4 Mild and selective labelling of malondialdehyde with 2-aminoacridone: assessment of urinary malondialdehyde levels

Based on:
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4.1 Abstract

Malondialdehyde (MDA) has become a well-established biomarker for oxidative stress. The most commonly used way to determine urinary MDA levels is the thiobarbituric acid (TBA) assay, which suffers from several drawbacks. In this manuscript, we describe a novel derivatisation strategy for the highly sensitive and selective fluorescence-based determination of MDA in urinary samples. The methodology is based on the mild labelling of MDA with 2-aminoacridone, which can be carried out in aqueous citrate buffer at 40 °C, yielding a highly fluorescent substance. No further sample preparation than mixing with the necessary chemicals is necessary. The formed MDA derivative can conveniently be separated from the label itself and matrix constituents by gradient LC in less than 5 minutes on acyano-based reversed-phase material. The method was validated with respect to matrix effects, linearity, selectivity and sensitivity (values as low as 1.8 nM for the LOD and 5.8 nM for the LOQ could be achieved). Standard addition quantitation was applied for the determination of MDA in human urine samples. Additionally, the protocol was applied to the measurement of a stability indicating analysis of MDA in urine at different storage conditions.

4.2 Introduction

Malondialdehyde (MDA) is a well-accepted biomarker for oxidative stress\textsuperscript{1}, arising from the peroxidation of polyunsaturated fatty acids\textsuperscript{2,3}. MDA levels have been used to monitor lipid peroxidation, induced by oxidative stress in different studies\textsuperscript{4,5}. MDA can be measured in urinary samples by a diverse set of analytical methods, including: liquid chromatography mass spectrometry (LC-MS/MS)\textsuperscript{6}, gas chromatography mass spectrometry\textsuperscript{7} and derivatisation-based techniques. Of the latter, the 2-thiobarbituric acid (TBA) assay\textsuperscript{8,9} is the most frequently used method.
The MDA levels found in human urine have been reported to be around 200 μmol mol⁻¹ creatinine when measured by the TBA assay⁸,⁹. However, the TBA assay intrinsically is not specific for MDA¹⁰ and therefore is frequently combined with LC separation and fluorescence detection of the formed products.¹¹ Still, the TBA assay including the LC step was reported to overestimate urinary MDA concentrations by almost 10-fold⁸. This might be related to the harsh conditions (100 °C, acidic conditions) needed to yield the coloured reaction product. Furthermore, if one wants a short chromatographic run time to be achieved, an additional liquid–liquid extraction step (LLE) has to be incorporated into the procedure¹¹. The limit of detection (LOD) of the TBA assay is 128 nM¹¹.

Other UV- and fluorescence-based methodologies for the measurement of MDA include the use of 2,4-dinitrophenylhydrazine (DNPH)⁸ or 9-fluorenylmethoxycarbonyl hydrazine (FMOC-hydrazine) ¹². Another reagent, which was especially used in combination with solid phase analytical derivatisation (SPAD), is dansylhydrazine.¹³,¹⁴ From these methods, only the TBA assay, showing the above described drawbacks, and the method employing DNPH⁸ have been described for the UV- or fluorescence-based determination of MDA in urine. The DNPH method requires a rather elaborate protocol for the preparation of the derivatisation solution as well as for the derivatisation reaction itself. Moreover, the method suffers from liquid chromatographic run times of typically more than 30 min per sample and the need of performing standard addition calibration, which in combination with the long run times renders the method quite inefficient for studies with large sample numbers. The limit of detection (LOD) achieved with this method was 54 nM (S/N = 3) for MDA measured in urine.
In this manuscript, we describe a mild and selective derivatisation of MDA with 2-aminoacridone (2-AA) for the determination of MDA in urinary samples. The reaction is based on the finding that MDA smoothly reacts with aromatic amines under mild conditions without the need to add any reducing agents, like NaCNBH$_3$. The reaction of MDA with 2-AA is outlined in Fig. 1.

