil were kept at constant temperature, inside the column oven. The knitted reaction coil allows for good mixing of the column eluate with the post-column reagent whilst minimising the amount of band-broadening that occurs.

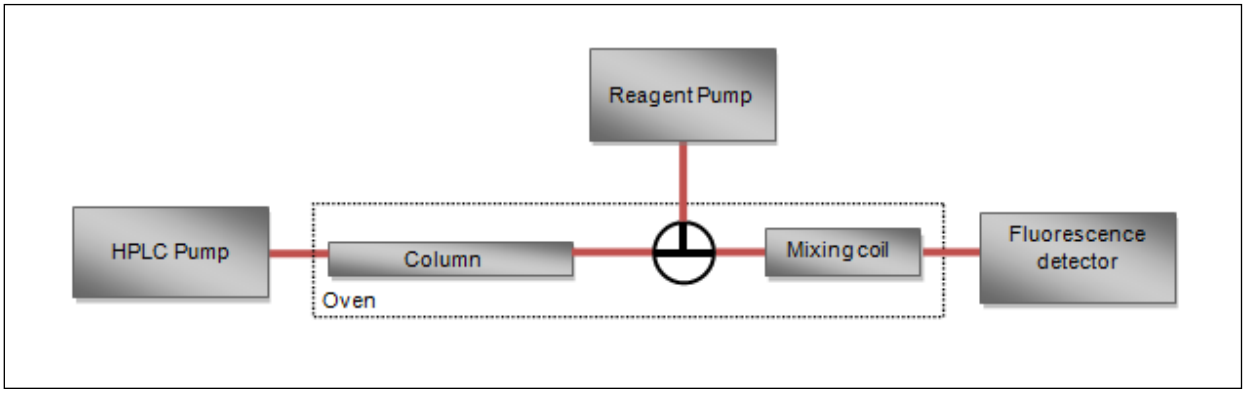


Figure 4: HPLC System

System suitability was checked using biotin and biocytin standards to ensure adequate separation and consistent retention times.

The HPLC conditions used are shown in the table below:

Table 2: HPLC Conditions used for the CEN procedure

Column:	Phenomex Luna C18 250 mm x 4.6 mm, 5 µm, 100 A
Mobile phase: (Isocratic)	Phosphate buffer, pH 6.0 : Methanol (80:20)
Flow rate	0.4 mL/min
Column oven temperature:	37°C
Detection:	Fluorescence Excitation wavelength : 490 nm Emission wavelength : 520 nm
Injection volume:	30µL
Knitted reactor coil)	10 m x 0.50 mm ID (2.0 mL)
Post-column Reagent	Avidin-FTIC in phosphate buffer
Flow rate	1.0 mL/min
Knitted reactor oven temperature:	37°C

3.2.4 Linearity, range and injection repeatability

The calibration was shown to be linear over a range from 0.05 to 0.35 µg/mL.

The calibration curve for biotin is shown below in Figure 5.

