Quantification of Methylmalonic Acid in Human Plasma with Hydrophilic Interaction Liquid Chromatography Separation and Mass Spectrometric Detection

Hans-Åke Lakso,1† Patrik Appelblad,2† and Jörn Schneede1*  

BACKGROUND: Measurement of methylmalonic acid (MMA) in serum or plasma is useful for diagnosing cobalamin deficiency. We developed a method for quantifying MMA in plasma based on hydrophilic interaction liquid chromatography (HILIC) and single-stage negative electrospray ionization (ESI) mass spectrometry.

METHODS: We deproteinized plasma samples (200 μL) with 800 μL acidified acetonitrile containing 0.17 μmol/L deuterated MMA (D3-MMA) internal standard, centrifuged the samples, and injected 4 μL of the supernatant into the LC-MS instrument. Separation was achieved within 3 min on a Merck SeQuant ZIC®-HILIC column with a mobile phase consisting of 4 volumes acetonitrile plus 1 volume 100 mmol/L ammonium acetate buffer, pH 4.5, at a flow rate of 400 μL/min. Subsequent column washing and reconditioning contributed to a total run time of 10 min. MMA and D3-MMA were quantified by single-ion monitoring (m/z 117.2 and 120.2, respectively) in negative ESI mode at a drying-gas flow rate of 10 L/min, 300 °C, and a capillary voltage of 3.0 kV.

RESULTS: The estimated limits of MMA quantification and detection were 0.09 μmol/L and 0.03 μmol/L, respectively, in plasma. The assay was linear to 200 μmol/L. Interassay and intraassay CVs were ≤5% at all tested concentrations. Recoveries were 90%–93%.

CONCLUSIONS: This robust assay allows analysis of MMA in human plasma without derivatization. Sample preparation is simple and suitable for automation.

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Methylmalonic acid (MMA)3 in serum/plasma or urine was introduced as a marker of cobalamin (vitamin B12) deficiency more than 20 years ago (1–6). The different methods that have been developed for measuring MMA in serum, plasma, urine, and cerebrospinal fluid include GC-MS, liquid chromatography–tandem mass spectrometry (LC-MS/MS), HPLC, and capillary electrophoresis (7–10). The main obstacles to overcome are related to the low physiological concentrations of MMA in human serum (11) and the fact that MMA is a hydrophilic, nonvolatile compound. Direct analysis with GC-MS is thus not feasible (7). Furthermore, separation of MMA on reversed-phase liquid chromatography columns is difficult because MMA shows poor retention, and the fact that MMA has a low molar absorption coefficient makes ultraviolet detection of low MMA concentrations impossible (9, 12). Lastly, the MMA structural isomer succinic acid (SA) may cause interference. Concentrations of SA in the serum are usually considerably higher than for MMA (11).

Current methods require extraction and derivatization steps to yield MMA derivatives that are compatible with GC-MS techniques (7) or for increased selectivity and sensitivity in HPLC/capillary electrophoresis methods (9, 10). More recently, investigators have introduced LC-MS/MS methods (8, 11, 13, 14), in which MMA and SA derivatives may be distinguished by differences in their fragmentation patterns (8). One of these methods can even measure MMA without derivatization (13). The majority of LC-MS/MS methods still require extensive MMA derivatization with different alcohols or silyl reagents and subsequent evaporation and/or solid-phase extraction steps. Consequently, the costs per MMA result are usually considerably higher.

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3 Nonstandard abbreviations: MMA, methylmalonic acid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; SA, succinic acid; HILIC, hydrophilic interaction liquid chromatography; ESI, electrospray ionization; PPT, protein precipitation; D3-MMA, deuterated MMA (methylmalonic acid–methyl-D3); E, 0.18 μmol/L endogenous MMA.
than for standard immunologic assays of vitamin B_{12}. This fact is possibly the main reason why B_{12} assays are still used as primary diagnostic tests (15).