![Proposed reaction for the labelling of MDA with 2-AA.](image)

**Figure 1**
Proposed reaction for the labelling of MDA with 2-AA.

A mesomerically stabilized Schiff base is formed between MDA and 2-AA. This renders the described protocol selective for the determination of MDA as other aldehydes require a reduction step in order to form stable products. Moreover, the method allows the measurement of free MDA rather than an overall MDA level, because the mild reaction conditions prevent the release of MDA from proteins or other endogenous substances to which MDA may be bound in urine. The direct incorporation of the MDA molecule into the conjugated $\pi$-system of the resulting derivative causes changes in the spectroscopic properties of the derivative compared to the reagent itself. This and the incorporation of a cyano based LC column allowed for short overall run time of less than 12 minutes per sample. Optimization of the labelling protocol, validation of the method, and its application to the determination of free MDA in urine samples are described. In addition, the stability of MDA in urine was studied.
4.3 **Materials and methods**

4.3.1 **Chemicals**

2-Aminoacridone (2-AA, purum, for fluorescence, ≥98%), 1,1,3,3-tetramethoxypropane (TMP, 99%), malondialdehyde (MDA) tetrabutylammonium salt (≥96%), acetic acid (p.a. 99.8%), formic acid (p.a. ≥ 98%), acetaldehyde (≥99%), pentanal (97%), concentrated hydrochloric acid (p.a. 37%), citric acid (reagent grade), dimethylsulfoxide (DMSO, spectrophotometric grade, ≥99.9%), DiscoveryCyano Supelguard cartridges (20 × 2.1 mm i.d., 5 μm), including a cartridge holder were purchased from Sigma Aldrich (Schnelldorf, Germany). Ammonium acetate (100%) and aqueous formaldehyde solution (37%) were from Mallinckrodt Baker (Deventer, The Netherlands). Sodium azide (≥99%) was from Merck (Darmstadt, Germany), and trisodium citrate dihydrate (99.5%) were from Gibco-BRL (Gaithersburg, MD, USA). MilliQ water was obtained from a MilliQ purification system (MilliQ, Amsterdam, The Netherlands). Acetonitrile (ACN, LC-MS grade) and methanol (MeOH, LC-MS grade) were from Biosolve (Valkenswaard, The Netherlands), while 3 kDa regenerated cellulose molecular-weight cut-off spin-filters were from Millipore (Bedford, MA, USA). 4-Hydroxynonenal (≥98%) was from Cayman Chemicals (Ann Arbor, USA). Citrate buffer was made by dissolving sodium citrate in MilliQ water and adjusting the pH by adding a 3 M solution of aqueous citric acid solution.

4.3.2 **Instrumentation and analysis conditions**

4.3.2.1 **LC-UV-FD instrumentation.**

The analytical instrumentation consisted of the following parts: two Shimadzu LC 20AD (’s Hertogenbosch, the Netherlands) pumps, a Shimadzu CTO 10AC column
oven, a Gilson 234 autosampler (Gilson, Villiers le Bel, France), equipped with a 20 µL injection loop, an Agilent 1050 DAD diode array detector (for UV spectra) and/or an Agilent FLD 1100 fluorescence detector (Agilent Technologies, Waldbronn, Germany). A Discovery Cyano Supelguard cartridge 20 × 2.1 mm, 5 µm was employed as separation column. The cartridge was thermostatted to 40 °C and protected by a VICI 1 µm in-line metal frit (Bester, Amstelveen, The Netherlands). Gradient elution was performed at 350 µL min⁻¹ of ammonium acetate buffer/MeOH/ACN (solvent A: 10 mM ammonium acetate, 5 mM sodium azide, pH 5.8 and solvent B: ACN : MeOH : H₂O 49.5 : 49.5 : 1, v/v/v) starting at 2.5% B increasing to 5% B in 2 min, ramping to 15% B at 5 min, followed by an increase to 90% B at 8 min, held for 1 min. For fluorescence measurements, excitation at 345 nm and emission at 500 nm were used.

When the system was operated for high throughput analysis an isocratic flow of 3% eluent B at a flow rate of 0.7 mL min⁻¹ was applied.