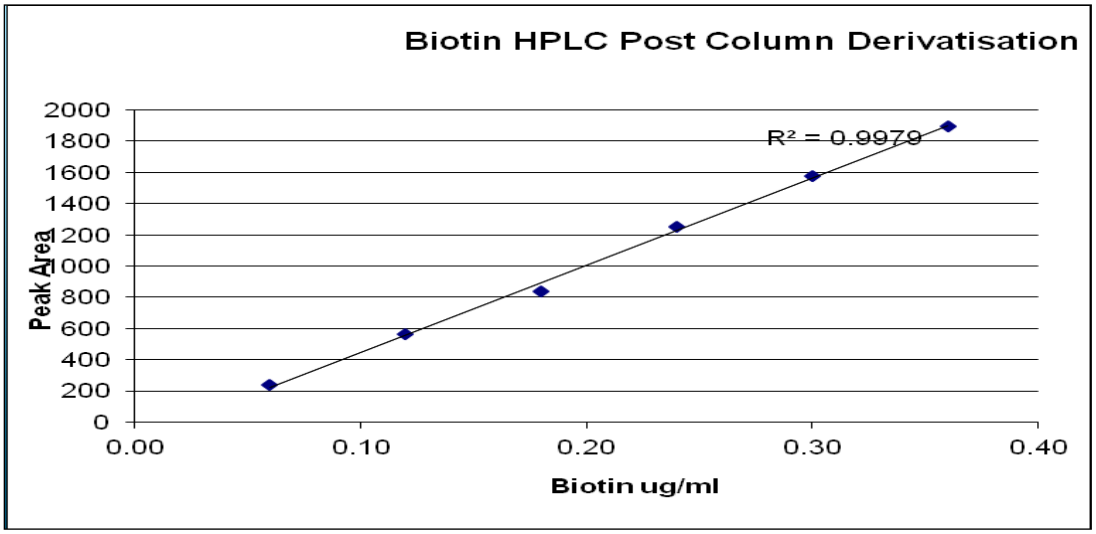


Figure 5 – Biotin calibration curve

The injection repeatability (RSD %) at 0.05µg/mL and 0.30µg/mL was <10%.

3.2.5 Limit of detection

The limit of detection obtained using the conditions described above was approximately 10 ng/ml in the injected solution. When using a sample weight of 10 g, and an extract volume of 50mL, this is equivalent to a biotin concentration of 5 µg/100g in the sample.

3.2.6 Method performance

A range of infant formula and follow-on formula samples were analysed to assess the method performance. Two of these samples were also analysed using a VitaFast®, microbiological test kit for comparison. A standard reference material and a sample of pig's liver were also analysed. The results were as shown in Table 3 and Fig 6 below:

Table 3: Analytical results

Sample ID	Sample Description	Label Declaration or expected amount (µg/100g)	Biotin Concentration CEN method (µg/100g)	Result reported by VitaFast® Method (µg/100g)
1	Milk Formula powder	*13*	12	13
2	Milk Formula powder	*17*	27	29
3	Milk Formula powder	*22*	25	-
4	Milk Formula powder	*24*	22 / 24	-
5	Milk Formula powder	*21*	35	-
6	Milk Formula powder	*18*	35	-
7	Milk Formula powder	*22*	25	-
8	Pigs' Liver	*~ 89 **	94	-
9	NIST CRM 1849 Adult formula powder	*192 ± 25*	194	-

