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On-line cleavage of disulfide bonds by soluble and immobilized tris-(2-carboxyethyl)phosphine using sequential injection analysis

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ABSTRACT

Reduction of disulfide bonds is – in many cases – a critical pretreatment step for the determination of thiols in real samples. This study reports the first systematic investigation of the potentials of the on-line reduction of disulfide bonds under flow conditions in a sequential injection setup. One of the most promising reducing agents, tris-(2-carboxyethyl)phosphine (TCEP) was selected for this purpose while the Ellman's disulfide (DTNB) was used as model compound. The study involved the investigation of several parameters that affected the kinetics and efficiency of the reaction, including stopped-flow experiments. Both soluble and immobilized TCEP on agarose beads were examined. The results confirmed that both forms of TCEP can be used as an advantageous on-line reducing reagent for disulfide bonds under flow conditions.

1. Introduction

The unique role of sulfhydryl-containing compounds in biological systems and their numerous applications in food, pharmaceutical, aroma industries etc. have rendered thiols as one of the most "attractive" group of analytes in analytical chemistry [1–3]. One of the most important properties of thiols is the oxidation of the –SH moiety under various conditions to form disulfide bonds (or bridges) (–S–S–). The formation of these bonds can occur both under physiological conditions – e.g. in the presence of sulphydryl oxidase enzymes – and chemically, using various oxidants (hydrogen peroxide, iodine in alkaline medium, trace metals etc.) [4]. Disulfide bonds are critical for the stabilization of the structures of proteins and play a key protective role for bacteria [5]. On the other hand, disulfides are considered as impurities of certain active pharmaceutical ingredients with the captopril/captopril disulfide being a typical example [6].

From an analytical chemistry point of view it is important to be able to determine not only the total thiol content of a sample, but the oxidation status/ratio of the analytes as well. A typical protocol for total thiol analysis involves a reductive pretreatment step that should ensure quantitative conversion of the disulfides. Additionally, preserving the reactive sulfhydryls of a protein in a reduced state is critical to the maintenance of function of many proteins [7]. "Popular" reducing agents for this purpose include e.g. mercaptoethanol, dithiothreitol (DTT) and several phosphines. The action of the first two reagents – that are thiols themselves – is based on the thiol-disulfide exchange reaction [8]. A major disadvantage is the necessity of often removal of their excess since they also react with the most widely used derivatization reagents for thiols. On the other hand, phosphines tend to oxidize easily, are not stable in solutions and are difficult to handle due to their odor [9].

Tris-(2-carboxyethyl)phosphine (TCEP, Fig. 1) was introduced as an advantageous reducing agent for disulfide bonds in 1969 by Levison et al. [10] but it was almost completely ignored by the scientific community until 1991 when Burns et al. proposed a convenient synthetic procedure [11]. It reacts fast even with the most stable disulfides in a wide pH range, it is stable in aqueous solutions and in buffers, it is odorless and safe to handle and it is commercially available in soluble and immobilized forms. A detailed comparison between TCEP and DTT for use in protein biochemistry revealed several advantages of the former including non-competitive behavior with protein sulfhydryls for attachment of thiol-reactive dyes [7]. It also forms relatively weak complexes of simple stoichiometries with metals and as a result it should be a much safer protecting agent for thiol compounds in the presence of metal ions than DTT [12]. The significantly greater thermostability of TCEP makes it an appropriate sulfhydryl reductant for use in high-temperature assays where DTT would oxidize within seconds [13]. Since TCEP is not a thiol itself, the removal of its excess is not necessary during pre-column derivatization protocols [14]. Recent studies suggest that TCEP may also reduce other compounds such as sulfoxides, sulfonylchlorides, N-oxides, and azides [15] or even dehydroascorbic acid in biological samples [16].

The scope of this work was to investigate – for the first time – the cleavage of disulfide bonds by both soluble and immobilized TCEP under flow conditions using sequential injection analysis (SI).

