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The rapid and direct determination of ATP-ase activity by ion exchange chromatography and the application to the activity of heat shock protein-90

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Abstract

Adenosine nucleotides are involved as substrates or co-factors in several biochemical reactions, catalyzed by enzymes, which modulate energy production, signal transduction and cell proliferation. We here report the development and optimization of an ion exchange liquid chromatography (LC) method for the determination of ATP, ADP and AMP. This method is specifically aimed at the determination of the ATP-ase activity of human heat shock protein 90 (Hsp90), a molecular chaperone that has emerged as target enzyme in cancer therapy. Separation of the three nucleotides was achieved in a 15-min run by using a disk shaped monolithic ethylene diamine stationary phase of small dimensions (2×6 mm i.d.), under a three-solvent gradient elution mode and UV detection at 256 nm. The described direct LC method resulted highly specific as a consequence of the baseline separation of the three adenosine nucleotides and could be applied to the determination of the enzymatic activity of ADP/ATP generating or consuming enzymes (such as kinases). Furthermore, comparison of the LOD and LOQ values of the LC method with those obtained with the malachite green assay, which is one of the most used indirect screening methodologies for ATP-ase activity, showed that the LC method has a similar range of application without presenting the drawbacks related to contamination by inorganic phosphate ions and glycerol, which are present in Hsp90 commercial samples.

Keywords

Heat shock protein 90 activity; ion-exchange liquid chromatography; UV detection; ADP/ATP/AMP determination; gradient mode elution

1. Introduction

ATP is fundamental for a broad range of biochemical reactions involving ATP-dependent enzymes. ATP is used as a substrate or co-factor by several key proteins involved in signal transduction pathways such as kinases and adenylate cyclases, in the regulation of protein

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folding such as heat shock proteins and in the modulation of transcription and DNA repair such as ATP-dependent chromatin remodeling factors and ATP-dependent DNA ligases.

The evaluation of ATP consumption by these enzymes is an approach to the study of the effect of endogenous and exogenous molecules on enzymatic activity and can be used in drug discovery for the screening of specific ligands.

Among ATP hydrolyzing enzymes, human heat shock protein 90 (Hsp90) has emerged as target in cancer drug discovery [1, 2]. Hsp90 is part of a family of molecular chaperones that play a crucial role in the normal folding, intracellular disposition, and proteolytic turnover of a vast array of factors involved in cell regulation. Hsp90s are overexpressed in cancer cells [3] and the increase of their expression and activity has been linked with the protection of oncoproteins from physiological clearance. The Hsp90 protein folding activity is ATP-dependent and ATP hydrolysis is a key aspect in the Hsp90 chaperone function. [3] Consequently, the inhibition of ATP hydrolysis is a validated avenue for the development of new anti-cancer therapies, whose efficacy has been already proved both *in vitro* and *in vivo*. [2, 4]

A variety of assays have been proposed to monitor the ATP hydrolyzing activity of ATP-dependent enzymes including the determination of ADP formation [5], ATP consumption [6] or Pi release [7] by coupled enzymes, the measurement of ^{32}P generated from [g- ^{32}P]ATP hydrolysis [8] and the determination of orthophosphate ions by colorimetric reactions [9–11]. An alternative approach utilizes the determination of ligand binding affinities using the displacement of a fluorescently labeled molecule, which binds specifically to the ATP-binding site [12].

In the specific case of Hsp90, only a few high throughput (HT) screening assays have been reported and most of these assays have been based upon the detection of inorganic phosphate ions (Pi), generated by the enzymatic hydrolysis of ATP. In these assays, the determination of Pi was achieved either through the formation of a phosphomolybdate complex and subsequent reaction with malachite green (MG) reagent [13] or by a coupled enzymatic assay in which the generated Pi triggers a three enzyme cascade reaction, which finally leads to the formation of the colored and fluorescent product resorufin. [14, 15] These Pi-based assays are complex and indirect, and are generally complicated by problems related to a high background signal caused by contamination with non-enzymatically produced Pi and other interfering components of the reaction mixture and/or by non-enzymatic hydrolysis of the substrate in the assay conditions. Moreover, in coupled enzymatic assays, further evaluation is required for any active inhibitors to exclude cross-interaction or interference with the activity of the secondary (or tertiary) enzyme.