4.3.2.2 **Mass spectrometric analysis of the MDA derivative.**

For the mass spectrometric analysis of the formed MDA derivative, 200 µL of a 200 µM solution of MDA was reacted under the below stated optimized conditions, with the same volume of a 200 µM solution of 2-AA. After injection into the analytical system, which in this case did not contain sodium azide in buffer A, the peak eluting at the retention time of labelled MDA was collected manually and infused into a MicroTOFQ mass spectrometer (MS) (Bruker Daltonics, Bremen, Germany). The MS scanned from m/z 100 to 700. Capillary voltage was set to 4.5 kV; nitrogen (99.9990%) was used as nebulizer gas at 1.6 bar and as drying gas at 8.0 L min⁻¹ (200 °C). Argon (99.9995%) was used as collision gas.
4.3.3 Optimized derivatisation reaction

A 5 mM stock solution of 2-AA in ACN (1.05 mg mL\(^{-1}\)) was prepared freshly on a weekly basis. 500 μL of water or urine containing the specified amounts of MDA were mixed with 460 μL of 1 M citrate buffer pH 4.0 (containing 1% DMSO when isocratic high through put analysis was applied) and made up to 1.0 mL by adding 40 μL of the reagent solution, resulting in a final reagent concentration of 200 μM 2-AA. The reaction mixture was allowed to react for 90 min at 40 °C before injection. After the reaction, samples should be analysed within a two hour period. The urine samples used in this study were collected from healthy volunteers.

4.3.4 Optimization experiments

4.3.4.1 pH optimization.

A 10 mM stock solution of MDA was prepared as described earlier.\(^{15}\) Briefly 17 μL of TMP were added to 10 mL of 0.1 M HCl, which was kept at 40 °C for 60 min, this solution was further diluted with water in an appropriate way to obtain the MDA working solutions. To 1000 μL of an aqueous solution, 40 μL of an MDA stock solution (10 μM) were added. 880 μL of either: 0.1 M HCl (pH = 1.2), 0.1 M formic acid (pH = 2.4), 0.1 M acetic acid (pH = 3.6), or 0.5 M citrate buffer (pH = 4.0, 4.5, or 4.7) were added and made up to 2.0 mL by adding 80 μL of the reagent solution. The mixture was immediately injected into the LC system (total delay time about 2 min). Subsequent injections were carried out every 12 min for a total of 2.5 hours. From these data, the kinetics of the derivatisation reactions as well as the overall fluorescence yield under the different pH conditions were determined.
4.3.4.2 Temperature optimization, derivative stability and kinetics investigation in different urine samples.

To 500 μL of a urine sample 20 μL of a MDA stock solution (10 μM) were added. 440 μL of 1 M citrate buffer pH = 4.0 was added and the solutions were kept at 20 °C or 40 °C by using a water-cooled sample rack of the employed auto-injector. The kinetics of the derivatisation was established as described for the pH optimization. To investigate the stability of the formed derivative, the solutions were injected approximately every 12 minutes over a period of 4.5 hours. The same experiment was carried out after spiking four different urine specimens with 200 nM MDA in order to assess differences in kinetics behaviour, when the reaction is carried out in different urine specimens.

4.3.5 Reagent excess.

To assess the influence of the reagent excess, a urine sample spiked with 200 nM MDA was reacted with a final concentration of 2 μM, 20 μM, 200 μM and 400 μM 2-AA, for 90 minutes at 40 °C.

4.3.6 Selectivity.

To investigate the selectivity of the developed protocol, an aqueous sample was spiked with 1 μM formaldehyde, acetaldehyde, pentanal and 4-hydroxynonenal. The column effluent was monitored for additional chromatographic peaks. Additionally, the area of an aqueous sample of 200 nM MDA with and without the aforementioned aldehyde mix was compared. In order to evaluate, whether MDA is released from proteins, or other sources, a urine sample was centrifuged using a regenerated cellulose 3 kDa cut-off filter before derivatisation (500 μL, 60 min, 13
000 × g). The results obtained for this sample were compared to a non-filtered sample treated under the same conditions.