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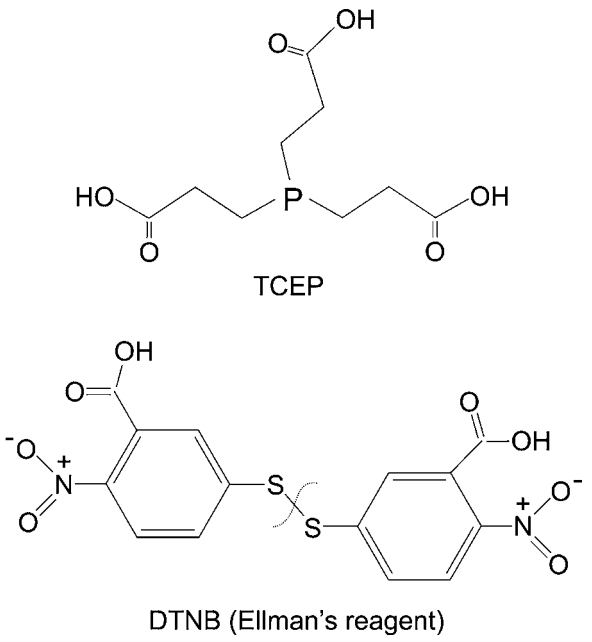

Fig. 1. Chemical structures of tris-(2-carboxyethyl)phosphine (TCEP) and Ellman's reagent (DTNB).

SI enables the automated handling of precisely defined volumes of samples and reagents and based on the computer-controlled operation offers the possibilities for stopped-flow experiments and "real time" monitoring of the kinetics of the reactions. A typical disulfide, namely the Ellman's reagent (DTNB, Fig. 1), was selected as model compound.

2. Experimental

2.1. Reagents and materials

All chemicals used throughout this study were of analytical-reagent grade. Ultra-pure quality water was produced by a Milli-Q system (Millipore, Bedford, USA).

The stock solution of 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent or DTNB, Sigma) was prepared at an amount concentration of 1.0 mmol L^{-1} in TRIS buffer (10 mmol L^{-1}, pH 7.5). This solution was kept refrigerated and found to be stable for at least one month. Diluted working solutions were prepared daily in water. Tris-(2-carboxyethyl)phosphine (TCEP, Sigma) stock solutions were prepared daily in water (c(TCEP) = 10 mmol L^{-1}). Working TCEP solutions were also prepared in water by appropriate dilutions. TRIS buffer (c = 100 mmol L^{-1}) was prepared by dissolution of a suitable amount of tris(hydroxymethyl)aminomethane (Sigma) in water. The pH was adjusted to the desired values by adding appropriate volumes of a HCl solution (c = 1.0 mol L^{-1}). Ethyl-propiolate (EP) and Cysteine (CYS)/Cystine (CSN) for the preliminary studies were prepared as mentioned elsewhere [17].

Immobilized TCEP was covalently linked to 4% cross-linked beaded agarose support and was supplied by Pierce. According to the manufacturer the gel-material has an effective (functional) TCEP concentration of >8 mmol L^{-1} (i.e., 8 μmol per mL of gel). The gel was slurry-packed in 1 and 2 cm long mini-columns (the internal volumes were 90 and 180 μL, respectively) (GlobalFIA) with the aid of a micro-pipette under suction. Non-metallic frits were used in order to avoid metal-catalyzed oxidation of the thiols.