** Calculated from food tables for raw pigs liver*

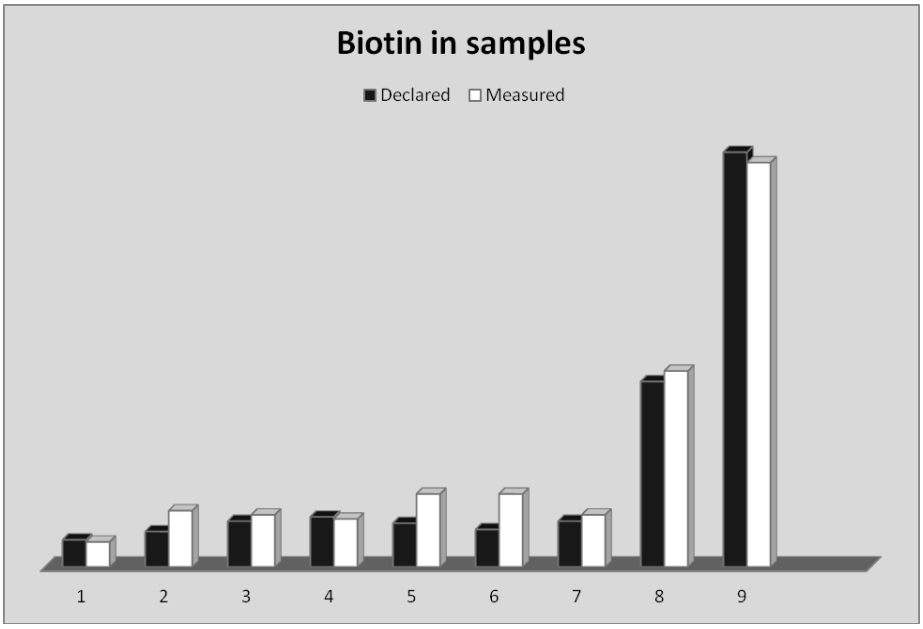


Figure 6: Comparison of Biotin results using the CEN method with declared values

The results obtained for the milk powder samples agreed well with the product declarations in most cases, although slightly high results were obtained in three of the samples. It is however, possible that these samples may have contained biotin in excess of their declared values. Also, the values obtained for two of the samples using microbiological assay agreed well with the HPLC values (See Table 3).

The expected biotin content of the pig's liver was estimated from UK food table data. The analytical result was consistent with the calculated value.

The results obtained for the NIST adult formula CRM material was in excellent agreement with the certified value.

Example chromatograms for biotin and biocytin standards and typical chromatograms for samples are shown below (Figs 7 - 10):