### 4.3.7 Validation

For the generation of calibration lines, the used MDA stock solution (10 mM) was prepared by dissolving 3.14 mg of MDA tetra-butyl ammonium salt in 1.0 mL 0.1 M HCl. All further dilutions of the stock solution were prepared with water. The stock solutions were stored at 4 °C and prepared freshly every week.

### 4.3.8 Repeatability and recovery.

Intra-day repeatability was determined from 5 urine samples, spiked with 100 nM MDA, analysed as described. For recovery determination the principle described in ref. 8 was adopted. A pooled urine sample (3 male donors) was divided into two aliquots and one aliquot was spiked with 200 nM MDA. Both aliquots were analysed in triplicate by standard addition quantification, spiking the samples with 0, 50, 100, 200 and 400 nM MDA, referring to the final solution.

### 4.3.9 Matrix effect, linearity and calibration.

To assess possible matrix effects and to determine linearity, calibration curves (blank, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, 250 nM and 500 nM MDA, referring to the final solution) were prepared in aqueous solution and in 4 different urine specimens (3 male, 1 female). The average slopes of the generated regression lines were compared by ANCOVA analysis (Graph Pad Prism 5).
4.3.10 **LOD and LOQ.**

LOD and LOQ were estimated from a standard addition calibration line (blank, 50 nM, 100 nM, 200 nM and 400 nM, referring to the final solution) measured in triplicate, by employing the following formulas to the resulting averaged calibration line: $3.3 \frac{s_{yx}}{b}$ and $10 \frac{s_{yx}}{b}$, with $s_{yx}$ being the standard deviation of the residuals and $b$ the slope of the calibration line.\(^\text{18}\)

4.3.11 **Stability indicating MDA analysis**

The MDA stock solution described in Section 2.5 was properly diluted with water, or urine, to reach a final concentration of 400 nM (spike in the case of urine). These solutions were subdivided into different aliquots and stored under different conditions: in the dark at room temperature (RT), in a refrigerator (4 °C), and in a freezer (−20 °C). Every day, over a period of 5 days, two aliquots of each sample and each storage condition were analysed with the optimized protocol.

4.3.12 **Standard addition procedure for the measurement of MDA in isocratic high-throughput mode**

In order to determine urinary MDA levels, samples are spiked with 0, 50, 100, 200 and 400 nM MDA (aqueous stock solution). The so prepared samples are derivatised according to Section 2.3 and analysed as described under Section 2.2.1.

4.4 **Results and discussion**

The described labelling protocol was optimized with respect to the following parameters: pH, temperature, reagent excess and selectivity. Subsequently, the complete protocol was fully validated for the determination of MDA in human urine. As matrix effects were observed, standard addition calibration had to be applied.
Linearity was proven in a range from 10–500 nM, in both urine and water. The found LOD (defined as 3.3 s_{y|x}/b) for MDA was 1.8 nM, being more than 20 times lower than described for the previously reported methods. The selectivity of the developed method was assessed by comparing ultrafiltrated (3 kDa cut off) and non-ultrafiltrated human urine samples, thereby addressing the possible release of MDA from higher molecular weight species during the labelling process. Moreover, the method was applied to study the stability of MDA under different storage conditions, as this is crucial especially in a clinical environment to guarantee meaningful data.

4.4.1 Spectroscopic (spectrometric) data and selection of excitation and emission wavelengths

A normalized comparison of the UV absorbance, as well as the emission spectra (excitation at 345 nm) obtained for 2-AA and labelled MDA can be found in Fig. 2. It can clearly be seen that the two UV spectra show significant differences, which allowed for a rather selective excitation of the labelled MDA derivative, yielding a strong fluorescence maximum at 485 nm. As urine is known to have a strong intrinsic fluorescence background, we investigated the background at different combinations of excitation and emission wavelengths. The lowest interference from additional signals was found at 345 nm and 500 nm for excitation and emission, respectively (data not shown). Standard chromatograms obtained from urine-samples analysed under the optimized conditions can be found in Fig. 3.
Figure 2
Normalized UV absorbance and emission spectra, UV spectra: 2-AA (200 μM) (blue) and MDA (100 μM) after labelling with 2-AA (red), upper right corner emission spectra of 2-AA (blue) and labelled MDA (red), excitation wavelength 345 nm.