Recently, the combination of hydrophilic interaction liquid chromatography (HILIC) with mass spectrometry has gained interest (16). In particular, the bonded zwitterionic stationary-phase ZIC®-HILIC chromatography medium (Merck SeQuant) is suitable for separating polar compounds such as MMA. Hydrophilic and weak electrostatic interactions between the stationary phase and MMA allow separation in the presence of high concentrations of organic solvent. A high percentage of organic solvents in the mobile phase is often a prerequisite for more efficient electro-spray ionization (ESI) (17). We describe a method for quantifying MMA in plasma that combines HILIC separation with single-stage negative ESI mass spectrometry. Sample preparation requires no derivatization and consists only of protein precipitation (PPT) and centrifugation.

**Materials and Methods**

**REAGENTS AND CHEMICALS**

MMA was purchased from Sigma–Aldrich, and SA (99.5%) was from Fluka (both are available from Sigma–Aldrich Sweden). Deuterated MMA (methylmalonic acid–methyl-D_{3}, 98%; D_{3}-MMA) was from Cambridge Isotope Laboratories via Larodan Fine Chemicals, and acetonitrile (LiChrosolv, HPLC grade) and acetic acid (p.a.) were purchased from Merck. Ammonium acetate (Baker Analyzed) was obtained from J. T. Baker. Reagent grade water was purified by Milli-Q equipment (Millipore) and had an electrolytic conductivity of <60 nS/cm. Pools of plasma samples with MMA concentrations within the reference range (0.05–0.26 mol/L) were acquired from anonymized volunteer blood donors from the local blood bank (Umeå University Hospital).

**INSTRUMENTATION**

The chromatographic system consisted of an Agilent 1100 LC/MSD instrument equipped with a degasser, quaternary pump, autosampler, thermostated column compartment, ESI interface, and a single-quadrupole mass spectrometer. Data processing was performed with Agilent ChemStation software (version A09.03). Isocratic separations were performed on PEEK ZIC®-HILIC zwitterionic columns [100 mm × 2.1 mm (i.d.)] with 3.5-μm/10-nm particles (Merck SeQuant). The injection volume was 4 μL, and the column compartment was thermostated at 30 °C. The mobile phase consisted of 4 volumes acetonitrile and 1 volume 100 mmol/L ammonium acetate buffer adjusted to pH 4.5 with formic acid, and the flow rate was 400 μL/min. The HPLC flow was directed to waste from 0–1.5 min and after 3.01 min to avoid contamination of the ion source. MMA and D_{3}-MMA were quantified by single-ion monitoring (m/z 117.2 and 120.2, respectively) in the negative ESI mode at a drying-gas flow rate of 10 L/min, 300 °C, and a capillary voltage of 3.0 kV. The MMA peak eluted within 3 min. After separation, the analytical column was washed for 6 min and then subjected to a reconditioning step for 1.0 min (Table 1). A Shimadzu model LC-10ADVp HPLC pump was used for postcolumn infusion experiments.

**STOCK SOLUTIONS, PLASMA CALIBRATORS, AND CONTROLS**

Individual stock solutions of 196 mmol/L D_{3}-MMA and 2.1 mmol/L MMA were prepared in Milli-Q water. The MMA stock solution was further diluted to yield working solutions of 21 μmol/L and 100 μmol/L. These working solutions were used to prepare the plasma calibrators and control samples. The plasma PPT solution was prepared by adding 43 μL of the 196-μmol/L D_{3}-MMA stock solution and 250 μL concentrated acetic acid to acetonitrile to yield a total volume of 50 mL. This PPT solution thus contained 5 mL/L acetic acid and 0.17 μmol/L D_{3}-MMA. We prepared 6 plasma calibrators from a pool of human plasma samples containing 0.18 μmol/L endogenous MMA (E) by spiking with 5 different MMA concentrations (0.05–1.0 μmol/L). The endogenous MMA concentration in the plasma pool was measured by the standard-addition technique. Control samples with low (E + 0.10 μmol/L), medium (E + 0.50 μmol/L), and high (E + 1.0 μmol/L) MMA concentrations were prepared in the same manner.

**SAMPLE PREPARATION**

Human plasma treated with EDTA or citrate (200 μL) was added to 800 μL of the PPT solution in 2-mL glass
autosampler vials. The vials were capped, placed on an orbital shaker for 5 min, centrifuged at 6400g for 10 min at 15 °C, and then placed in the autosampler of the LC-MS instrument.

