



Setting standards  
in analytical science



## Government Chemist Dissemination Event 2008

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## Developments in Vitamin Analysis



# Vitamin Legislation in Food



- European Union (EU) Directive 2002/46/EC on food supplements.
- Regulation (EC) 1925/2006 on addition of vitamins and minerals and of certain other substances to foods.
  - In both cases, there are restrictions on the vitamin forms allowed and provisions to set minimum and maximum amounts



# Establishment of Upper and Lower limits



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## Upper Limits:

- Risk assessment of safe upper limits based on toxicity for all groups.
- Intake of vitamins from other sources.
- Population reference intakes

## Lower Limits:

- Minimum amount must be set for supplements.
- Food claims based on the concept of “significant amount” – currently proposed as 15% RDA per daily dose.
- Tolerances are currently wide, poorly defined and vary. Minimum amount must be present for whole shelf-life.



# Techniques for vitamin analysis



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Method	Microbiological Assay	HPLC	Biacore	ELISA	Other
Principle	Metabolism of microorganisms	HPLC UV/vis/FI	Surface Plasmon resonance	Antigen-antibody	Photometry, colorimetry, etc
Equipment reqd	Lab suitable for microbiology Photometer	HPLC system	Biacore system	Test kit /system Photometer	Photometer
Equipment costs	Low-Medium	Medium	High	Low	Low
Personnel costs	High (Set-up & maintenance)	High	Low -medium	Low	Medium
Speed	Slow	Rapid	Rapid	Rapid	Slow



# Application of techniques



## Fat soluble vitamins

Mainly HPLC

## B-group vitamins -

Microbiological assay - All b-group vitamins

ELISA - Folic acid, Vitamin B<sub>12</sub>, Biotin

Biacore - B<sub>2</sub>, B<sub>12</sub>, folic acid, biotin, pantothenic acid

HPLC - B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, folic acid,

Immunoaffinity - Folic acid, Vitamin B<sub>12</sub>

## Vitamin C

Titration, Spectrophotometric, Enzymatic, HPLC



# Sources of analytical methods



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- Official methods:

- **AOAC**

- Methods for vitamins , A,  $\beta$ -carotene D, E & K in various matrices (13)

- **CEN / ISO**

- Methods for vitamins A,  $\beta$ -carotene D, E & K in various matrices

- **USP-NF**

- Methods for water and fat-soluble vitamins in supplements

- Many newer methods published which have no official status





European Committee for Standardization  
Comité Européen de Normalisation  
Europäisches Komitee für Normung



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## CEN Standardisation

CEN Technical Committee TC275

– "Food Analysis" - Horizontal methods

Working Group 9

– **Vitamins and carotenoids in food and food supplements**



# CEN Standards for fat-soluble vitamins



- EN 12821:2000 Foodstuffs - Determination of **vitamin D** by HPLC -
  - Measurement of cholecalciferol (D<sub>3</sub>) and ergocalciferol (D<sub>2</sub>)
- EN 12822:2000 Foodstuffs - Determination of **vitamin E** by HPLC -
  - Measurement of alpha-, beta-, gamma-, and delta-tocopherols
- EN 12823-1:2000 Foodstuffs - Determination of vitamin A by HPLC-Part1:
  - Measurement of **all-trans-retinol and 13-cis-retinol**
- EN 12823-2:2000 Foodstuffs - Determination of vitamin A by HPLC – Part 2:
  - Measurement of **Beta-carotene**
- EN 14148:2003 Foodstuffs - Determination of **vitamin K1** by HPLC