One alternative to the determination of enzymatically produced Pi, is the measurement of adenosine diphosphate (ADP), the initial product of ATP hydrolysis. Although this approach may offer the general advantage of being insensitive to background levels of orthophosphate or other contaminants, to our knowledge, only one HT Hsp90's activity assay based upon the detection of ADP formation has been recently published [16]. This method was an immunoassay based on the displacement of labeled ADP molecules from an anti-ADP monoclonal antibody by the enzymatically generated ADP. [16] However ADP vs ATP selectivity of the anti-ADP antibodies is only around two orders of magnitude, which needs to be taken into account when slow ATP-ases are investigated in the presence of a high concentration of substrate as is the case with human Hsp90. In this case, the low percentage conversion of substrate into product, especially in the presence of an inhibitor, might lead to a cross reaction between the antibody and the tracer system that needs to be correctly accounted.

Taking into account all these issues, we here report the development and optimization of an ion exchange high performance LC method for the direct separation, identification and determination of the adenosine nucleotides using a small disk shaped monolithic ethylene diamine column (2×6 mm i.d.). To our knowledge, this is the first time that human Hsp90α's ATPase activity has been successfully monitored by a LC method using a mini-monolithic column. The reverse phase HPLC separation of ATP, ADP and AMP has been previously reported for non enzyme-related purposes [17, 18] and for the evaluation of the sarcoplasmic reticulum Ca²⁺-ATPase activity [19]. In the latter case, a 150 mm long C18 column and an ion-pair reverse-phase chromatographic approach were used for nucleotides separation [19]. In the present study, the ion exchange LC method was optimized for the determination of the off-line activity of human Hsp90α, which is a very slow ATP hydrolyzing enzyme. [20] Although being the effective target in Hsp90 based cancer therapies, the low turnover number has strongly limited its use in screening campaigns. Therefore, on the basis of these limitations, chromatographic conditions were optimized to achieve a suitable separation of substrate and product even in the presence of a large excess of substrate and low levels of product, using enzyme-friendly conditions, which should also allow the in-line coupling of the analytical column with a Hsp90-IMER. The feasibility of developing such an IMER was previously demonstrated.[21]

To evaluate advantages and disadvantages of the LC method over the available assays, this method was compared with the malachite green (MG) colorimetric assay [13], one of the most frequently applied methods for the determination of ATP-ase's activity. However, since most of published works on Hsp90 with MG have been performed with other isoforms of Hsp90, an initial optimization of the MG assay for the human alpha isoform was required and performed.

2. Experimental part

2.1 Materials

Human recombinant Hsp90α (heat shock protein 90-α) was purchased from three different suppliers: Stressgen (2.2 mg/mL; purity >80%; DPBS containing glycerol 10%), StressMarq (0.5 mg/mL; purity >95%; in glycerol 10%, NaCl 0.3 M, Tris-HCl pH 7.5 50 mM, beta-mercaptoethanol 5 mM) (Canada) and Abcam (0.5 mg/mL; purity >80%; in glycerol 10%, NaCl 0.3 M, Tris-HCl pH 7.5 50 mM, beta-mercaptoethanol 5 mM) (UK). Malachite Green dye, poly(vinyl alcohol), magnesium chloride hexahydrate and HEPES [4-(2-hydroxyethyl)-1-piperazineethansulfonic acid], adenosine 5'-monophosphate (AMP) and adenosine 5'-triphosphate (ATP) were from Sigma Aldrich (Italy). Adenosine 5'-diphosphate (ADP), ammonium molybdate tetrahydrate and potassium chlorate were from Fluka (Italy). Tris(hydroxymethyl)aminomethane (Tris), potassium chloride, sodium chloride, glycerol, potassium dihydrogen phosphate and citric acid were purchased from Carlo Erba (Italy). Dibasic sodium phosphate and sodium hydroxide were from Riedel-de Haën (Germany).

2.2 Colorimetric determination of ATPase activity

The colorimetric determination of ATPase activity was performed by a Malachite Green assay using the procedure previously described by Rowlands *et al.* [13], slightly modified. In brief, the malachite green (MG) reagent was freshly prepared every day and consisted of a mixture of MG solution (0.00812%, w/v in bidistilled water), polyvinyl alcohol aqueous solution (2.32%, w/v), ammonium molybdate (5.72%, w/v) in HCl 6M, and bidistilled water in a 2:1:1:2 ratio. The MG reagent was left standing for 2h to get a stable green/golden solution, which was filtered through 0.45 μm regenerated nitrocellulose syringe filters (Phenex, Germany) before use.