**EXPERIMENTAL DESIGN AND MULTIVARIATE ANALYSIS**

We used experimental-design and multivariate analysis in MODDE 4.0 software (Umetrics) to optimize PPT conditions with regard to ionization efficiency. We evaluated the effects of changes in the relative proportions of plasma and PPT solution (1 volume plasma to 4, 3, and 2 volumes of PPT solution), as well as the concentration of acetic acid (3, 5, and 7 mL/L) in the PPT solution, according to a predefined model.

**ASSAY CALIBRATION AND PERFORMANCE**

We calibrated the assay during method-validation experiments with the plasma calibrators, control samples, and unknown samples and analyzed each sample in triplicate; however, method-validation experiments showed that singlet analysis yielded virtually the same results as analysis of triplicates. We therefore performed only single measurements in routine analyses of patient samples.

We measured endogenous concentrations in calibrator samples and in the low-, medium-, and high-concentration control samples (6 separate analyses) to estimate intra- and interassay variation, reproducibility, imprecision, and recovery for the method.

**LINEARITY AND LIMIT OF DETECTION**

To evaluate the linear dynamic range of the assay, we spiked plasma samples to MMA concentrations ranging from E to E plus 0.05–200 μmol/L. We prepared calibration curves from the results of analyses of 6 plasma calibrators (E, E + 0.10 μmol/L, E + 0.25 μmol/L, E + 0.50 μmol/L, and E + 1.0 μmol/L) on 6 separate occasions and plotted the peak area ratio of MMA to D₃-MMA against the concentration of added MMA. The slope, the intercept, and the coefficient of linear correlation (r²) were calculated for each calibrator analysis.

The limits of detection and quantification were estimated from the SD of the mean response for one of the calibrator plasma samples containing the low MMA concentration (E, 0.18 μmol/L). The limit of detection was defined as 3 times the SD, and the limit of quantification was defined as 10 times the SD of the interassay results (Table 2).

**RECOVERY**

MMA recoveries were estimated from control samples with known concentrations at 4 different concentrations: E, E + 0.10 μmol/L, E + 0.50 μmol/L, and E + 1.0 μmol/L.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control E</th>
<th>Control E + 0.10 μmol/L</th>
<th>Control E + 0.50 μmol/L</th>
<th>Control E + 1.0 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.170</td>
<td>0.268</td>
<td>0.655</td>
<td>1.101</td>
</tr>
<tr>
<td>2</td>
<td>0.195</td>
<td>0.294</td>
<td>0.633</td>
<td>1.062</td>
</tr>
<tr>
<td>3</td>
<td>0.171</td>
<td>0.267</td>
<td>0.640</td>
<td>1.105</td>
</tr>
<tr>
<td>4</td>
<td>0.182</td>
<td>0.279</td>
<td>0.640</td>
<td>1.116</td>
</tr>
<tr>
<td>5</td>
<td>0.178</td>
<td>0.279</td>
<td>0.640</td>
<td>1.118</td>
</tr>
<tr>
<td>6</td>
<td>0.181</td>
<td>0.286</td>
<td>0.613</td>
<td>1.073</td>
</tr>
<tr>
<td>Mean</td>
<td>0.180</td>
<td>0.279</td>
<td>0.637</td>
<td>1.096</td>
</tr>
<tr>
<td>SD</td>
<td>0.009</td>
<td>0.010</td>
<td>0.014</td>
<td>0.023</td>
</tr>
<tr>
<td>CV, %</td>
<td>5.0</td>
<td>3.7</td>
<td>2.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Interassay CVs of single measurements of the first of the 6 separately prepared samples at 4 different concentrations on 6 different days during a 10-week period.

METHOD COMPARISON

We used the present HILIC mass spectrometry method to reanalyze EDTA-containing plasma samples from 67 anonymized individuals that had been assayed for MMA by a GC-MS method (7) at the University Hospital in Bergen, Norway. The samples were shipped on dry ice and stored at −20 °C until reanalysis. MMA concentrations ranged from 0.10–47.5 μmol/L according to primary analysis at the laboratory in Bergen.

