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Short communication

Derivatization of thiols under flow conditions using two commercially available propiolate esters

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ABSTRACT

In the present study we report new data on the reaction of three representative thiols – captopril (CAP), cysteine (CYS) and N-acetylcysteine (NAC) – with two commercially available propiolate esters – methyl-propiolate (MP) and butyl-propiolate (BP) – under flow conditions. The reactions were investigated on-line using sequential injection analysis (SI) and the formed derivatives were monitored spectrophotometrically at 285 nm. The effect of critical parameters of the reaction such as the pH, the temperature and the amount concentration of the reagents were studied in detail including stopped-flow (SF) experiments. Both reagents were found to be suitable for the automated derivatization of thiols, although MP offered faster kinetics compared to BP. The applicability of the procedures was demonstrated by the development of SI methods for the dissolution studies of CAP tablets with satisfactory results.

1. Introduction

Thiols are an important group of compounds that continue to attract the interest of analytical scientists due to their unique role in biological systems, food, pharmaceutical and aroma industries. Apart from the several review articles on the analysis of thiols [1–5] and the many research articles that appear in the literature, the analytical challenge can be even pointed out by a very recent special issue of *Journal of Chromatography B* (Elsevier) under the characteristic title "*Analysis of thiols*" (Volume 877, Issue 28, 2009).

In a previous study we explored – for the first time from an analytical chemistry point of view – the reaction of thiols with ethyl-propiolate (EP) under flow conditions [6]. The experimental work that was carried out clearly demonstrated the feasibility and advantages of this new reagent for analytical applications: (i) the reaction is simple, (ii) it proceeds in aqueous solutions, (iii) it reacts rapidly and selectively with thiols under mild conditions, (iv) the reaction rate can be controlled effectively by adjusting the pH, (v) it offers adequate sensitivity at the $\mu g\,L^{-1}$ level using simple UV instrumentation, (vi) the blank signals due to the native absorbance of the reagent are negligible and is therefore suitable for post-column applications, (vii) it is commercially available at a low cost compared to many derivatizing reagents for thiols, and (viii) the reaction can be readily carried out under flow conditions.

The scope of the present report is to expand current knowledge on this topic by studying the reaction of thiols with two other commercially available propiolic esters, namely methyl-propiolate (MP) and butyl-propiolate (BP) under flow conditions. For this purpose we employed sequential injection (SI) as the preferred technique, since it offers the advantages of strict control of the experimental conditions via software, minimum sample and reagent consumption and the potential of carrying out efficiently stopped-flow experiments through precise timing. Due to the different hydrophobic character of the derivatives of low molecular weight thiols with the three commercially available propiolate esters (MP, EP and BP) one could expect variations in the behavior when combined with separation techniques such as liquid chromatography and capillary electrophoresis. The kinetic behavior of these reactions and the potential analytical figures of merit should provide significant data on the basis of future work towards the aforementioned direction.

2. Experimental

2.1. Instrumentation

On-line derivatization experiments were carried out using a SI analyzer built-in house. It consisted of the following parts: a micro-electrically actuated 10-port valve (Valco, Switzerland); a peristaltic pump (Gilson Minipuls3, France); a SPD-10AV flow-

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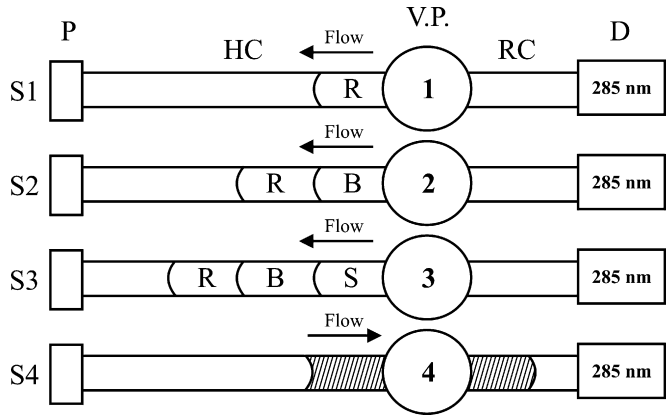