Optimized assay solution consisted of 3 μg (33.3 pmol) of Hsp90 solution (either dialyzed or not dialyzed), ATP (1mM) and Tris-HCl (pH 7.4; 100 mM) buffer containing KCl (20 mM) and magnesium chloride (6 mM). Assay volume was 170 μL . Spontaneous hydrolysis of ATP and interference from enzyme solution and buffer were accounted by preparing the corresponding blanks. Activity assays were carried out at 37°C. Before the spectrophotometric determination, assay solutions were diluted to 800 μL by addition of 417.5 μL of assay buffer, 200 μL of MG reagent and 12.5 μL of sodium citrate aqueous solution (34%, w/v) to limit ATP hydrolysis under acidic conditions [13]. Diluted solutions were left standing at room temperature for 30 min to get the stable formation of the colored species. Finally absorbance at 638 nm and 799 nm were determined and the Abs_{corr} value was determined following formula:

$$\text{Abs}_{\text{corr}} = \text{Abs}_{638 \text{ nm}} - \text{Abs}_{799 \text{ nm}}.$$

The concentration of Pi ions produced by Hsp90 α was determined by a standard curve prepared by incubation of increasing Pi concentrations (range 40–480 μM) in the same assay conditions but in the absence of Hsp90 α . The enzyme activity expressed as pmoles of substrate hydrolyzed per time unit (min) was calculated as follow:

$$\text{pmol/min} = \frac{[\text{Pi}] (\mu\text{M})}{t_{\text{incub}} (\text{min})} \times V_{\text{assay}} (\mu\text{L})$$

2.3 Enzyme pretreatment

A Hsp90 α sample (91 μL , 2.2 $\mu\text{g}/\mu\text{L}$) containing 200 μg of enzyme was dialyzed by Slide-A-Lyzer Mini Dialysis Units with 10,000 MWCO (Thermo Scientific, Illinois, USA) against 4×500 mL of HEPES buffer (pH 7.4; 50 mM) at 4 °C for 4 h under gentle stirring. Hsp90 concentration after dialysis was determined by comparison of circular dichroism (CD) spectra of the Hsp90 solution before and after dialysis.

Alternatively 60 μL of enzyme were loaded into a Microcon YM–50 tube with 50,000 MWCO (Millipore, Italy), and treated as suggested by the manufacturer protocol for buffer exchange. In brief, 60 μL of HEPES buffer (pH 7.4; 50 mM) were added to the Hsp90 solution, the tube was spinned at 12,879 x g (Centric 150, TEHTNICA ELEZNIKI d.o.o., Slovenia) for 10 min and the protein sample was recovered from the sample reservoir by 60 μL of HEPES buffer (pH 7.4; 50 mM).

2.4 Circular dichroism studies

CD measurements were carried out with a Jasco J-810 Spectropolarimeter. Direct CD spectra were recorded in the spectral range 195–260 nm using a 0.5 mm pathlength cell ($V = 150 \mu\text{L}$, Hellma Italia, Italy) at room temperature. Spectra were recorded at 0.2 nm intervals, time constant 2 sec, scanning speed 10 nm/min and spectral bandwidth 1 nm. Baselines were performed with corresponding Tris HCl buffers (100 mM) without enzyme.

2.5 Chromatographic conditions for adenosine nucleotides separation and determination

Chromatographic analyses were carried out with a Hewlett Packard HP-Ti-1050-Series HPLC system equipped with a 20 μL loop. Data were acquired and processed at 256 nm with HP-CORE ChemStation. Separations were performed with a monolithic EDA CIM® mini-disk (2 x 6 mm i.d.) column (BIA Separations, d.o.o., Slovenia).