Figure 3
Standard chromatograms, obtained from a labelled urine sample (blank—black line) spiked with 50 (red), 200 (blue) and 400 nM (green) MDA.
High resolution MS analysis confirmed the identity of a single-labelled MDA derivative, showing a measured accurate $m/z$ value of 265.0966 [$\text{M+H}^+$], while the calculated $m/z$ value for $\text{C}_{16}\text{H}_{13}\text{N}_2\text{O}_2$ is 265.0972. The main fragment observed was $m/z$ 247.0863 ($\text{C}_{16}\text{H}_{11}\text{N}_2\text{O}$), resulting from a loss of water.

### 4.4.2 Optimization results

#### 4.4.2.1 pH.

The influence of the pH of the reaction medium on the reaction kinetics is shown in Fig. 4. The highest signal intensity and fastest reaction speed were achieved by carrying out the reaction with the addition of 0.5 M citric acid buffer pH 4.0, while 0.1 M HCl as a reaction additive only gave a minor reaction yield. An additional advantage of the use of a citric acid buffer was its ability to reliably adjust the pH of diverse urinary samples.

#### 4.4.2.2 Temperature optimization, stability and kinetics.

In order to assess the effect of reaction temperature and to define optimum reaction times, the kinetics determination was carried out at different temperatures and in different urine specimens (Fig. 5). It was found that the reaction kinetics can be affected by differences in the employed urine specimen. Therefore, it is necessary to react the samples with 2-AA for at least 90 minutes, in order to compensate for such matrix effects. Furthermore, it was found that maximum yield of the reaction being performed at 40 °C was slightly higher and that maximum signals are reached faster than at 20 °C. As the formed derivative shows a slow decrease over time, a sample should be measured within 2 hours after being reacted (40 °C).
Figure 4
Kinetic curves of the reaction between MDA and 2-AA at different pH values. All employed citrate buffers were of 0.5 M concentration. Area refers to the measured area of the labelled MDA peak at the different conditions; red triangles (▼) citrate buffer pH 4.0; green triangles (▲) acetic acid 0.1 M; black squares (■) citrate buffer pH 4.7; orange diamonds (◆) citrate buffer pH 4.5; green squares (■) formic acid 0.1 M; blue circles (●) HCl 0.1 M.
4.4.2.3 Reagent excess.

The reaction between MDA and 2-AA only takes place when a high excess (>100 times) of 2-AA is being used. Although a final concentration of 400 μM label gave a slightly higher peak height than a concentration of 200 μM, interferences eluting very close to the analyte peak started to cause problems with peak integration and selectivity, thereby compromising the achieved $S/N$ ratio. Therefore, a final concentration of 200 μM 2-AA was chosen for all further experiments.

**Figure 5**
Comparison of reaction kinetics in different urine specimens and at different temperatures. The two dotted lines show the end of the reaction time at 90 min and the measurement window of 2 hours. Black circles (●) urine sample 1, reacted at 40 °C; green squares (■) urine sample 2, 40 °C, repetition 1; red triangles (▲) urine sample 2, 40 °C, repetition 2; blue triangles (▼), urine sample 2, 20 °C; black diamonds (◆) urine sample 3, 40 °C; orange squares (■) urine sample 4, 40 °C.
4.4.2.4 Selectivity.

The addition of 1 μM each of formaldehyde, acetaldehyde, pentanal and 4-hydroxynonenal did neither cause additional peaks in the recorded chromatogram after derivatisation, nor influence the peak areas obtained for a 200 nM aqueous sample of MDA; peak areas measured with and without aldehyde mix do not differ significantly (Student’s t-test, \( p < 0.05, n = 3 \)).