# CEN Standards for Water-soluble vitamins



- EN 14122:2003 Foodstuffs - Determination of **vitamin B<sub>1</sub>** by HPLC
- EN 14152:2003 Foodstuffs - Determination of **vitamin B<sub>2</sub>** by HPLC
- ENV 14164:2002 Foodstuffs - Determination of **vitamin B<sub>6</sub>** by HPLC
- ENV 14166:2001 Foodstuffs - Determination of **vitamin B<sub>6</sub>** by microbiological assay
- EN 14663:2005 Foodstuffs - Determination of **vitamin B<sub>6</sub>** (including its glycosylated forms) by HPLC
- EN 14131:2003 Foodstuffs - Determination of **folate** by microbiological assay
- EN 14130:2003 Foodstuffs - Determination of **vitamin C** by HPLC



# Standards under development/consideration



- The determination of niacin in foods by HPLC
- The determination of biotin (& biocytin) in foods by HPLC
- The determination of pantothenic acid in foods by HPLC.
- The determination of folates in food by HPLC after conversion to 5MeTHF.
- Carotenoids in foods (Currently canthaxanthin and astaxanthin in fish)
- Methods for the determination of vitamins in Supplements
- CRM's for Vitamin Analysis
- Tolerances for vitamins



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# Methods for supplements - Fat soluble vitamins



- Method for simultaneous determination of vitamins A, E & D in supplements developed with FSA funding.
- saponification,
- solvent extraction of A, E & D with ethyl acetate/pet ether
- analysis of vitamins A & E in one run using HPLC with UV detection (Fluorescence possible)
- HPLC clean-up and analysis of vitamin D