**Results**

**LC-MS ANALYSIS**

We achieved isocratic separation of MMA in plasma on HILIC columns within 3 min (Fig. 1). The total assay time, including column washing and reconditioning, was 10 min. At pH 4.5, SA elutes with the void volume during the first 0.5 min.

We assessed matrix effects and ion suppression in postcolumn infusion experiments (see information on the experimental setup in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue12) by injecting both unspiked and SA-spiked protein-precipitated plasma samples into the column (18). We observed no ion suppression in the time window for mass spectrometric evaluation of the MMA and D₃-MMA peaks.

**OPTIMIZATION OF SAMPLE PREPARATION**

Evaluation of 2 variables, the acetic acid concentration in the PPT solution and the ratio of the plasma and PPT solution volumes, was crucial for achieving a high ionization efficiency. To evaluate potential interaction
effects, we used a partial least-squares analysis on a 2^2 full-factorial design to optimize the sample-preparation procedure. After removing nonsignificant factors, we obtained $R^2$ and $Q^2$ values of 0.95 and 0.83, respectively, in evaluation of the MMA peak area. These results show that the projected model is valid and that the predictive power is adequate. The partial least-squares analysis demonstrated that the highest yield could be achieved with a ratio of 3 volumes PPT solution to 1 volume plasma sample (Fig. 2). We decided to use a PPT solution–plasma sample ratio of 4:1, however, to increase column lifetime.

**RECOVERY AND IMPRECISION**

MMA recoveries were between 90% and 93% (Table 3). Intraassay CVs were $\leq$5% on all 6 days (Table 3); interassay CVs of single measurements (the first of the 6 separately prepared samples at 4 different concentrations on each of the 6 days) were also $\leq$5% (Table 2). The CVs of triplicate analyses were not different from the intraassay CVs for 6 separately prepared samples, indicating that instrument variation, not the sample-preparation step, was the main contributor to the total analytical CV. Therefore, we carry out all routine analyses of patient samples as singlets in daily practice.

**LINEARITY AND LIMIT OF DETECTION**

The limit of quantification (10 × SD) for MMA was 0.09 μmol/L, and the limit of detection (3 × SD) was 0.03 μmol/L (Table 2). To estimate the linear range of the assay, we repeated the analysis of the calibration curves on 6 different occasions over a 10-week period. Least-squares linear regression analysis of peak area ratios vs analyte concentrations indicated that the assay behaves linearly ($y = 0.99x + 172; r^2 = 0.999; n = 6$) from the endogenous MMA concentrations in the plasma pool up to 1.0 μmol/L MMA. Furthermore, experiments with extended calibration curves (up to 200 μmol/L) demonstrated a linear dynamic range of the assay over at least 4 orders of magnitude (data not shown).

**METHOD COMPARISON**

We compared the present LC-MS method with an established GC-MS method (7) by analyzing 67 plasma samples with both methods. Linear regression analysis of the MMA concentrations (in micromoles per liter) obtained with the 2 methods indicated the results to be highly correlated [$y = 0.891x - 0.0014$ μmol/L; $r = 0.99; S_{yx} = 0.025$ (data not shown; $S_{yx}$, standard error of the estimate)]. A Bland–Altman plot (19) of the
measurements illustrates the degree of agreement between the 2 methods (Fig. 3). We observed a minor systematic difference between the 2 methods: Values obtained with the LC-MS method were higher by a mean of 0.03 μmol/L. The difference between the 2 methods is expected to be 0.06 μmol/L in 95% of the cases.

**SAMPLE THROUGHPUT**

Approximately 130 MMA samples can be analyzed as singlets within a 24-h period on a single LC-MS instrument. Although chromatographic separation could be achieved within 3 min, the total run time was 10 min because of the washing and reconditioning steps that constituted a major fraction of the total run time.