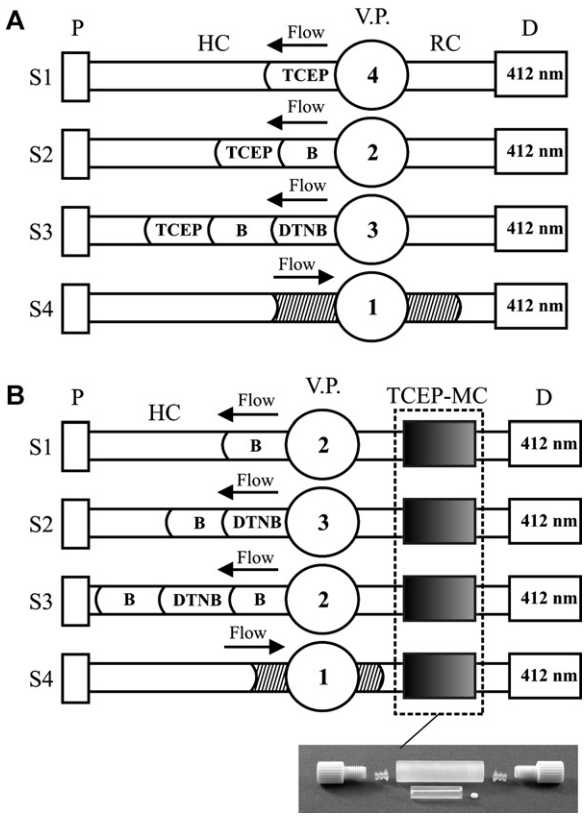

Fig. 2. Sequential injection protocol for the reduction/cleavage of the disulphide bond of DTNB by soluble (A) and immobilized (B) TCEP: S1–S4, SI steps; P, peristaltic pump; HC, holding coil (300 cm/0.7 mm i.d.); V.P., valve position; RC, reaction coil (60 cm/0.5 mm i.d.); TCEP-MC, mini-column containing TCEP immobilized on agarose beads; D, flow-through UV-Vis detector; B, tris buffer (100 mmol L^{-1}); the carrier was de-ionized water in all cases.

2.2. Instrumentation

The SI analyzer was consisted of the following parts: a micro-electrically actuated 10-port valve (Valco, Switzerland); a peristaltic pump (Gilson Minipuls3, France); a SPD-10AV flow through UV-Vis spectophotometric detector with a flow-cell volume of 8 μL (Shimadzu, Japan). System control was achieved by means of a special program written in LabVIEW® 5.1.1 (National Instrument, US). Data acquisition was performed through the Clarity® software (DataApex, Czech Republic). The flow system used 0.5 mm i.d. PTFE tubing throughout except for the holding coil (HC, 3.0 m × 0.7 mm i.d.). A FIAstar 5101 thermostat (Tecator, Sweden) was used to thermostat the reaction coil at the desirable temperature when necessary. Off-line spectrums were recorded by a V-530 UV-Vis spectrophotometer (Jasco, US) equipped with 1 cm quartz cuvettes.

2.3. SI protocol

The SI sequence for the study of the reduction of disulfide bonds using soluble TCEP was based on a three-zone approach and was consisted of four steps and is shown schematically in Fig. 2A. When immobilized TCEP was used, the reduction column was positioned between the multi-position valve (via port 1) and the detector (Fig. 2B). In the latter approach, DTNB was "sandwiched" between two zones of buffer. In both cases the reaction products were monitored on-line at 412 nm.

During stopped-flow (SF) experiments, the more concentrated section of the reaction mixture was kept at the flow-cell of the

detector for a defined time period, in order to investigate the kinetic behavior of the reaction.

3. Results and discussion

3.1. Preliminary off-line experiments

Preliminary off-line experiments were carried out selecting the Cysteine (CYS)/Cystine (CSN) thiol/disulfide pair. A typical experiment included off line mixing of the following:

- 200 μL CSN (50 μmol L^{-1}) or CYS (100 μmol L^{-1})
- 300 μL of TRIS buffer (100 mmol L^{-1}, pH 7.5)
- 100 μL TCEP (10 mmol L^{-1})
- dilution up to 2000 μL with de-ionized water.