HPLC separation of adenosine nucleotides was achieved using a three-solvent gradient elution. Specifically buffer A was Tris-HCl buffer (pH 7.4; 100 mM); buffer B was Tris-HCl buffer (pH 7.4; 100 mM) containing KClO_3 (150 mM); buffer C was Tris-HCl buffer (pH 8.5; 100 mM) containing KClO_3 (200 mM). The optimized elution program was as follows: 0 min 100 % A, flow rate 1.0 mL/min; 2.0 min 100% A flow rate 1.0 mL/min; 2.30 min 100 % B flow rate 1.4 mL/min; 9.0 min 100 % B, flow rate 1.4 mL/min; 9.05 min 100% C, flow rate 1.4 mL/min; 18.0 min 100% C, flow rate 1.4 mL/min. Column was reconditioned with buffer A for 10 min before the following analysis.

2.6 Calibration graph

Mixture of ATP and ADP at increasing concentrations (range 20–1000 μM) were prepared in the mobile phase. A 20 μL volume of each standard solution was injected in triplicate and the resulting peak areas were plotted against the corresponding analyte concentration to obtain the calibration graphs.

2.7 Off-line evaluation of HSP90 α 's ATPase activity

Assay solution consisted of 6 μg of Hsp90 solution, ATP (0.5 mM) and Tris-HCl (pH 7.4, 100 mM) buffer containing KCl (20 mM) and magnesium chloride (6 mM). Assay volume was 250 μL . Spontaneous hydrolysis of ATP and interference from enzyme solution and buffer were accounted by preparing the corresponding blanks. Activity assays were carried out in a Termomixer Confort (Eppendorf, Italy) at 37°C, setting the following stirring program: 1 min stirring at 350 rpm every 60 min. After incubation, assay solutions were centrifuged at 7,245 x g (Centric 150, TEHTNICA ELEZNIKI d.o.o., Slovenia) for 5 min before 20 μL injections. Separation and determination of substrate and product were achieved by following the optimized chromatographic conditions with UV detection at 256 nm (section 2.5). The concentration of ADP and ATP produced and consumed by Hsp90 were determined by interpolating the respective peak areas in the calibration curves.

3. Results and discussion

3.1 ATP-ase activity determination by colorimetric assay

The MG assay is a widely used colorimetric assay for ATP-ase activity that is based on the release of inorganic phosphate (Pi) from ATP hydrolysis [3]. Due to the low turnover number of the human form, yeast Hsp90 and the truncated form of Hsp90 β endowed with a higher catalytic activity [3, 22, 23] have been often used instead. Therefore, since previously reported colorimetric studies were optimized for other forms of Hsp90, the MG method by Rowland *et al.*[13] was partially re-optimized. In particular, in the optimization of the MG assay, citrate concentration was evaluated to avoid the spontaneous hydrolysis of ATP in acidic conditions. The optimal final concentration of citrate was 0.531 % (w/v) while MG was used at 0.0203% (w/v) and ammonium molybdate was 1.43% (w/v). Good linearity of the calibration curve ($y=0.05102x-0.01207$, $r^2 = 0.9970$) and a not significant Y intercept, confirmed a linear dependence of the absorbance at 638 nm from the concentration of Pi in the range 2.5–30 μM . The optimized MG method has a submicromolar LOD value equals to 0.4 μM and a LOQ value of 1.34 μM .

The assay requires 2h a day for the initial preparation of the MG reagent which must be freshly prepared, and 35 minutes for the development of a stable color.

The MG assay was then applied to the evaluation of the ATP-ase activity of commercial samples of human recombinant Hsp90 α , which may differ in storage buffer and purity. Therefore samples from three different suppliers were used for a better evaluation of critical issues. Indeed, the type and composition of the storage buffer in which the enzyme is

delivered is a key aspect when a Pi-based assay is performed to avoid contamination by Pi (from buffers of commercial ATP) or interference from other components such as glycerol. Specifically, the commercial samples were delivered in Dulbecco's phosphate buffer (DPBS) containing glycerol (10%) (Stressgen) or in a MG-compatible buffer which however contained a certain amount of glycerol (Abcam and StressMarq). Consequently, all the samples needed a pre-treatment to remove glycerol and/or Pi ions and achieve a low background signal as required for an optimal activity determination.

Hsp90 α from Stressgen, being the most problematic samples from the MG assay point of view, was selected as case study for the evaluation of the assay feasibility. This sample showed a very high intrinsic absorbance value at 638 nm (1.12 AU) in the absence of the substrate, mainly due to the presence of phosphate ions (9.6 mM) in the delivery buffer (DPBS). The high background signal completely hindered the release of Pi from ATP catalyzed by Hsp90.