Urine was reacted with and without previous filtration through a 3 kDa molecular-weight cut-off filter (500 μL, 60 min, 13 000 \( \times g \)) (\( n = 4 \)). The obtained peak areas from the two datasets were compared by a paired two-tailed Student’s t-test (\( p < 0.05, n = 3 \)) and proved not to be significantly different. This proves for the fact that under the developed protocol no MDA was released from >3 kDa molecular species present in the urine.

4.4.3 Validation and assessment of urinary MDA levels

4.4.3.1 Matrix effects and assessment of MDA levels in human urine

The relative matrix effects observed between different urine specimens rendered an external standard calibration impossible. In order to address matrix effects in more detail, we compared the slopes of 4 calibration lines, generated in different urine specimens (10–500 nM, 3 male, 1 female). As expected, the slopes of the generated calibration lines showed significant differences as proven by ANCOVA analysis (Graphpad Prism 5). When comparing the slopes of the urinary calibration lines to an aqueous calibration line a decrease in the obtained slope values of approximately 20% was found (Table 1). Hence, standard addition has to be applied for quantification of MDA from urinary samples, in order to compensate for matrix effects between different urine specimens. The standard addition data for the
assessments of linearity were also used to quantify the free MDA concentrations in the used urine samples. The native MDA concentrations found in the tested urine samples ranged from 210 to 310 nM (not corrected for creatinine levels), as determined by standard addition quantitation.

Table 1
Overview of validation parameters, all data were generated in urine as matrix, RSD relative standard deviation.

<table>
<thead>
<tr>
<th>Linearity $R^2$ ($n = 4$)</th>
<th>Intra-day repeatability ($n = 5$)</th>
<th>LOD</th>
<th>LOQ</th>
<th>Recovery ($n= 3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9979–0.9994</td>
<td>2.8% RSD</td>
<td>1.8 nM</td>
<td>5.8 nM</td>
<td>101.4 ± 3.9%</td>
</tr>
</tbody>
</table>

*Data generated in different urine specimens at different days.*

4.4.4 Linearity, repeatability, recovery, LOD and LOQ

Linearity was determined in a range from 10 nM to 500 nM in aqueous as well as urinary samples, resulting in overall acceptable $R^2$ values. Intra-day repeatability showed a good relative standard deviation (RSD) of 2.8%. As shown in Section 5, MDA is significantly degrading over time, which renders the determination of an inter-day repeatability not useful as either matrix effects would dominate (different urine samples) or the data would be affected by a significant MDA degradation. LOD and LOQ values were estimated from a standard addition calibration line measured in triplicate. A LOD as low as 1.8 nM and a LOQ as low as 5.8 nM could be achieved. It has to be noted that LOD and LOQ can be affected by matrix effects and hence might significantly differ in different urine samples. The estimated LOD was more than a factor of twenty lower than previously published results (LOD = 54 nM, $S/N = 3$, DNPH) for the measurement of MDA by DNPH labelling in urine, based on standard addition⁸. When comparing the here described method to a triple–quadrupole based LC-MS/MS methodology⁶, the LOD values are in the same order of magnitude. The
LOD values in LC-MS/MS, estimated from blank measurement, were about 1.2 nM\(^6\). An overview of the obtained validation parameters is given in Table 1. Recovery was determined in triplicate according to ref. 8 from a pooled urine sample and was found to be 101.4 ± 3.9%.