The mixture was left to react for a certain period of time (typically 5–10 min) and was analyzed by a SI method based on the selective derivatization of thiols with ethyl propiolate (EP) [17]. The main SI steps and parameters (chemical – instrumental) can be found in [17]. The cleavage yield was calculated by the CSN/CYS signals ratio. The kinetics of the off line reaction was investigated by analyzing aliquots of the mixture (after 5 min reaction) by SI at 2 min intervals. No differences were observed indicating fast kinetics, promising for on-line transfer. Quantitative cleavage (97.2–98.7%) was achieved in a pH range of 7.5–9.0 and for amount concentrations of TCEP in the range of 250–1000 μmol L^{-1}. Linearity was obeyed up to 100 μmol L^{-1} CSN (mA.U. = 761.09 [CSN] + 9.45/r = 1) and quantitative cleavage was demonstrated by the 2:1 ratio of the slopes compared to CYS curves (slopes values of 761.09 vs 383.81, respectively). Further demonstration of the validity of the procedure included analysis of CSN/CYS mixtures at various amount concentration ratios in the range of 25–100 μmol L^{-1}. Recoveries varied between 95.7 and 104.9%.

The last series of preliminary experiments involved the transfer of the above-mentioned chemical system in an on-line mode using SI. Zones of EP, TCEP (+buffer, pH 10) and CSN (and/or CYS) were aspirated in the holding coil and propelled to the detector through a suitable reaction coil. Stopped-flow experiments at elevated concentrations of TCEP (0–3.0 mmol L^{-1}) showed a promising 60% reduction/cleavage within 5 min.

3.2. On-line cleavage of S–S bonds by soluble TCEPs

The main disadvantage of the CYS/CSN pair for further investigation under flow conditions was the necessity of an additional reaction in order to measure the formed thiols, requiring the distinguishing between the kinetics of the two reactions. For this reason, subsequent on-line experiments were carried out by using Ellman's reagent (DTNB) as model disulfide. The reduction product (TNB) can be monitored directly at 412 nm (Fig. 3). On-line SI experiments confirmed no inhibition of the excess of DTNB on the photometric analysis.

In the typical SI sequence of Fig. 2A, equal volumes (50 μL) of TCEP (500 μmol L^{-1}), buffer (100 mmol L^{-1} TRIS, pH 8.0) and DTNB (50 μmol L^{-1}) were aspirated in the holding coil and reacted on passage through a 60 cm long (0.5 mm i.d.) reaction coil (RC). During stopped-flow experiments the most concentrated section of the reaction mixture was isolated in the flow-cell of the detector by precise timing and the reaction was monitored "real-time" for 300 s. It should be noted that since TNB is not commercially available, the reduction yield was estimated by comparison versus off-line reduction of DTNB (50 μmol L^{-1}) by TCEP (500 μmol L^{-1}, pH 8.0, t = 15 min) followed by identical SI analysis at 412 nm.

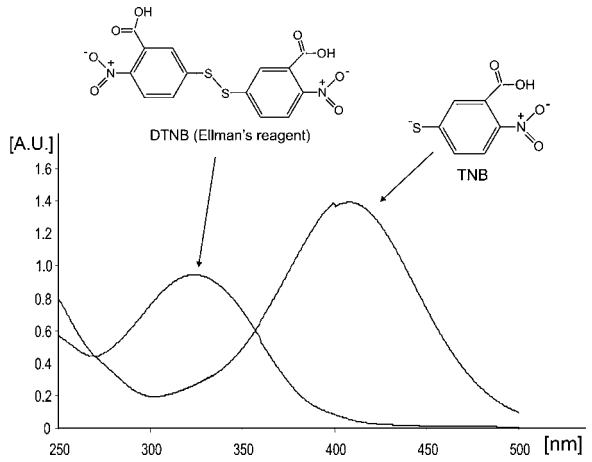

Fig. 3. UV–vis spectrum of the Ellman's reagent (DTNB) and its reduced form (TNB).