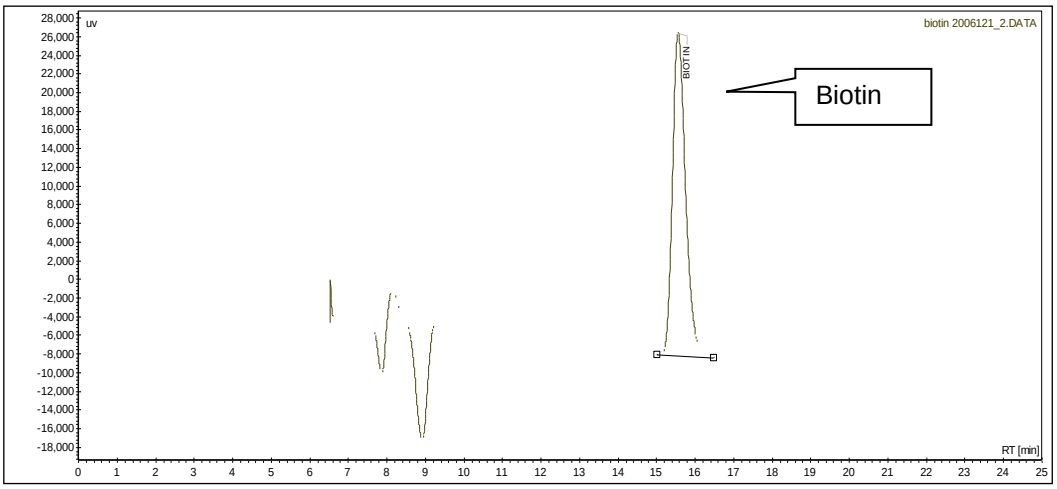


Figure 7: HPLC chromatogram of biotin standard @ 0.3 µg/mL

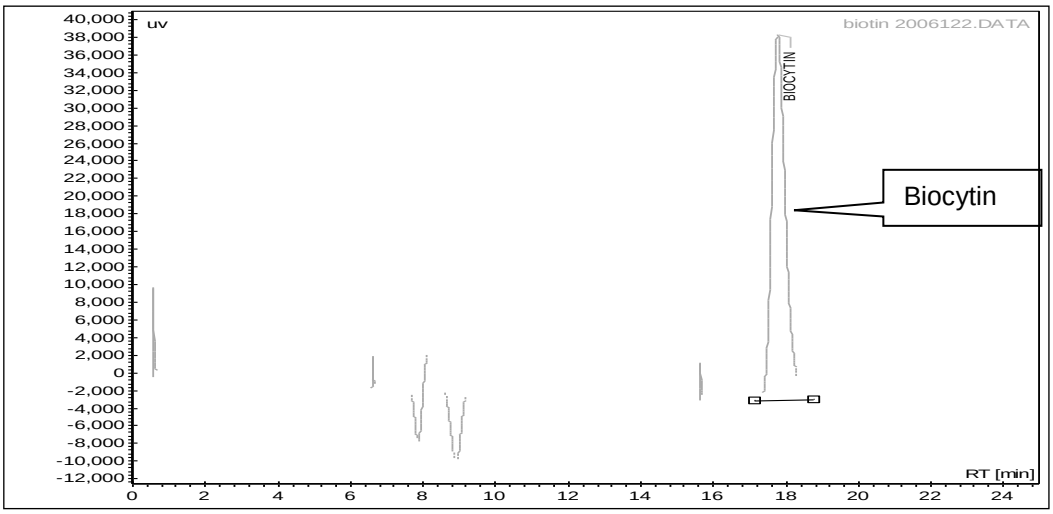


Figure 8: HPLC chromatogram of biocytin working standard @ 0.3 µg/mL

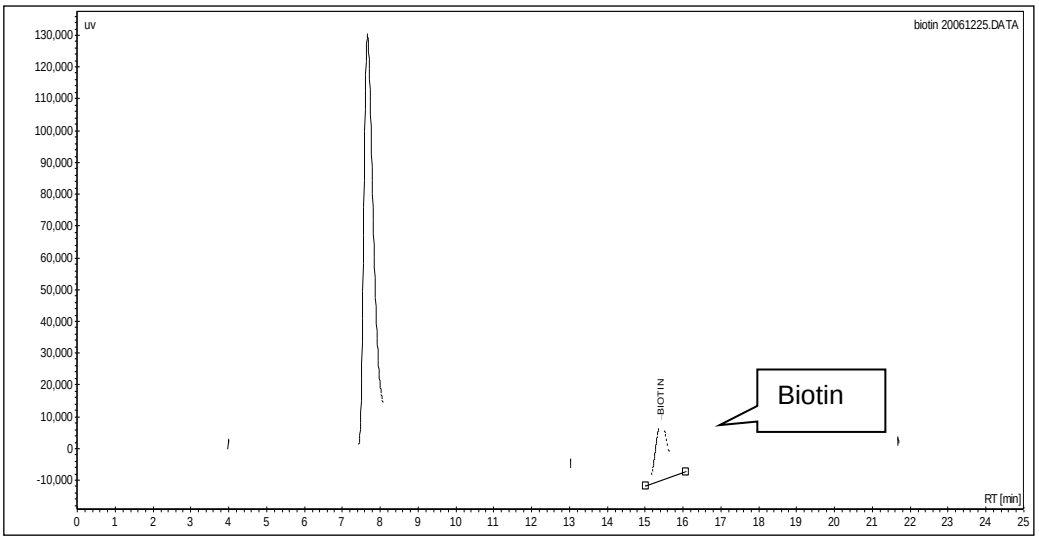


Figure 9: HPLC chromatogram of infant formula milk powder containing biotin at 0.2 µg/g