# Collaborative trial of oil-soluble vitamins method

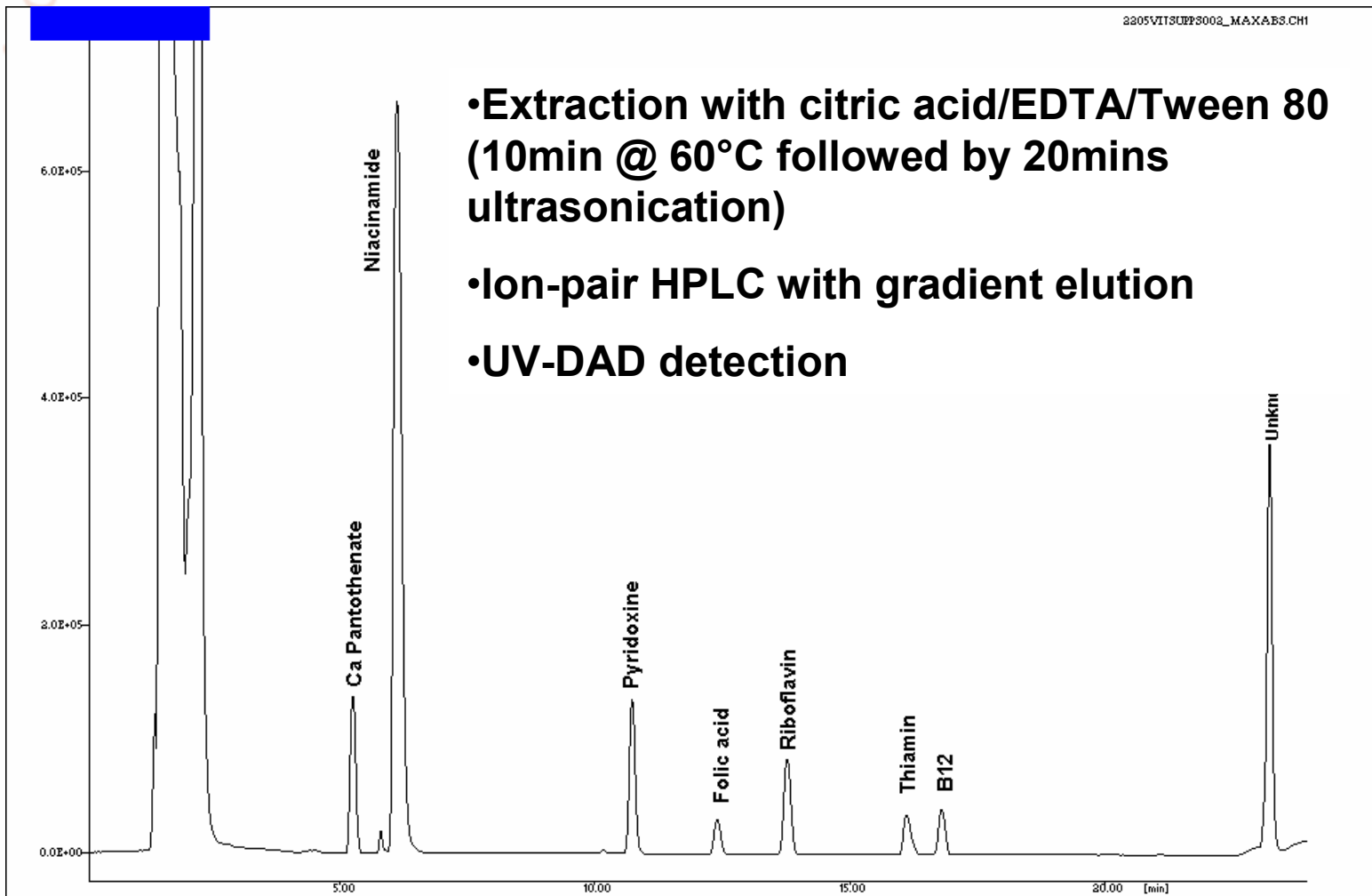


- 16 participants
- 5 duplicate samples

Sample	1	2	3	4	5	6
A	Ho <sub>r</sub> 1.8 Ho <sub>R</sub> 2.3	Ho <sub>r</sub> 1.6 Ho <sub>R</sub> 2.1	Ho <sub>r</sub> 2.6 Ho <sub>R</sub> 2.7	Ho <sub>r</sub> 2.0 Ho <sub>R</sub> 2.0	Ho <sub>r</sub> 2.0 Ho <sub>R</sub> 2.0	Ho <sub>r</sub> 3.1 Ho <sub>R</sub> 2.2
	Ho <sub>r</sub> 0.9 Ho <sub>R</sub> 1.1	Ho <sub>r</sub> 0.9 Ho <sub>R</sub> 1.0	Ho <sub>r</sub> 1.0 Ho <sub>R</sub> 1.0	Ho <sub>r</sub> 1.4 Ho <sub>R</sub> 0.9	Not added	Ho <sub>r</sub> 2.1 Ho <sub>R</sub> 1.7
E	Ho <sub>r</sub> 3.5 Ho <sub>R</sub> 2.7	Ho <sub>r</sub> 1.7 Ho <sub>R</sub> 4.0	Ho <sub>r</sub> 2.4 Ho <sub>R</sub> 3.9	Ho <sub>r</sub> 3.4 Ho <sub>R</sub> 2.8	Ho <sub>r</sub> 3.2 Ho <sub>R</sub> 4.9	Ho <sub>r</sub> 3.2 Ho <sub>R</sub> 4.8



# Method for thiamin, riboflavin, niacin, & B6 (and pantothenate) in supplements



# Collaborative trial of water-soluble vitamins method



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Fifteen laboratories.

Five test samples

Sample	1	2	3	4	5
<b>Thiamin</b>	Hor 1.1 HoR 1.1	Hor 1.3 HoR 1.4	Hor 0.8 HoR 1.1	Hor 0.4 HoR 0.4	Hor 0.5 HoR 1.4
<b>Niacinamide</b>	Hor 1.6 HoR 1.5	Hor 1.3 HoR 1.4	Hor 1.4 HoR 0.9	Hor 0.5 HoR 0.9	Hor 1.0 HoR 1.0
<b>Vitamin B6</b>	Hor 1.4 HoR 1.5	Hor 1.2 HoR 1.2	Hor 1.0 HoR 0.9	Hor 0.8 HoR 0.7	Hor 0.7 HoR 0.9
<b>Riboflavin</b>	Hor 1.1 HoR 2.8	Hor 0.6 HoR 2.2	Hor 1.7 HoR 2.6	Hor 0.9 HoR 0.9	Hor 2.1 HoR 3.5