The effect of the pH (6.0–10.0) on the efficiency of the reaction is depicted in Fig. 4A. Without stopped-flow (t = 0 s) the percent cleavage was >95% in the pH range of 8.0–9.0, while by increasing the reaction time to 300 s, a 95% reaction yield was achieved even at a lower pH value of 7.0. The fast kinetics of the DTNB–TCEP reaction for pH values > 7.0 were confirmed by the stopped-flow plots shown in Fig. 4B.

The effect of the amount concentration of TCEP was investigated in the range of 0.1–1.0 mmol L^{-1} at a pH range of 6.0–10.0. The experiments confirmed fast and quantitative reduction (>95%) above 0.2 mmol L^{-1} TCEP and pH values 7.0–9.0. The effect of the amount concentration of the reducing reagent was more intense at more acidic environment (pH 6.0), as can be seen in the stopped-flow plots of Fig. 5. In a similar manner, variation of the reaction temperature in the range of 25–60 °C had a negligible effect for pH values >7.0.

The linearity of the procedure was investigated at a pH value of 8.0, using an amount concentration of TCEP of 0.5 mmol L^{-1}.

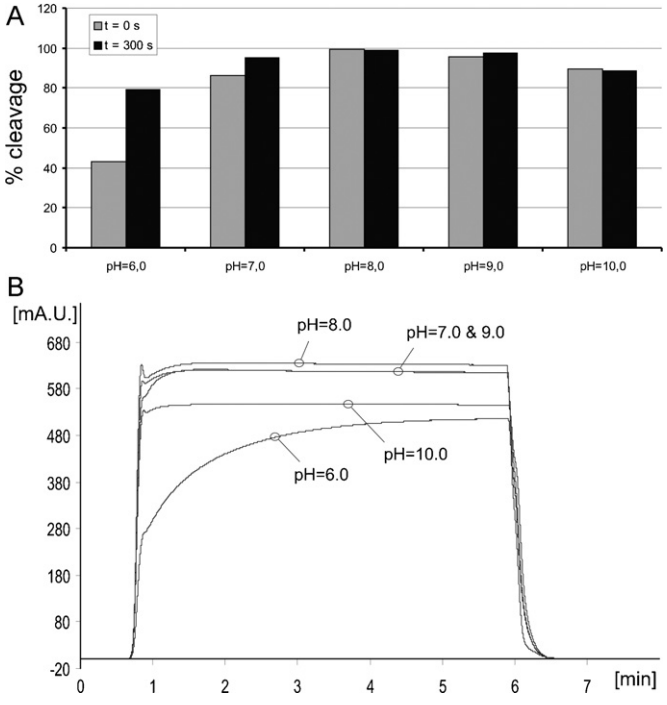

Fig. 4. Effect of the pH on (A) the efficiency and (B) the kinetics of the on-line reduction/cleavage of the disulphide bond of DTNB by soluble TCEP.

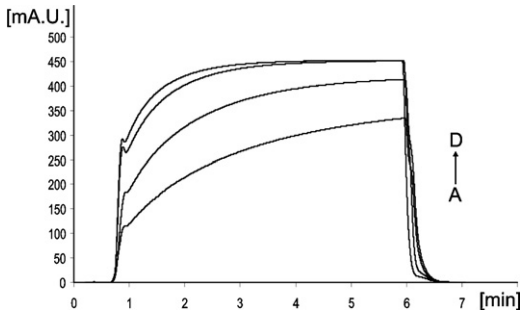

Fig. 5. Effect of the amount concentration of TCEP on the kinetics of the on-line reduction of DTNB (50 μmol L^{-1}) at pH 6.0: (A) c(TCEP) = 0.1 mmol L^{-1}; (B) c(TCEP) = 0.2 mmol L^{-1}; (C) c(TCEP) = 0.5 mmol L^{-1}; (D) c(TCEP) = 1.0 mmol L^{-1}.

The temperature was maintained at 25 °C. Satisfactory linearity (r > 0.999) was achieved up to 75 μmol L^{-1} DTNB, indicating uniform reduction in a wide range of concentrations. The precision was evaluated by replicates (n = 12) at various concentration levels. The relative standard deviations (s_r) were better than 2.5% in all cases.