### 4.4.5 Stability study of MDA

The stability of MDA in urine is a very critical factor, especially when bigger cohorts of samples have to be analysed. Therefore, we performed a stability indicating analysis for MDA in aqueous and urinary samples at different storage conditions over a period of 5 days. As can be seen in Fig. 6, MDA can be stored without a significant decrease for at least 48 hours in aqueous samples at room temperature, or 4 °C. Surprisingly, storage at -20 °C, hence involving freezing and thawing, strongly affected the MDA concentration in both aqueous and urinary samples. In the case of urine, it is obvious that urinary samples should be stored at 4 °C until analysis. However, still a 10% degradation of the MDA concentration occurs during a 24 h storage period. It can be concluded that preferably immediate sample analysis should take place for the determination of urinary MDA levels. If this is not possible, storage at 4 °C is advised, still demanding analysis within 24 hours after collection. Otherwise, one will underestimate real MDA concentrations. These results are in good agreement with the earlier published observation that MDA levels in urinary samples significantly decrease when being frozen (50% reduction within 3 weeks)\(^8\). Moreover, other authors report the highest stability in an unfrozen state, at low temperatures (0 °C), and also recommended analysis of urinary samples within 24 hours\(^8\).
Figure 6
Stability indicating analysis of MDA under different storage conditions in urine and water. All measurements were carried out in duplicate, starting values were determined in triplicate. Yellow line squares (■) water at room temperature (RT), green line triangles (▲) water at 4 °C, black line triangles (▼) water at −20 °C, black line circle (●) urine at RT, blue line star (*) urine at 4 °C, red line squares (■) urine at −20 °C.
4.4.6 Analysis in high throughput mode (isocratic elution)

In order to speed up the analysis and initially test the capabilities of the system to function in a high throughput analysis mode, we chose for isocratic elution at a flow rate of 0.7 mL min\(^{-1}\). This resulted in an overall analysis time of 4 minutes, with a retention time of the labelled MDA of 2.7 min. It has to be noted that a loss in sensitivity by a factor of approximately 5 was obtained under these conditions. But as standard addition calibration has to be applied, this is not necessarily a drawback for the determination of free MDA in urine under isocratic conditions as MDA levels due to the addition of the analyte are usually high enough for their accurate detection, especially as the sensitivity of the developed method is very high.

Preliminary tests showed that, after the subsequent injection of 30 urine samples, no deterioration of the resulting chromatograms, no shift in retention times and no significant trend within the resulting areas of the labelled MDA peak were found. Furthermore, we analysed a standard addition calibration line of a urine sample in triplicate, injected in duplicate (=30 injections) directly followed by the injection of a blank sample. No carry-over was detected. Two chromatograms resulting from the injection of an aqueous sample and a urine sample, spiked with MDA can be found in Fig. 7.
Figure 7
Comparison between an aqueous sample (black) and a urine sample (orange), spiked with 200 nM MDA, analysed in high throughput mode. The inset in the upper right corner shows the standard addition calibration of a human urine sample fortified with 0, 50, 100, 200 and 400 nM MDA. The endogenous MDA concentration in the sample was determined to be 45 nM.

4.5 Conclusion and perspectives

A method allowing the highly sensitive and selective determination of the oxidative stress biomarker MDA in urinary samples has been developed. The presented method is based on the mild and selective labelling of MDA with 2-AA, not requiring a reducing agent, as a mesomerically stabilized Schiff base is formed. Clear advantages of the presented method in comparison to earlier published methods for the fluorescence-based determination of MDA in urinary samples are: the very simple and mild protocol, not requiring more than two pipetting steps; the very fast
separation of interfering matrix substances in less than 5 min; the very simple LC separation system, not demanding more than a precolumn cartridge and finally the higher sensitivity, showing a LOD as low as 1.8 nM. The method was fully validated, giving overall acceptable results. If free and total MDA levels are to be measured, hydrolysis as usually carried out when GC-MS detection after derivatisation with phenylhydrazine is employed should be applied before making use of the here described protocol\textsuperscript{20}.

Initial results and the following facts make the method ideal for its further development and possible application in the high throughput analysis of urinary MDA levels: (a) the very simple derivatisation protocol which can easily be automated, and (b) the very simple and fast chromatography which can even be enhanced and accelerated by the use of cartridges packed with sub-2 μm material. Furthermore, the presented selective labelling with the obtained changes in the UV absorbance spectra might even allow the development of a plate-reader assay for the high-throughput screening of MDA in patient samples. Moreover, the described labelling might be applicable to the visualization of MDA in living systems under certain circumstances.

4.6 Acknowledgements

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4.7 References