Fig. 1. Sequential injection protocol for the derivatization of thiols under flow conditions. S1–S4: SI steps; P: peristaltic pump; HC: holding coil (300 cm/0.7 mm i.d.); V.P.: valve position; RC: reaction coil (100 cm/0.5 mm i.d.); D: flow-through UV–vis detector; R: MP or BP reagents (50 μL); B: Britton–Robinson buffer (50 μL); S: thiol compounds (50 μL); the carrier was de-ionized water in all cases.

through UV–vis spectrophotometric detector with a flow-cell volume of 8 μL (Shimadzu, Japan). The SI system was controlled by means of a special program built-in the laboratory using the LabVIEW® 5.1.1 instrumentation software package (National Instrument, USA). 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.). Tygon pump tubing was used for aspirating and delivering the solutions.

A FIAstar 5101 thermostat (Tecator, Sweden) was used to thermostate the reaction coil at the desirable temperature when necessary. Off-line spectrums of derivatives were recorded on a V-530 UV–vis spectrophotometer (Jasco, USA) equipped with 1 cm quartz cuvettes.

2.2. 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).

Working solutions of methyl-propiolate (MP) and butyl-propiolate (BP) (Sigma, MO, USA) were prepared daily by appropriate dilution of the liquid reagents in water. Standard stock solutions of captopril (CAP) (Fluka, Switzerland), cysteine (CYS) (Merck, Germany) and N-acetylcysteine (NAC) (Merck, Germany) were also prepared daily at an amount concentration of 1.0×10^{-3} mol L^{-1} using 1.0 mmol L^{-1} EDTA (Merck, Germany) pre-purged with nitrogen. Working standard solutions of the thiols were prepared prior to analysis by appropriate dilution of the stocks in water.

The Britton–Robinson buffer consisted of a mixture of H_3PO_4, CH_3COOH and H_3BO_3 (0.04 mol L^{-1} each). The pH was adjusted to the desired values by adding appropriate volumes of a NaOH solution (c = 1.0 mol L^{-1}).

2.3. SI protocol

The SI sequence for the on-line derivatization reactions was consisted of four steps and is shown schematically in Fig. 1. In brief, after the sequential aspiration of the zones of the derivatizing reagents (R), the buffer (B) and the thiol samples (S) through ports 1–3 of the valve respectively in the HC, the reaction mixture was propelled to the detector (λ = 285 nm) through port 4 at a flow rate of 0.6 mL min^{-1}. The derivatives were formed on passage through a 100-cm long reaction coil (RC). During stopped-flow (SF) experiments, the more concentrated section of the reaction mixture was trapped 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 experiments were carried out in order to investigate the reaction of the two propiolate esters (MP and BP) in an off-line mode. Captopril (CAP), cysteine (CYS) and N-acetylcysteine (NAC) were selected as model compounds. On this basis, 1 mL of each thiol ($c = 1.0 \times 10^{-4}$ mol L^{-1}), 1 mL of each reagent (c = 10 mmol L^{-1}) and 1 mL of B–R buffer (pH 9.0) were mixed and left to react for 15 min. The spectra of the reaction mixtures were recorded in the range of 225–345 nm in a 1-cm cuvette against a reagent blank. The experiments confirmed that MP and BP react with the selected thiols to form UV-absorbing derivatives having maxima in the range of 280–285 nm. Besides the two esters, we also investigated the potential reaction of propiolic acid (PA) with the thiols and confirmed that no UV-absorbing reaction products were observed (245–345 nm), indicating that only the alkyl esters of PA are suitable for analytical applications.