3.3. On-line cleavage of S–S bonds by immobilized TCEP on agarose beads

The on-line cleavage of the S–S bond of DTNB by immobilized TCEP was investigated by using commercially available TCEP immobilized on agarose beads packed in 1 and 2 cm long mini-columns. The SI sequence involved "sandwiching" of 25 μL of DTNB (c = 25 μmol L^{-1}) between two zones of buffer solution (2 × 50 μL, 100 mmol L^{-1} TRIS, pH 8.0) in the holding coil of the SI setup. Prior to entering the reduction column, the zones were allowed to overlap on passage through a 30 cm-long mixing coil (MC) at a flow rate of 0.2 mL min^{-1}. It should be noted that the practice showed that the SI carrier solution (water) should be filtered (0.45 μm filters) in order to avoid excessive back-pressure and leakage phenomena.

The effect of the pH was studied in the range of 6.0–10.0 using both columns. Quantitative cleavage (compared to off-line reaction) was achieved in the range of 7.0–8.0, while similar results were obtained for either 1 or 2 cm long columns, indicating fast reaction (Fig. 6). What is worth mentioning is the performance of the immobilized TCEP at a slightly acidic pH value of 6.0, where a ca. 80% cleavage was achieved. These values are dramatically better compared to soluble TCEP, where the same percentage could be obtained only after 300 s of stopped-flow (ca. 40% without stopped-flow).

When solid-phase reactors are employed in flow-based methods, a critical parameter is the flow rate of the carrier stream that determines the contact time between the soluble sample and the

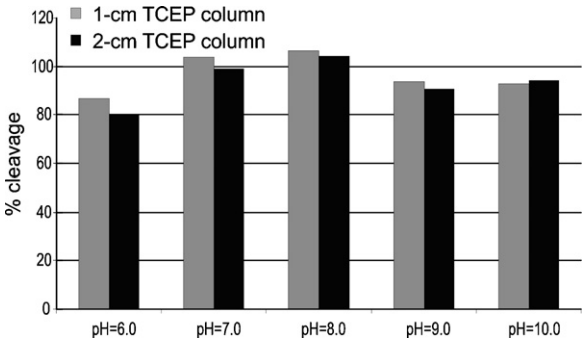

Fig. 6. Effect of the pH on the efficiency of the on-line reduction/cleavage of the disulphide bond of DTNB by TCEP immobilized on agarose beads.

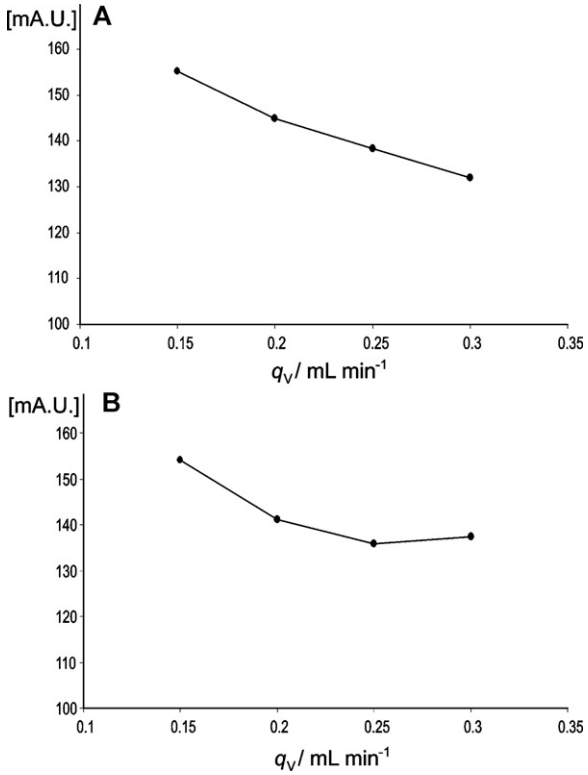

Fig. 7. Effect of the flow rate on the on-line reduction of DTNB by immobilized TCEP: (A) 1 cm long column and (B) 2 cm long column.

