LC Solutions for Vitamin Analyses

Fast Analysis of Fat-Soluble Vitamins in Infant Milk Powder by Heart Cutting 2D-LC

Fast Multi-LC-Method for Analysis of High and Low-dosed Water-soluble Vitamins

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Agilent Technologies, Germany, Waldbronn
Fast Analysis of Fat-Soluble Vitamins in Infant Milk Powder by Heart Cutting 2D-LC

Fat-soluble vitamins, including vitamins A, D and E are frequently used as additives. The amounts of Vitamin A and E are relatively large and easy to determine. Amount of Vitamin D is relatively small and much more difficult to determine due to weak UV absorption and serious matrix interference. Therefore sample clean up is the key for Vitamin D determination. Clean up procedure using semi prep normal phase LC is laborious as well as time and organic solvent consuming.

Development of Online 2D-LC (heart cutting) method for the simultaneous determination of Vitamin A, E and D including automated sample clean up.
2D-LC – Heart-cutting

Heart-cutting 2D-LC (LC-LC):

Heart cutting using 2PS/6PT with Enrichment Column
Sample Preparation

According to Chinese National Standard GB5413.9-2010

- Saponification (KOH and ascorbate)
- Extraction (petroleum ether)
- Concentration (rotary evaporator)
- Dilution

- Sample Clean by 1st dimension LC
2-Dimensional LC (Heart-Cutting)

System configuration

Pump → Autosampler → Column 1 → Detector

Pump 2

Injector

Column 2

Detector
10 mm Max-Light cartridge cell

Detector
60 mm Max-Light cartridge cell
# Heart Cutting Parameter

<table>
<thead>
<tr>
<th></th>
<th>1st Dimension</th>
<th>2nd Dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flow rates</strong></td>
<td>1 mL/min</td>
<td>0.6 mL/min</td>
</tr>
<tr>
<td><strong>Mobile phase:</strong></td>
<td>A: H2O, B: MeOH</td>
<td>A: ACN, B: MeOH</td>
</tr>
<tr>
<td></td>
<td>0 min 90 % B</td>
<td>0 min 5 % B</td>
</tr>
<tr>
<td></td>
<td>3 min 100 % B</td>
<td>10 min 5 % B</td>
</tr>
<tr>
<td></td>
<td>10 min 100 % B</td>
<td>10.1 min 100 % B</td>
</tr>
<tr>
<td></td>
<td>10.1 min 90 % B</td>
<td>12 min 100 % B</td>
</tr>
<tr>
<td></td>
<td>14 min 90 % B</td>
<td>12.1 min 5 % B</td>
</tr>
<tr>
<td><strong>Injection volume ALS</strong></td>
<td>10 µL</td>
<td></td>
</tr>
<tr>
<td><strong>Column</strong></td>
<td>Agilent Zorbax RRHT Extend-C18</td>
<td>Agilent Zorbax Eclipse PAH</td>
</tr>
<tr>
<td></td>
<td>4.6 x 50 mm, 1.8 µm</td>
<td>2.1 x 100 mm, 3.5 µm</td>
</tr>
<tr>
<td><strong>Enrichment column</strong></td>
<td>Agilent Zorbax Extend-C18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.6 x 13.5 mm, 5 µm</td>
<td></td>
</tr>
<tr>
<td><strong>Valve Timing</strong></td>
<td>0 to 4.4 min Position 1-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>4.4 to 4.6 min Position 1-6</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.6 to 10 min Position 1-2</td>
<td></td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>35 °C</td>
<td>35 °C</td>
</tr>
<tr>
<td><strong>Detector</strong></td>
<td>10 mm Max-Light flow cell</td>
<td>60 mm Max-Light flow cell</td>
</tr>
<tr>
<td></td>
<td>0 to 3.5 min 325 nm (4 nm, Ref off)</td>
<td>265 nm (4 nm, Ref off)</td>
</tr>
<tr>
<td></td>
<td>3.5 to 14 min 290 nm (4 nm, Ref off)</td>
<td>20 Hz</td>
</tr>
<tr>
<td></td>
<td>20 Hz</td>
<td></td>
</tr>
</tbody>
</table>
Valve Switching for 2D-LC Heart-Cutting

Step 1: Injection
Vit A determination

Step 2: Vit D clean up
Trapping

Step 3: Vit E determination 1st dim.
Vit D determination 2nd dim

0 to 4.4 min
4.4 to 4.6 min
4.6 to 10 min
Separation Vit A, D and E with 2D-LC Heart-cutting
Performance Data