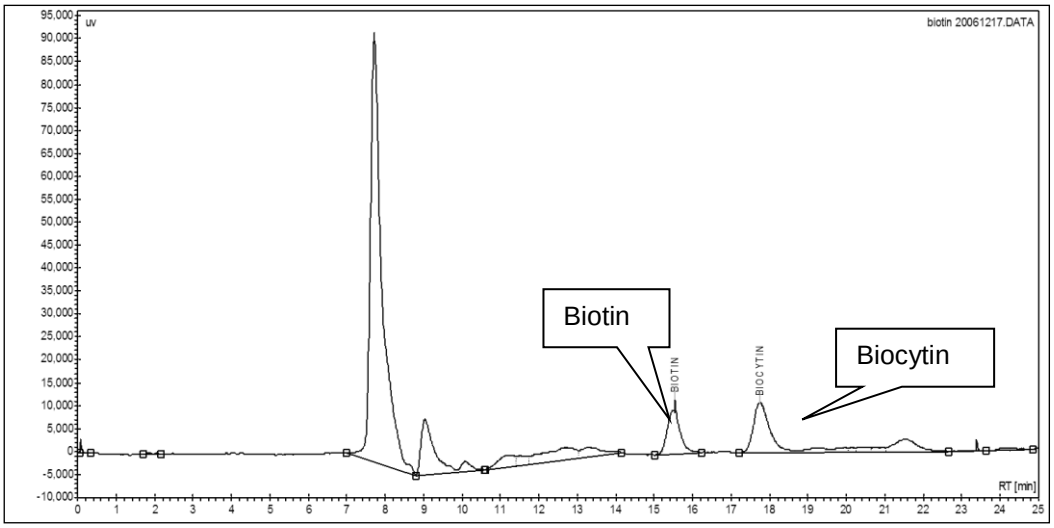


Figure 10: HPLC chromatogram of lyophilised pig's liver containing biotin and biocytin

3.2.7 Repeatability

The method repeatability was evaluated by analysing two different infant formula powders, and a milk-based, meal-replacer powder in triplicate on three different days.

The data obtained is shown in the table overleaf:

Table 4: Determination of Biotin (µg/100g) in three different formula powder matrices using the EN standard method

	Infant Formula 1	Infant Formula 2	Meal replacer powder
Day 1	31	30	36
	31	29	29
	34	32	30
Mean	*32*	*30*	*32*
σ	*1.73*	*1.53*	*3.79*
RSDr	*5.4*	*5.0*	*12.0*
Day 2	37	22	35
	37	24	37
	42	19	35
Mean	*39*	*22*	*36*
σ	*2.89*	*2.52*	*1.15*
RSDr	*7.5*	*11.6*	*3.2*
Day 3	28	34	37
	29	30	37
	28	36	32
Mean	*28*	*33*	*35*
σ	*0.58*	*3.06*	*2.89*
RSDr	*2.0*	*9.2*	*8.2*
Mean All days	***33***	***28***	***34***
STDEV All days	***4.8***	***5.7***	***3.1***
RSD All days	***15***	***20***	***9***

There was some variation in repeatability both between samples and between days. The repeatability calculated using the Horwitz calculation for 0.3 ppm and multiplying by 0.67 to estimate the within laboratory precision is 12.75%. All of the within-day repeatability values were less than this value. The between-day repeatabilities were higher, as would be expected. The %RSD ranged between 9 and 20%. The repeatability shown in the published procedure for infant formula was however 5.24ug/100g with a mean of 16ug/100g, which is equivalent to 32.8%RSD. The overall repeatability in these samples was therefore acceptable.