immobilized reagent. By varying the flow rate of the carrier in the range of 0.15–0.30 mL min^{-1}, the signals decreased by only 15% and 10% for the two columns respectively, confirming the fast kinetics of the reaction. Additionally, no variation was observed for the 2 cm column in the range of 0.25–0.30 mL min^{-1} (Fig. 7).

Both columns showed excellent linearity for DTNB in the range of 375–875 pmol DTNB (r > 997) with relative standard deviations (s_r) better than 4% in all cases. Their within-day stability of the solid phase reactors was evaluated by performing repetitive injections over a period of 8 h. The R.S.D. was less than 10% in all cases. A useful feature of the immobilized reagent was that it could be regenerated up to three times – with less than 5% activity variations – by overnight storage at 0 °C in a TCEP solution (1 mmol L^{-1}, pH 8.0).

4. Conclusions

The results of this study demonstrate the usefulness of both soluble and immobilized TCEP for the reduction of disulfide bonds under flow conditions. Rapid, quantitative reduction of a model disulfide was achieved in a sequential injection manifold at alkaline medium and under mild reaction conditions. This promising behavior may become the basis of the development of automated, on-line sample pretreatment protocols for the simultaneous determination of thiols/disulfide pairs in real samples (pharmaceuticals, biological etc.) by coupling SI to HPLC. Further investigation is carried out towards this direction.

References

[1] T. Toyo'oka, J. Chromatogr. B 877 (2009) 3318–3330.
[2] N.R. Srinivas, R.N.V.S. Mamidi, Biomed. Chromatogr. 17 (2003) 285–291.
[3] K. Kusmierek, G. Chwatko, R. Głowacki, P. Kubalczyk, E. Bald, J. Chromatogr. B 879 (2011) 1290–1307.
[4] D. Witt, Synthesis 16 (2008) 2491–2509.
[5] C.S. Sevier, C.A. Kaiser, Nat. Rev. Mol. Cell Biol. 3 (2002) 836–847.

[6] U.S. Pharmacopoeia, 30th ed., –NF 25, Captopril monograph, 2007, pp. 1612–1613.
[7] E. Burmeister Getz, M. Xiao, T. Chakrabarty, R. Cooke, P.R. Selvin, Anal. Biochem. 273 (1999) 73–80.
[8] H.F. Gilbert, Methods Enzymol. 251 (1995) 8–28.
[9] Interchim Ltd, TCEP: A reducing agent that is odorless and more effective than DTT, URL: http://www.interchim.fr/ft/2/242214.pdf (accessed 4/2011).
[10] M.E. Levison, A.S. Joseph, D.M. Kirschenbaum, Experientia 25 (1969) 126–127.
[11] J.A. Burns, J.C. Butler, J. Moran, G.M. Whitesides, J. Org. Chem. 56 (1991) 2648–2650.
[12] A. Krezel, R. Latajka, G.D. Bujacz, W. Bal, Inorg. Chem. 42 (2003) 1994–2003.
[13] S.S. Rhee, D.H. Burke, Anal. Biochem. 325 (2004) 137–143.
[14] J.C. Han, G.Y. Han, Anal. Biochem. 220 (1994) 5–10.
[15] A.M. Faucher, C. Grand-Maitre, Synth. Commun. 33 (2003) 3503–3511.
[16] Y. Sato, T. Uchiki, M. Iwama, Y. Kishimoto, R. Takahashi, A. Ishigami, Chem. Pharm. Bull. 33 (2010) 364–369.
[17] C.K. Zacharis, P.D. Tzanavaras, D.G. Themelis, J. Pharm. Biomed. Anal. 50 (2009) 384–391.