Therefore, 100 μ L aliquots of the commercial sample were either dialyzed against HEPES buffer (50 mM, pH 7.4) or loaded into Microcon YM-50 centrifugal filters for buffer exchange (endpoint buffer: HEPES 50 mM, pH 7.4). In both cases, the post-treatment concentration, the conformational stability and MG-related background value at 638 nm were evaluated. The loss of protein resulted acceptably limited (negligible with the dialysis approach and about 38.6% with the filtration approach), without any significant conformational alterations as showed by the circular dichroism spectra superimposition before and after the treatment. However the treatment with Microcon centrifugal filters resulted the most efficient in removing background interference. The intrinsic absorbance value of treated enzyme solutions by MG method in the absence of substrate was 1.0 AU and 0.099 AU for dialysis-treated and Microcon-treated samples, respectively.

To determine the amount of Pi produced by enzymatic hydrolysis, the corrected absorbance value was interpolated into a standard curve prepared by incubation of various phosphate concentrations in the optimized assay conditions in the absence of Hsp90 α . Under optimized conditions and after enzyme pretreatment, the enzyme velocity could be determined and was 1.80 ± 0.03 pmol/min while k_{cat} value was 0.054 ± 0.001 min⁻¹, a value that is in agreement with previously published data [20].

3.2 ATP-ase activity determination by ion exchange chromatography

Adenosine nucleotides separation was achieved by a disk shaped monolithic ethylene diamine column (2x6 mm i.d.) using a three-component gradient condition and UV-Vis detection. This small monolithic column offers advantages of high stability and rapid conditioning time (5 min) due to low limitations to mass transfer and low backpressure.[24] Mobile phase composition and flow rate were optimized in order to (i) preserve enzyme activity, (ii) achieve high selectivity and resolution values and (iii) have a suitable analysis time, by using standard mixtures of adenosine nucleotides. More in details, organic modifiers were avoided and mobile phases were selected to be compatible with Hsp90 stability for the next step consisting in the in-line coupling with the Hsp90-IMER, in view of automated screening campaigns. Moreover, commercially available samples of ADP may contain small amounts of AMP and samples of ATP may contain low amounts of ADP, which therefore need to be separated and determined for a proper enzyme activity evaluation. Consequently, the LC method was optimized for the separation of ATP, ADP and AMP. Detection at 256 nm was selected on the basis of the higher signal-lower baseline principle.

In the optimized conditions adenosine nucleotides were separated in a 15-min run (Figure 1). Capacity factors of the three analytes were: $k'(\text{AMP}) = 3.63$, $k'(\text{ADP}) = 27.1$, $k'(\text{ATP})$

= 82.8. This method showed good selectivity: the α value for ADP/ATP was found to be 3.03, with a resolution (R_s) value of 10.1.

Conformational enzyme stability in the three mobile phases was evaluated by CD in order to evaluate the compatibility with the next development of Hsp90-IMER. The CD spectra of the enzyme were carried out soon after dilution (t_0) and after 2 h and 5 h. No significant changes of the CD spectra were observed thus supporting the conformational stability of the enzyme on the time (data not shown). Furthermore, the perfect overlapping of spectra recorded in Tris HCl buffer at pH 7.4 and 8.5 confirms the conformational stability of Hsp90 α in the selected pH range of the mobile phase (Figure 2).

Noteworthy, run length may be further shortened while maintaining acceptable selectivity and resolution. However, at this stage, chromatographic parameters were set up to achieve ADP quantitation even in the presence of a low substrate conversion rate as expected for Hsp90 α ; indeed, high substrate concentration was used to increase the signal window. More specifically, since published K_m value for the substrate ranges from 100 to 850 μM [13, 20, 25] the amount of substrate was fixed at 1.0 mM as suggested by Rowlands *et al.* [13]. Moreover, due to the very low turnover number of the human isoform, percent substrate converted is quite low. Therefore the ion exchange method was optimized to quantitatively retain low amount of ADP even in the presence of a large excess of ATP.