3.2. Investigation of the reactions under flow conditions

The reaction of MP and BP with the selected thiols was investigated under flow conditions, using the general SI protocol shown in Fig. 1. The amount concentration of each thiol was set at 2.5×10^{-4} mol L^{-1} and of each reagent at 0.01 mol L^{-1}. A pH value of 9.0 was used for these initial studies according to our previous findings with EP [6]. The effect of the pH, the temperature and the amount concentration of the reagents was investigated under the above-mentioned conditions. When necessary, the kinetic behavior of the reactions was further studied by stopped-flow experiments (SF-SI).

3.2.1. Effect of the pH

The mechanism of the derivatization reaction is based on the nucleophilic attack of the thiolate ion to the α-carbon atom of the triple bond of the ester [7]. It is therefore expected that the pH will play an important role in the reaction rate since it affects the dissociation of the sulfylhydryl group and consequently its ability to act as nucleophilic reagent.

The theoretically expected behavior of the chemical systems was confirmed by studying the effect of the pH in the range of 8.0–12.0 using suitable B–R buffers. All six reactions were favored at alkaline pH values above the pK_a of the thiols (pK_a: 9.8 (CAP), 8.3 (CYS) and 9.5 (NAC) [6,8,9]). For MP, the signals practically leveled-off in the range of 10.0–12.0 while for BP in the range of 11.0–12.0. Characteristically, the reaction rate of MP–CAP was increased 5.6-fold in the range of 8.0–10.0, of MP–NAC 5.9-fold in the same range and of BP–CYS and BP–NAC 2.5-fold and 18.7-fold in the pH range of 8.0–11.0 respectively.

Stopped-flow experiments confirmed the accelerated reaction kinetics in highly alkaline pH in all cases. For example, the reaction between MP and NAC was leveled-off at pH values of 10.0 and 11.0 and was not affected by the SF period. On the other hand, BP showed slower kinetics compared to MP in all cases. This behavior could be attributed to stereochemical phenomena due to the structure of the BP molecule. Even at pH values of 11.0 and 12.0 the reactions for example between BP and CAP or NAC required at least 240 s for reaching equilibrium. A typical set of SF curves from the reaction of BP with the three model thiols in the range of 8.0–11.0 are depicted in Fig. 2.