Linearity: Correlation coeff.: 0.99996 (Vit A), 0.99984 (Vit E), 0.99988 (Vit D3)

Reproducibility of RT and Area of infant formula (3 runs)

<table>
<thead>
<tr>
<th></th>
<th>Vitamin A</th>
<th></th>
<th>Vitamin E</th>
<th></th>
<th>Vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention</td>
<td>Peak area</td>
<td>Retention</td>
<td>Peak area</td>
<td>Retention</td>
</tr>
<tr>
<td></td>
<td>time (min)</td>
<td></td>
<td>time (min)</td>
<td></td>
<td>time (min)</td>
</tr>
<tr>
<td>1</td>
<td>2.064</td>
<td>4645.55273</td>
<td>4.951</td>
<td>2301.32178</td>
<td>8.953</td>
</tr>
<tr>
<td>2</td>
<td>2.063</td>
<td>4658.86182</td>
<td>4.961</td>
<td>2301.61694</td>
<td>8.991</td>
</tr>
<tr>
<td>3</td>
<td>2.064</td>
<td>4660.52637</td>
<td>4.965</td>
<td>2301.80396</td>
<td>9.005</td>
</tr>
<tr>
<td>RSD%</td>
<td>0.03</td>
<td>0.18</td>
<td>0.14</td>
<td>0.01</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Limits of detection:

Vitamin D3 (S/N = 3): 0.01 µg/mL
10 µg/kg in milk powder
Summary

- Simultaneous analysis of vitamins A, E, and D2/D3 by heart-cutting 2D-LC
- Highly sensitive and specific for Vitamin D
- Higher productivity and lower cost per sample due to automated sample clean up
Fast Multi-LC-Method for Analysis of High and Low-dosed Water-soluble Vitamins
Water-Soluble Vitamins

Background

• Current regulatory and standard method are based on microbiological (50 years old), spectrophotometric or fluorimetric techniques or HPLC methods (USP, AOAC) often for analysis of only one vitamin at a time.

Development of a Fast Multi-Vitamin Method with LC
Water-soluble vitamins analyzed

Vitamin C

Vitamin B5
Panthotenic Acid

Vitamin B6
Pyridoxine

Vitamin B2
Riboflavine

Vitamin B3
Niacine

Vitamin B1
Thiamine

Vitamin B12
Cyanocobalamin
Spectra of analyzed Vitamins

**Vitamin C**

**Niacinamid B3**

**Vitamin B1**

**Vitamin B6**

**Pantothenic acid B5**

**Vitamin B2**

**Vitamin B12**

Red: 220 nm
Blue: 265 nm
Green: 360 nm
Chromatographic Separation of Water-Soluble Vitamins

**Chromatographic conditions**

**Column**

For method development: ZORBAX Eclipse Plus C18, 50 mm × 2.1 mm, 1.8 μm
For routine testing: ZORBAX Eclipse Plus C18, 150 mm × 4.6 mm, 5 μm

**Mobile phases**

Phase A: Dissolve 1.03 g hexane sulfonic acid and 6.8 g potassium dihydrogen phosphate in 1000 mL water. The pH value should be adjusted to pH = 2.3 with phosphoric acid.

Phase B: Acetonitrile

**Gradient (linear):**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>6</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/B (%/%)</td>
<td>100%/0%</td>
<td>80%/20%</td>
<td>50%/50%</td>
<td>100%/0%</td>
</tr>
</tbody>
</table>

Confidentiality Label
June 21, 2016

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Chromatographic Separation of Water-Soluble Vitamins

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Column
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Gradient (linear):

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>100% A/0% B</th>
<th>80% A/20% B</th>
<th>50% A/50% B</th>
<th>100% A/0% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100% A/0% B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>80% A/20% B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>50% A/50% B</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

More Information:
5990-4379EN
5990-7950EN
## Concentration Levels in Vitamin Tablets

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Injected amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>60 mg/tablet</td>
<td>6000 ng</td>
</tr>
<tr>
<td>Vitamin B3</td>
<td>18 mg/tablet</td>
<td>1800 ng</td>
</tr>
<tr>
<td>Vitamin B5</td>
<td>6 mg/tablet</td>
<td>600 ng</td>
</tr>
<tr>
<td>Vitamin B6,</td>
<td>2 mg/tablet</td>
<td>200 ng</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>1.6 mg/tablet</td>
<td>160 ng</td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>1.4 mg/tablet</td>
<td>140 ng</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>2 µg/tablet</td>
<td>0.2 ng</td>
</tr>
</tbody>
</table>
Linearity, linear range and dynamic range
1200 Series Diode Array Detector, 10 mm flow cell