**COSTS OF ANALYSIS**

Assuming that a column has a lifetime of 400–500 analyses, that 80 patient samples (in addition to control samples and calibrators) can be analyzed unattended outside the working hours of the laboratory staff (i.e., 5 PM to 8 AM), and that sample preparation and evaluation of chromatographs can be performed within 2 h, we estimate a current cost of US $5–$6/analysis, not including any costs for capital equipment and rental of laboratory and office space. This cost compares with current reagent costs for immunologic cobalamin assays of serum samples (on a “cost-per-test leases” basis) of about US $10–$12.

**Discussion**

The present single-stage mass spectrometric assay allows measurement of MMA in human plasma. Sample preparation requires no derivatization or solid-phase/liquid-liquid extraction steps but only PPT with acidified acetonitrile and centrifugation before injection. MMA is a nonvolatile aliphatic dicarboxylic acid of low molecular mass and thus is highly hydrophilic (20). It may be detected on LC-MS instruments with atmospheric pressure ionization in negative mode (21). Physiological concentrations of MMA in serum/plasma

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### Table 3. Analytical recovery for the assay.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control E&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Control E + 0.10 μmol/L</th>
<th>Control E + 0.50 μmol/L</th>
<th>Control E + 1.0 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMA, μmol/L</td>
<td>CV, %</td>
<td>n</td>
<td>MMA, μmol/L</td>
</tr>
<tr>
<td>1</td>
<td>0.168 (0.005)</td>
<td>3.1</td>
<td>6</td>
<td>0.256 (0.007)</td>
</tr>
<tr>
<td>2</td>
<td>0.181 (0.006)</td>
<td>3.1</td>
<td>6</td>
<td>0.261 (0.009)</td>
</tr>
<tr>
<td>3</td>
<td>0.178 (0.005)</td>
<td>2.9</td>
<td>6</td>
<td>0.268 (0.007)</td>
</tr>
<tr>
<td>4</td>
<td>0.194 (0.009)</td>
<td>5.0</td>
<td>5</td>
<td>0.279 (0.005)</td>
</tr>
<tr>
<td>5</td>
<td>0.178 (0.005)</td>
<td>2.9</td>
<td>6</td>
<td>0.268 (0.007)</td>
</tr>
<tr>
<td>6</td>
<td>0.180 (0.008)</td>
<td>4.4</td>
<td>5</td>
<td>0.288 (0.009)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.180</td>
<td>0.270</td>
<td></td>
<td>0.630</td>
</tr>
<tr>
<td>Recovery, %</td>
<td>NA</td>
<td>90.1</td>
<td></td>
<td>90.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> MMA data are presented as the mean (SD). Intraassay imprecision estimated from control plasma and plasma calibrators containing 0–1.0 μmol/L MMA, assayed on 6 different days over a 10-week period. Total number of experiments for each concentration, n = 34.

<sup>b</sup> E, 0.18 μmol/L, endogenous MMA; NA, not applicable.
are very low, however, and the structural isomer of MMA, SA, is often indistinguishable from MMA (14) with reversed-phase columns (22). Although some investigators have recently succeeded in separating MMA on octadecyl silane columns (13), chromatographic selectivity and detection remain the main challenges to be overcome during method development.

Most previously published mass spectrometry methods include MMA derivatization, either to improve selectivity and/or sensitivity or to yield volatile gas chromatography–compatible derivatives. In the majority of cases, derivatization means esterification of the MMA carboxylic moieties (14), preferably in water-free solvents to avoid hydrolysis (11). Consequently, most assays demand additional extraction and/or evaporation steps that often impede automation of the sample workup (8, 11, 14). In addition, the different MMA derivatization products may show considerable variation in ionization efficiency, which may produce variation in the sensitivity of the different MMA and SA diesters. Thus, the preferred derivatization technique is often a trade-off between sensitivity and specificity (8). Such trade-offs may not be an obstacle with modern tandem mass spectrometry instruments, which normally have superior analytical performance, but it is certainly a problem with single-stage mass spectrometry (23, 24).

Our main efforts during method development were thus related to improving chromatographic selectivity for separating MMA from SA while simultaneously achieving the highest possible ionization efficiency. We discovered that not only is the composition of the separation buffer (i.e., pH, ionic strength, type and proportion of organic modifier) crucial for ionization efficiency in negative ESI, but the PPT conditions are also critical (17, 23). By applying multivariate analysis and systematically adjusting the experimental design (25), we found the optimal proportions of plasma and PPT solution as well as the optimal acid concentration.

