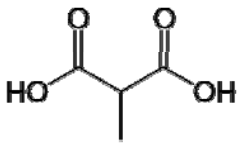
# Methylmalonic Acid (MMA)

## – B12 deficiency biomarker

Methylmalonic acid (MMA) in serum, plasma and urine is used to monitor cobalamin (vitamin B12) deficiency and methylmalonic acidemia. Measurements of metabolites, i.e. methylmalonic acid and homocysteine have proven to be more sensitive in the diagnosis of vitamin B12 deficiency than measurement of serum B12 levels alone. Different methods for MMA in serum, plasma, urine and cerebrospinal fluid have been developed including GC-MS, LC-MS-MS, HPLC, and capillary electrophoresis (CE). The main problems to overcome in method development are related to the low physiological concentrations (100-500 nM) of MMA in human serum, and the fact that MMA is a hydrophilic non-volatile compound. Retention and separation of MMA on reversed phase liquid chromatographic columns is difficult as MMA shows poor retention and the structural isomer succinic acid (SA) may interfere (ion suppression) since serum concentrations of SA are usually considerably higher than MMA. Many labs use methods that require extraction and derivatization steps to yield MMA-derivatives that are compatible with GC-MS-techniques or reversed phase mode based liquid LC-MS/MS methods where derivatives of MMA and SA may be differentiated due to different fragmentation pattern. As a consequence the costs per MMA-test are usually considerably higher than standard immunological assays for B12, and this is possibly the main reason why serum B12 assays still are used in clinical routine.

We have developed a method that combine hydrophilic interaction liquid chromatography (HILIC) with single stage negative ESI-MS. that allows analysis of up to 130 serum samples per 24 hours.* The new method take advantage of the bonded zwitterionic stationary phase ZIC®-HILIC that is an ideal tool for separation of polar compounds, such as MMA in serum/plasma. In this compilation we illustrate how to optimize protein crash for sample preparation of serum samples using experimental design and multivariate data analysis. We also show that this method successfully can be transferred from a LC-MS platform to a LC-MS/MS platform. Both methods have been fully validated, and are currently in use for clinical routine analysis of serum/plasma MMA at several hospitals in Europe and US.




Methylmalonic Acid (MMA)

*" Quantification of Methylmalonic Acid in Human Plasma with Hydrophilic Interaction Liquid Chromatography Separation and MS Detection" Hans-Ake Lakso, Patrik Appelblad, and Jorn Schneede, Clinical Chemistry 54:12, 2028–2035 (2008)

# Methylmalonic Acid (MMA) in Plasma

## SeQuant® ZIC®-HILIC

Recommended column:

| | |
|---|---|
| SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 100 × 2.1 mm | (1.50441.0001) |

Recommended solvents and reagents

| | | |
|---|---|---|
| Acetonitrile: | hypergrade for LC-MS LiChrosolv® | (1.00029) |
| Water: | Water for chromatography LiChrosolv®<br>or freshly purified water from Milli-Q® water purification system | (1.15333) |

Ammonium acetate (HPLC grade) or in-situ prepared buffer from ammonia and acetic acid

| | |
|---|---|
| Ammonia solution 28-30% for analysis EMSURE® ACS, Reag. Ph Eur | (1.05423) |
| Acetic acid 96% for analysis EMSURE® | (1.00062) |
| Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur | (1.00264) |

**Mobile phase preparation**

A 100 mM ammonium acetate buffer was prepared by adjusting the pH with concentrated formic acid. The isocratic mobile phase consisted of acetonitrile and 100 mM ammonium acetate buffer adjusted to pH 4.5 with formic acid (80:20, v/v) and the column cleaning between injections were achieved by increasing the salt concentration by addition of salt from mobile phase B (100 mM ammonium acetate buffer adjusted to pH 4.5 with formic acid).

**Protein precipitation solution:**

The plasma protein precipitation (PPT) solution was prepared by adding 43 µl of the 196 µmol/L D3-MMA stock solution and 250 µl concentrated acetic acid to acetonitrile to yield a total volume of 50 ml. This PPT solution thus contained 0.5 volume-% acetic acid and 0.17 µmol/L D3-MMA.