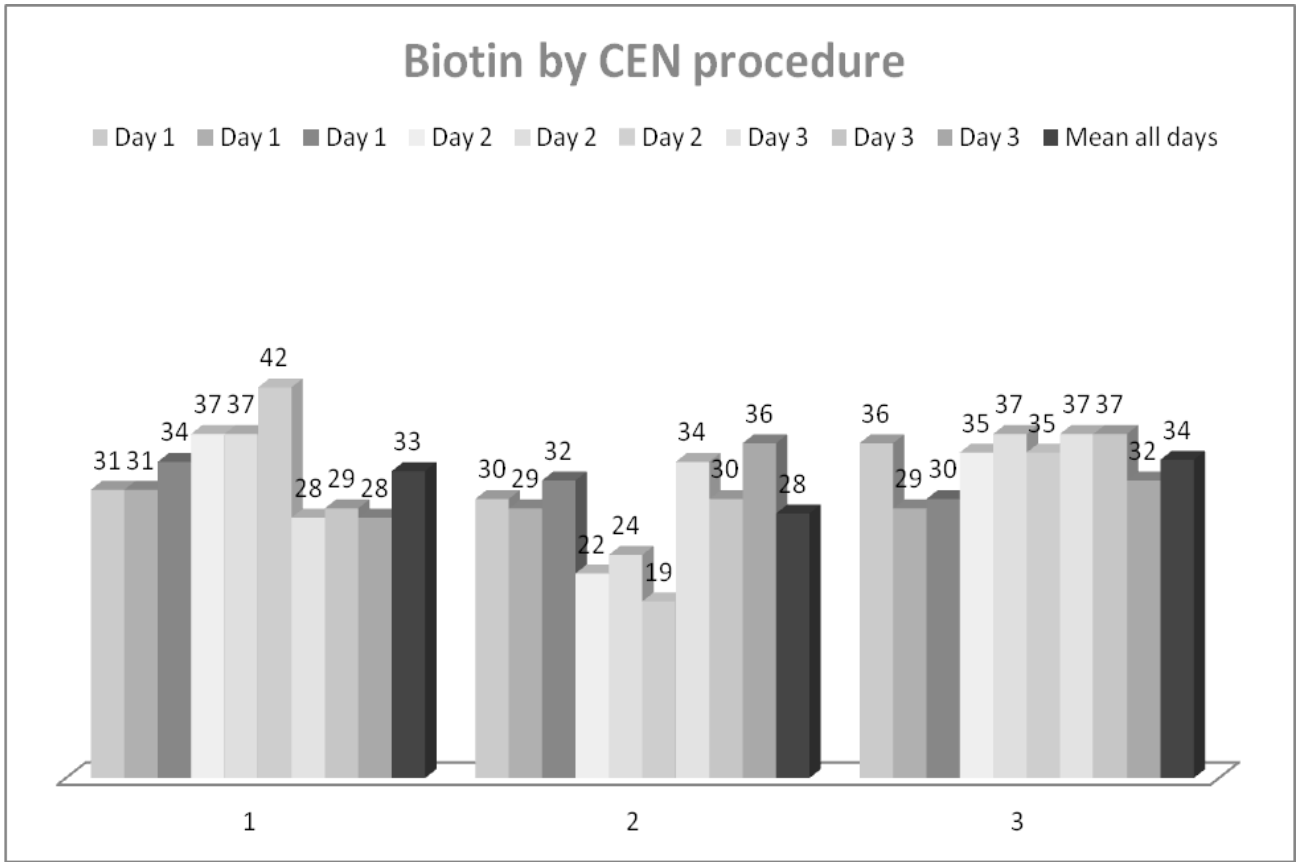


Figure 11: Biotin results for all samples and all days

4 Conclusion

Microbiological assay, ELISA and protein binding methods can be used to determine biotin in foods at natural levels but chromatographic methods are often preferred because of their wide applicability and availability. The analysis of biotin in foods using liquid chromatographic techniques however, remains challenging due to the low concentrations found in natural foodstuffs and the potential for interference from other matrix components.

The availability of commercial immunoaffinity extraction cartridges allows for selective separation of biotin from foods and concentration of the biotin so that HPLC using UV detection can be used. A limitation of this approach is the limited availability of information relating to the sample extraction required for different food matrices and the need to include biocytin for some sample types. Additional validation is necessary for sample matrices that are not included in the kit instructions.

The use of fluorescence labelled avidin to enhance the detection sensitivity of biotin is beneficial although the limit of quantitation using this technique was only approximately twice that obtained using the immunoaffinity clean-up.

The method using the immunoaffinity clean-up is easy to use and reasonably rapid but requires additional validation for the extraction of other food or feed matrices. This however, should be fairly straightforward to achieve. The cartridges are only available from one supplier but there are some similar products on the market from at least one other manufacturer.

The CEN standard method uses similar extraction principles (enzymatic hydrolysis) which have been applied to a wider range of foodstuffs. The procedure does however require post column derivatisation which can be complicated to set up, especially for small numbers of analyses. The price of the fluorescently bound avidin reagent and it's poor stability in use, are limitations for routine use.

Both methods have been successfully transferred in-house and are available for future use. Additional validation will be required when analysing unknown matrices however, this can be addressed as needed.

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6 Acknowledgements

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Mention of a commercial analytical product in this report does not imply endorsement by the Government Chemist.