Calibration curves were derived for the substrate ATP ($y = 8.529x - 51.82$, $r^2 = 0.9998$) and product ADP ($y = 8.730x - 63.96$, $r^2 = 0.9989$). LOD and LOQ values were calculated and compared with those of the MG assay (Table 1). Taking into account that detected product species in the LC and colorimetric assay differ, it can be stated that the two methods have quite similar sensitivity (compare $\text{LOQ}(\text{Pi}) = 1.34 \mu\text{M}$ and $\text{LOQ}(\text{ADP}) = 2.30 \mu\text{M}$).

The LC method was applied to the off-line determination of the ATP-ase activity of Hsp90 samples obtained from different manufacturers. Contrary to MG method, the Hsp90 solutions could be used with the LC method without pretreatment. Enzyme incubations with substrate in the same assay conditions used for the colorimetric assay were carried out. After the incubation, samples were simply centrifuged before injection to avoid column clogging. Figure 3 shows representative overlaid chromatograms for the off-line ATPase determination by the LC method.

Enzyme k_{cat} values could be determined for all the enzymes from different sources and resulted to be 0.030 min^{-1} (StressGen), 0.059 min^{-1} (StessMarq), 0.045 min^{-1} (Abcam). The lower k_{cat} value of the sample from Stressgen could be ascribed to the presence in the storage buffer of the commercial sample of one or more components which might partially inhibit the enzyme ATP-ase activity as confirmed by MG assay (data not shown).

4. Conclusions

The ATPase activity of human Hsp90 α was successfully monitored by a purposely developed and optimized ion exchange liquid chromatographic method with UV detection. This approach offered advantages over the available MG colorimetric assay since it was unaffected by the experimental conditions used for enzyme activity assay. Thus, a screening campaign can be optimized for the specific requirements of the project rather than adjusted to the differences in the commercially available Hsp90. Moreover, the simultaneous determination of ATP and ADP reinforces the reliability and specificity of this method. The absence of interferences from common components of enzyme matrix allowed the direct use of the commercial enzymes without any pre-treatments. This translates into time and money saving. Moreover, advantages in terms of time saving are also related to shorter analysis time and no need for reagent preparation.

The optimized chromatographic conditions are also suitable for the direct coupling with a future bioreactor containing immobilized Hsp90, being the enzyme stable in the LC experimental conditions as shown by circular dichroism studies.

Finally, by allowing the simultaneous determination of ATP, ADP and AMP, the LC method can be generally applied to the determination of the hydrolyzing activity of other adenosine nucleotide-dependent enzymes.

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Abbreviations

Hsp90	90 kDa Heat Shock Protein
IMER	immobilized enzyme reactor
DPBS	Dulbecco's phosphate buffer
MG	Malachite green

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Highlights

A reliable and highly specific ion exchange LC method was developed for the evaluation of the enzymatic activity of ADP/ATP generating or consuming enzymes. The LC method was applied to the off-line determination of the ATPase activity of human heat shock protein-90. The LC approach offered advantages over the frequently used malachite green method. These advantages are mainly related to the absence of interferences from common components of enzyme matrix, and time and money saving.

Figure 1. Representative chromatogram for separation of adenosine 5'-phosphate nucleotides. [AMP] = [ADP] = [ATP] = 160 μ M. Chromatographic conditions as in par. 2.5.

Figure 2.
Overlaid CD spectra of human Hsp90α samples in Tris HCl buffer (0.1 M) at pH 7.4 and pH 8.5.

Figure 3.

Off-line determination of ATP-ase activity of Hsp90 α . Samples were incubated in eppendorf tubes at 37°C with saturating concentration of substrate before being analyzed by ion exchange liquid chromatography. Chromatograms of incubation assay mixture in the absence (straight line) and in the presence (dotted line) of Hsp90 α are overlaid. Chromatographic conditions as in par. 2.5.

Table 1

Comparison of assay parameters for the MG and LC assays.

	Malachite Green method	Ion exchange LC method
Detected species	Inorganic phosphate	ADP (or ATP content)
Pre-treatment of Hsp90	Yes, if Pi containing buffers or glycerol are present	no
Analysis time	35 min	15 min
Required starting time	MG reagent must be freshly prepared (2.5h)	Column conditioning (10 min)
LOD (detected species)	0.40 μM	0.93 μM
LOQ (detected species)	1.34 μM	2.30 μM