**HPLC Sample Preparation**

Human EDTA or citrate plasma (200 µL) was added to 800 µL of the protein precipitation (PPT) solution in 2 ml autosampler glass vials. The vials were capped and allowed to stand on an orbital shaker for 5 minutes before centrifugation at 6200 rpm for 10 min at 15 °C and then placed in the autosampler of the LC/MS instrument.

# Methylmalonic Acid (MMA) in Plasma

## SeQuant® ZIC®-HILIC

**Optimization of sample preparation – use of protein precipitation**

The protein precipitation conditions were optimized with regard to ionization efficiency using experimental design and multivariate analysis. Acetic acid was chosen as stimulant for the protein precipitation since an acid was target molecule for the extraction. The effects of changes in the relative volume portions of plasma and PPT solution (1:4, 1:3 and 1:2) plus the concentration of acetic acid (0.3, 0.5 and 0.7 volume-%) were evaluated. Partial least squares (PLS) analysis demonstrated that highest yield could be achieved at a 3:1 ratio between PPT solution and plasma/serum sample, see figure 1. Yet, with a 4:1 ratio, it is possible to increase the column lifetime substantially and thereby attain more economical analysis. Recoveries for MMA range between 90-93% and both within-day and between-day RSD's are $\leq$ 5 %.

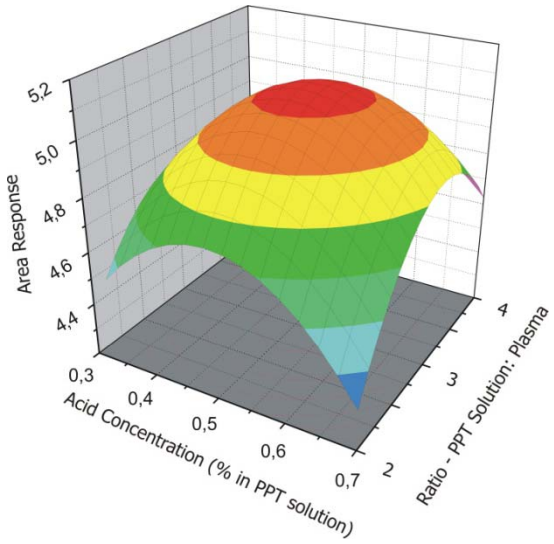




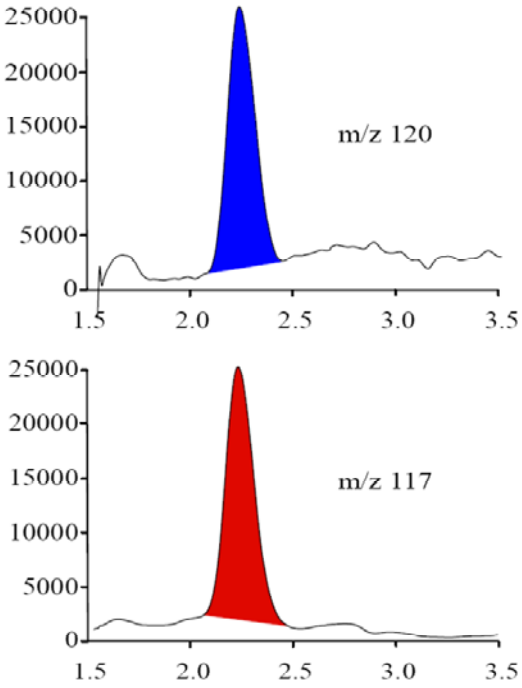
Figure 1. Response surface model, based on partial least square analysis, illustrating the extraction efficiency as a function of acid concentration in the protein precipitation (PPT) solution and the volumetric relationship between plasma and PPT solution.

# Methylmalonic Acid (MMA) in Plasma – LCMS

## SeQuant® ZIC®-HILIC

### Chromatographic Conditions

| | | |
|---|---|---|
| Column: | SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 100 × 2.1 mm | (1.50441.0001) |
| Injection: | 4 µL in mobile phase | |
| Detection: | LC-MS, negative ESI mode, SIM; (m/z 117.2 and 120.2) | |
| Flow Rate: | See table | |
| Mobile Phase: | Acetonitrile and 100 mM ammonium acetate, pH 4.5 (80:20 v/v). Total ionic strength: 20 mM | |
| Temperature: | 30 °C | |
| Sample: | Patient plasma sample treated according to sample preparation protocol. | |
| Pressure Drop: | 90 Bar (1305 psi) | |