# Improvements required for Oil-soluble vitamins methods



- Cheaper and faster methods
  - Direct extraction/SFE/ASE/GPC
  - Replacement of liquid/liquid extraction by SPE (Oasis, Chromabond XTR, Extralut)
  - Multi-analyte procedures
  - Use of Ultra performance Liquid chromatography
  - Use of LC-MS or MS/MS techniques.
- Sample test portion sizes
- Methods for carotenoids in food
- Need to harmonise activity factors used
- Suitability of methods to identify the form of vitamin used.



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# Improvements required for water-soluble vitamins



- Classical microbiological assay to be refined or replaced by other techniques (e.g HPLC)
- Multi-analyte methods (Fortified products or total?)
- Cheaper and more rapid methods
- HPLC or HPLC-MS procedures developed
- Development of specific immunoassay extractions using antibodies or binding proteins.
- Validation of multi-vitamin extraction procedures.



# Microbiological assay



- Classical methods – All b-group vitamins but slow and variability quite high
- Micro-titre plate assays can improve throughput & handling
- Vitafast<sup>®</sup> (R-Biopharm) available commercially but wider validation required.
- Also RIDASCREEN ELISA assays for biotin, folic acid and vitamin B<sub>12</sub>



**Biacore**®



- Surface plasmon resonance biosensor technology available.
- For B<sub>2</sub>, folic acid, vitamin B<sub>12</sub>, riboflavin and pantothenic acid.
- AOAC Performance tested methods
- Advantages: Rapid technique for limited range of products and vitamins
- Disadvantages: Not all vitamins available, Not totally specific, Response to vitamin forms varies, Equipment expensive and manufacturer specific.



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# Thiamin and Riboflavin



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- Thiamine, Thiamine HCl, Thiamine mononitrate, TTP, TPP, TMP.
- Two CEN Standards available using HPLC methods
- Acid extraction followed by enzyme hydrolysis
- Conversion of thiamine to thiochrome
- HPLC of riboflavin and of thiochrome with fluorescence detection
- GC project looked at enzyme hydrolysis, pre/post column derivatisation, optimisation of thiochrome conversion  
Report on Government Chemist website.



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# Niacin



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- Nicotinic acid, Niacinamide. NAD, NADP, Bound (unavailable) forms.
- Total Niacin [Bound NAD] ; Available Niacin (acid extractable)
- Draft CEN Standard being prepared for an HPLC procedure for nicotinic acid and nicotinamide.
- Acid (or enzymatic) extraction followed by HPLC. Post-column derivatisation with ultra-violet light and  $H_2O_2$  and  $Cu^{2+}$ . Fluorescence detection. LC runtime about 45mins.
- Limited validation data for milkpowder, ham, choc. cereals, wheat flour and green peas.



# Vitamin B6



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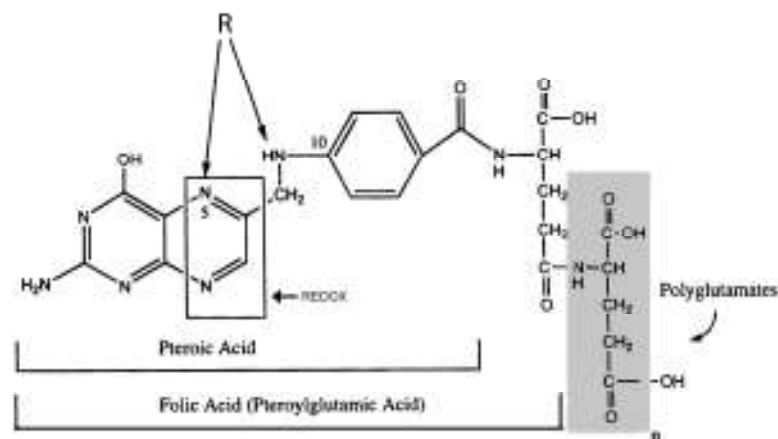
- Pyridoxine, Pyridoxal, Pyridoxamine, and their phosphates. B6 glycosides largely unavailable.
- 3 current CEN procedures:
  - B6 by MBA
  - **Total B6 by HPLC after conversion to pyridoxine.**
  - B6 Isomers (with optional glycosides)
- Acid extraction, enzymatic dephosphorylation and chemical conversion to PN. HPLC with fluorimetric detection. Validated on foods and dietetic foods.