Detector Signal [AU]

Upper limit 2.0

Detector noise $7 \times 10^{-6}$

Slope = Response Factor (Sensitivity)

Linear range

Dynamic range

$Linear\_range = \frac{Upper\_Limit}{(3 \times Detector\_Noise)} \approx 10^5$
The Challenge of widely different concentration Levels

Analysis on 1260 Infinity DAD with 10mm path length cell

2000 mAU linearity limit

Peak Height = 0.145998 mAU
PtoP Noise = 0.03282 mAU
S/N = 4.4

Vitamin C
Vitamin B3
Vitamin B5
Vitamin B6
Vitamin B1
Vitamin B2
Vitamin B12

Red: 220 nm
Blue: 265 nm
Green: 360 nm

Agilent Technologies
Linearity, linear range and dynamic range
1200 Series Diode Array Detector, 10 mm flow cell

Detector Signal [AU]

Upper limit 2.0

Detector noise 7*10^-6

Slope = Response Factor (Sensitivity)

Linear range

Dynamic range

LOQ (10 x S/N)

Conc (upper limit) linear range

Conc (upper limit) dynamic range

HDR-DAD

HDR-DAD

March 25, 2015
30x Wider Linear Range with HDR-DAD
All sample information in one run

For samples with widely different concentration levels
no time consuming
- reanalysis
- recalibration
- sample preparation
- higher precision/accuracy for labile compounds (Vit. C and B12)*

Comparision Flow Cell Technologies
- The Key for High Dynamic Range

Conventional flow cells:

Lower Light Transmission
Larger flow cell volume
Optical and chromatographic performance depends on path-length
(noise, dispersion or peak broadening)

Max-Light Cartridge Cell

Optofluidic waveguides
(total internal reflection)

High Light Transmission
Small flow cell volume ~ 1/10
Optical and chromatographic performance nearly independent on path-length
(noise, dispersion or peak broadening)
30x Wider Linear Range with HDR-DAD
3.7 mm and 60 mm and Max-Light flow cell

Computing the signals from
60 mm path-length for the low concentration
3.7 mm path-length for the high concentration

Detector Signal
[AU/cm]

Conc [µg/mL]

March 25, 2015
Agilent Confidential
Analysis with 1260 Infinity II HDR-DAD

5400 mAU linearity limit
Typically 6700 mAU

Peak Height = 0.126319 mAU/cm
PtoP Noise = 0.007922 mAU/cm
S/N = 16

Red: 220 nm
Blue: 265 nm
Green: 360 nm
30x Wider Linear Range with HDR-DAD
3.7 mm and 60 mm and Max-Light flow cell

- 30x lower Limit of Detection (LOD) for impurity analysis
  - for more reliable automated peak integration
  - higher area precision

- 10x higher sensitivity
- ~3x higher upper limit
- 30x lower Limit of Detection (LOD)

Detector Signal [AU/cm]
Conc [µg/mL]
Summary

• The High Dynamic Range DAD (HDR-DAD) provides a 30x wider linear dynamic range and a up to 30x higher sensitivity.

• The analysis of all water soluble vitamins incl. Vitamin C (60 mg/tablet) and Vitamin B12 (2 µg/tablet) was possible in one run within short time (< 12 min).

• This delivers higher productivity as well as higher accuracy/precision for labile compounds (Vit. C and Vit. B12)
Next Generation of fast Amino Acid Analysis
Next generation of fast Amino Acid Analysis

Combining the advantages of the latest developments in LC instrumentation and column technology with proven pre-column derivatization chemistry.

- Ready to use reagents and standards
- Infinity 1260 LC
  More performance at lower cost
- Poroshell 120 HPH-C18
  Fast and rugged amino acids separation
- Application Support including on-site training and application set up
Online derivatization with OPA and FMOC

- Fully automated in the Autosampler

Ortho-phthalaldehyde (OPA) for primary AA

9-fluorenylmethyl chloroformate (FMOC) for secondary AA

Automated in ALS

- Increase precision
- Eliminates manual process

Derivatization for:
1. Separation of polar AA on RP-phase
2. Detection by UV and FL
Fast and rugged amino acids separation

- The Poroshell 120 columns provides the speed and resolution like a sub-2 μm column with up to 50% less backpressure
- More forgiving for dirty samples, due to 2 μm frits
- Unique-chemically modified for high pH stability and column life time
- Guard column options that reduce your operating costs by extending the life of Poroshell 120 columns
Next Generation of fast Amino Acid Analysis

Guaranteed Solution

Available June 2016
Thanks for your attention!

Questions?