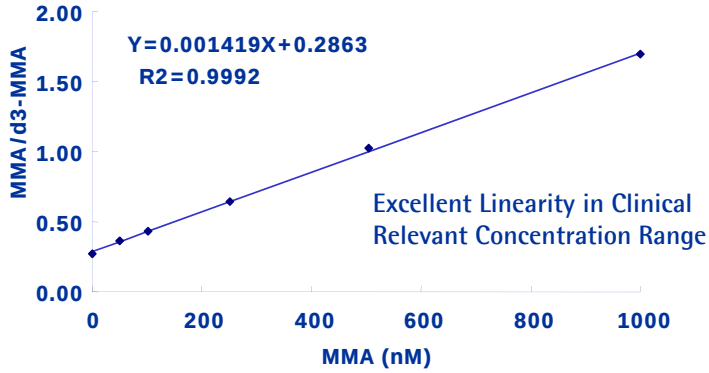

| Time (min) | A (%) | B (%) | Flow Rate (mL/min) | Flow Direction |
|---|---|---|---|---|
| 0.00 | 80 | 20 | 0.40 | Waste |
| 1.50 | 80 | 20 | 0.40 | Ion source |
| 3.00 | 80 | 20 | 0.40 | Ion source |
| 3.01 | 55 | 45 | 0.80 | Waste |
| 5.00 | 55 | 45 | 0.80 | Waste |
| 5.01 | 80 | 20 | 0.80 | Waste |
| 9.00 | 80 | 20 | 0.80 | Waste |
| 9.01 | 80 | 20 | 0.40 | Waste |
| 10.00 | 80 | 20 | 0.40 | Waste |

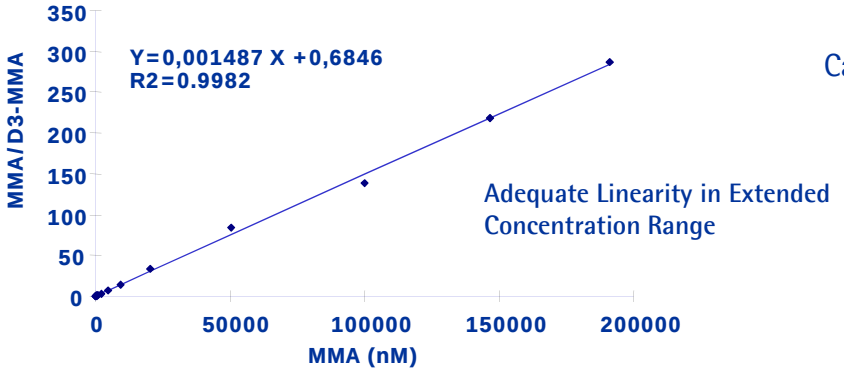
### Chromatographic Data

| No. | Compound | Retention Time (min) | m/z |
|---|---|---|---|
| 1 | Void volume | 0.5 | - |
| 2 | D3-MMA (I.S.) | 2.20 | 120.2 |
| 3 | MMA | 2.22 | 117.2 |

# Methylmalonic Acid (MMA) in Plasma – LCMS

## SeQuant® ZIC®-HILIC




**Quantitation of MMA in plasma LC-MS (negative ESI)**

Limit of Detection = 30 nM
Limit of Quantitation = 90 nM

Can be used for routine analysis

| | Control Endogenous | | | Control E+ 100 nM | | | Control E + 500 nM | | | Control E + 1000 nM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DAY | Conc (mM) | S.D | n | Conc (mM) | S.D | n | Conc (mM) | S.D | n | Conc (mM) | S.D | n |
| 1 | 169.5 | 4.7 | 18 | 256.0 | 5.1 | 18 | 629.1 | 17.6 | 18 | 1104.6 | 23.6 | 18 |
| 2 | 179.8 | 5.7 | 18 | 264.0 | 7.9 | 18 | 643.3 | 16.2 | 18 | 1126.9 | 30.5 | 18 |
| 3 | 178.7 | 4.3 | 18 | 270.3 | 8.3 | 18 | 621.8 | 10.8 | 18 | 1144.7 | 52.9 | 18 |
| 4 | 177.8 | 10.7 | 15 | 277.7 | 10.1 | 14 | 622.8 | 14.8 | 15 | 1083.2 | 22.7 | 15 |
| 5 | 169.5 | 4.7 | 18 | 256.0 | 5.1 | 18 | 629.1 | 17.6 | 18 | 1104.6 | 23.6 | 18 |
| 6 | 179.8 | 3.4 | 15 | 289.7 | 8.7 | 15 | 629.9 | 13.7 | 15 | 1100.7 | 40.1 | 15 |
| Average | 175.8 | | | 269.0 | | | 629.3 | | | 1110.8 | | |
| %RSD | 2.6 | | | 3.1 | | | 1.2 | | | 1.9 | | |