# Folic Acid

Folic acid synthetic. Natural folates are reduced H<sub>4</sub>-folates substituted at C<sub>5</sub>/C<sub>10</sub> and are conjugated to glutamic acid. e.g 5MeH<sub>4</sub>-folate.

- Few methods for natural folates (mainly MBA)
- Folates extracted in neutral buffer (not acid-stable). Enzymatic hydrolysis need for natural folates. Trienzyme extraction with amylase, protease and foyl deconjugase is required.
- Reduced folates fluoresce but folic acid does not. UV and fluorescence detection required.



# Folate by HPLC



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- Extraction in neutral or mildly alkaline buffer at 100°C.
- For natural folates, enzymatic hydrolysis required.
- Clean-up using SAX cartridge, FBP columns or IAC.
- Affinity extraction cartridge now available from R-Biopharm – (folic acid only)
- HPLC using fluorescence detection and UV for folic acid.
- Draft CEN standard under evaluation: Mild alkaline extraction followed by enzymatic hydrolysis where required. Chemical conversion of all forms to 5-Me THF. HPLC with fluorescence detection. Further development and validation required.



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# Biotin



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- Biotin is typically determined by microbiological assay or ELISA techniques.
- It has no chromophore therefore does not respond to HPLC detectors
- Draft CEN procedure for D-Biotin and D-biocytyn under elaboration:
  - Enzymatic hydrolysis with papain and takadiastase .
  - HPLC with post-column derivatisation with avidin fluoresceine isothiocyanate (Avidin –FITC) and fluorescence detection.
  - Tested on a range of foods but initial variability too high, therefore further validation required.



# Pantothenic acid



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- Can be determined by HPLC with UV detection only at high levels, e.g. premixes.
- Draft CEN procedure under evaluation:
  - Enzymatic hydrolysis with pepsin, alkaline-phosphatase and pantetheinase. Ion-exchange clean-up. HPLC with fluorescence detection after post column derivatisation with NaOH/OPA/MPA.



# Vitamin B<sub>12</sub>



- Present at very low levels, even in supplements.
- Analysed by microbiological assay or RIA.
- Can be determined by HPLC with UV detection in premixes.
- Extraction in pH 4.5 buffer followed by concentration using SPE and immuno columns.
- Immunoaffinity cartridge available from R-Biopharm.
- HPLC with UV detection



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# Vitamin C



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- L-Ascorbic acid & it's salts; dehydroascorbic acid ascorbyl palmitate
- Analysed by redox- titration, OPD derivatisation, enzymatic methods or by HPLC
- Extraction should be acidic, chelate metals, protect against AA oxidase and precipitate starch & protein.
- **HPLC** Two approaches :
- DHAA reduced to AA and HPLC with UV detection (CEN)
- Or// AA oxidised to DHAA , derivatised with OPD and HPLC with fluorescence detection.



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## Conclusion



- Methods for oil-soluble vitamins by HPLC are largely in place but improvements are needed to improve speed, cost and throughput.
- Methods for water soluble vitamins exist but HPLC methods still under development and required further validation.
- MS techniques using LC-MS , MS-MS or isotope dilution show promise but remain complex.



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