Some of the experimental conditions were predetermined. First, MMA has 2 pKₐ values in an aqueous matrix (pKₐ₁ = 2.83; pKₐ₂ = 5.69), and the difference between these pKₐ values is considerably greater than for SA (pKₐ₁ = 4.19; pKₐ₂ = 5.57). From these pKₐ values for the 2 carboxylic groups of MMA and SA, we expected a mobile-phase pH in the interval between pH 3 and pH 5 to be optimal for separation (10, 26). pKₐ values may change in mixtures of aqueous and organic solvents, however, and are difficult to predict (27). Therefore, we started at pH 6.8 and observed 2 separated peaks at baseline with the same m/z ratio of 117, which corresponded to MMA and SA. Gradually lowering the pH of the mobile phase produced progressively shorter retention times for the SA peak while the MMA peak continued to be retained. We finally chose a pH of 4.5 for the separation buffer. At this pH and in the presence of 800 mL/L acetonitrile, SA elutes in the void volume, whereas MMA elutes within 3 min. Second, mass spectrometric detection required the use of volatile acids as buffer components, and the buffer needed to have buffering capacity in the desired pH interval (23). Third, we wanted the concentration of the organic solvent used in the PPT solution to be equal to or greater than that in the mobile phase for the subsequent liquid chromatography separation in order to avoid incompatibilities between the injected material and the mobile phase (28). In contrast to reversed-phase columns, HILIC columns show retention of negatively charged hydrophilic analytes, even in the presence of a high concentration of organic solvents in the mobile phase (16). Lastly, plasma samples contain lipids and phospholipids that could lead to rapid column deterioration (29). Therefore, we wanted a concentration of organic modifier in the separation buffer that was as high as possible, both to prevent undesired lipid/phospholipid adsorption to the columns and to increase ionization efficiency in negative ESI at the same time (17, 23).

Increasing the buffer pH and the acetonitrile concentration in the mobile phase produced stronger MMA retention and consequently longer run times. Optimization of separation conditions was thus a trade-off between these opposing effects of the mobile-phase composition, and we found that combining 800 mL/L acetonitrile with the volatile ammonium acetate buffer (100 mmol/L, pH 4.5) yielded not only stable and short retention times (<3 min) but also a high ionization efficiency.

Column lifetimes exceeded 400–500 injections of plasma samples under the rather harsh experimental conditions we used. The high acetonitrile concentration used for PPT and in the mobile phase may have prevented adsorption of phospholipids to the zwitterionic stationary phase. In addition, at the end of each run, we washed the column at an increased flow rate for 2 min with a decreased acetonitrile concentration to remove potentially additional hydrophilic material that might have been adsorbed on the column. Unfortunately, this rather long (7 min) washing/reconditioning step increased the total run time considerably; however, this limitation of the assay may be overcome by switching between 2 or more analytical columns and performing the washing step offline.

Ion suppression can be a serious problem in mass spectrometry (18), especially with single-stage methods (30). We tested ion suppression in postcolumn infusion experiments [described elsewhere (31–33)]. We did not observe any signs of ion suppression for MMA and D₃-MMA despite the very simple sample prepara-
tion, nor did we detect any interference or ion suppression by SA, not even in plasma samples spiked with high SA concentrations. One possible explanation is that plasma samples were diluted with 4 volumes of PPT solution, which could have influenced the matrix/analyte ratio positively (18). Furthermore, the absence of ion suppression could be due to the selectivity afforded by the HILIC columns. At pH 4.5, the majority of matrix constituents may either be uncharged or show low retention; however, other compounds (e.g., small multivalent cations) may be strongly retained (34) and would probably be eluted during the column-washing step.

In conclusion, we have developed and validated a single-stage mass spectrometric assay for the measurement of MMA. Sample preparation requires no derivatization and consists only of PPT with acidified acetone-nitrite and centrifugation. This method is characterized by comparatively low costs, both for instrumentation and for consumables. Isocratic separation can be achieved on HILIC columns to produce stable and reproducible retention times.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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