Intra-day variability estimated from patient samples, controls and standards containing 0-1.0 µmol/L, at six different days of analysis over a ten weeks period. The analytical recovery rates were between 90 and 93 %. Total number of experiments for each concentration level, n=34. More than 3000 samples, controls and standards were analysed during development and validation.

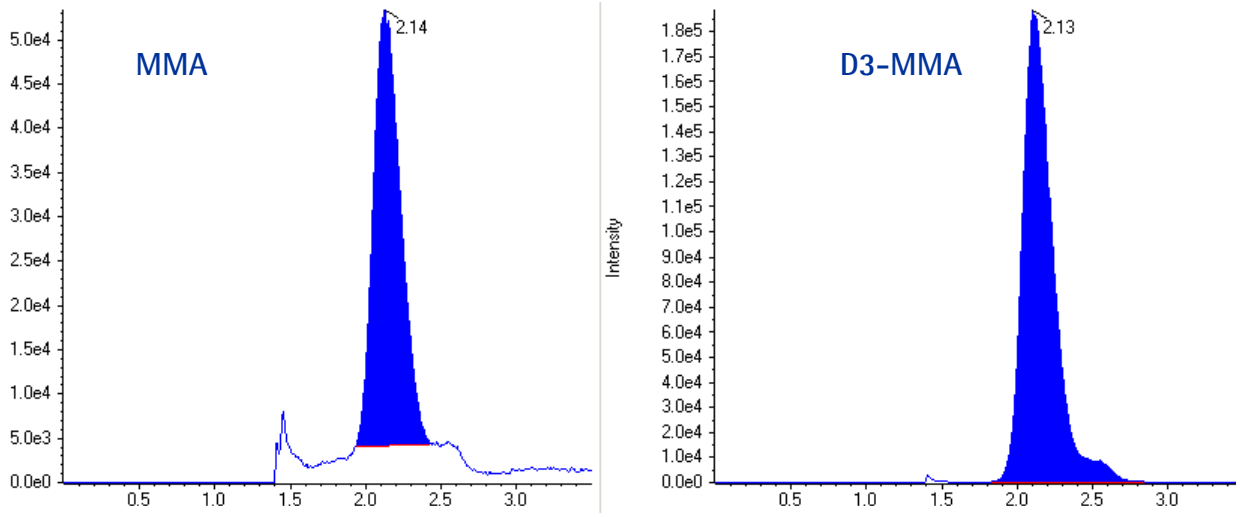
# Methylmalonic Acid (MMA) in Plasma
# Method Transfer from LC-MS to LC-MS/MS

## SeQuant® ZIC®-HILIC

### Chromatographic Conditions

| | | |
|---|---|---|
| Column: | SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 100 × 2.1 mm | (1.50441.0001) |
| Injection: | 7 µL in mobile phase | |
| Detection: | LC-MS/MS, negative ESI mode, MRM; (m/z 117.1→73.0; 117.1→55.1 and 119.9→75.9) | |
| Flow Rate: | See table on page 14. | |
| Mobile Phase: | Acetonitrile and 100 mM ammonium acetate, pH 4.5 (80:20 v/v). Total ionic strength: 20 mM | |
| Temperature: | 40 °C | |
| Sample: | Patient plasma sample treated according to sample preparation protocol. | |
| Pressure Drop: | 90 Bar (1305 psi) | |

**Quantitation of MMA in plasma LC-MS/MS (negative ESI)**
Limit of Detection = 5 nM
Limit of Quantitation = 15 nM




### Chromatographic Data

| No. | Compound | Retention Time (min) | m/z |
|---|---|---|---|
| 1 | Void volume | 0.5 | - |
| 2 | D3-MMA (I.S.) | 2.13 | 119.9→75.9 |
| 3 | MMA | 2.14 | 117.1→73.0 |