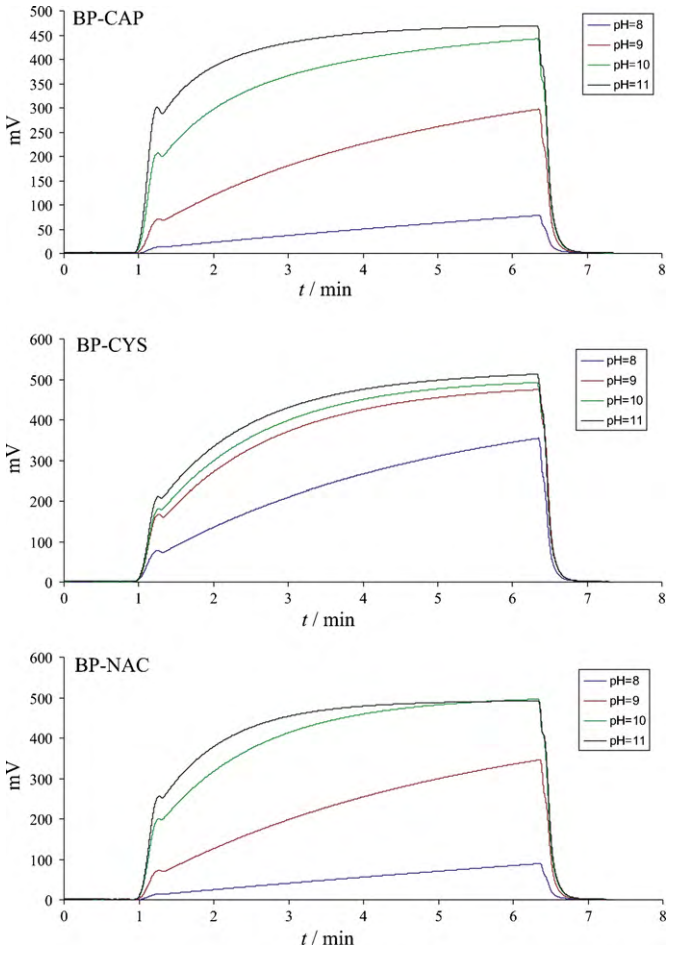

Fig. 2. Effect of the pH on the kinetics of the reaction of BP with CAP, CYS and NAC using stopped-flow; c(BP) = 0.01 mol L^{-1}, c(CAP) = c(CYS) = c(NAC) = 2.5 × 10^{-4} mol L^{-1}, T = 25 °C.

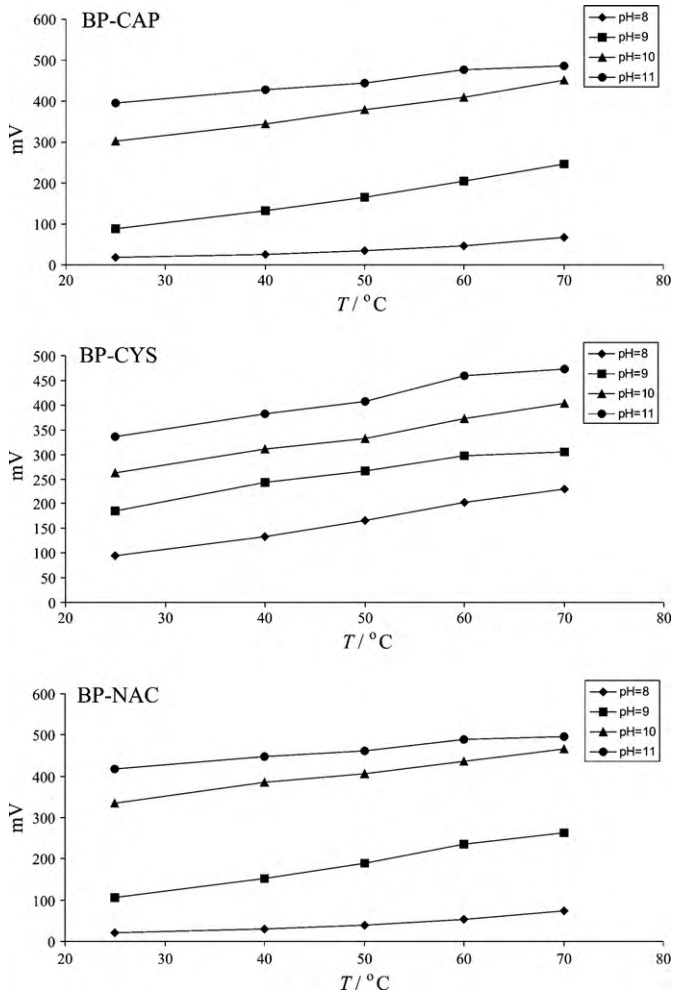

Fig. 3. Effect of temperature on the reaction of BP with CAP, CYS and NAC at various pH values; c(BP) = 0.01 mol L^{-1}, c(CAP) = c(CYS) = c(NAC) = 2.5 × 10^{-4} mol L^{-1}.

3.2.2. *Effect of temperature*

The effect of the temperature on the derivatization reaction was investigated in the range of 25–70 °C by thermostating the reaction coil. The experimental results for MP showed a generally drastic effect of the temperature on the reaction only at low pH values where the reaction rate is low. For example, for the MP–CAP system, variation of the temperature within the range mentioned above resulted in an increase in the reaction rate of 420% at pH 8.0, of 91% at pH 9.0, of 10% at pH 10.0, while the signals were practically unaffected (<2%) at pH 11.0. The respective values for e.g. the BP–CAP reaction were 285% (pH 8.0), 65% (pH 9.0), 49% (pH 10.0) and 23% (pH 11.0). Compared to MP, the reactions of BP were affected more intensely by the temperature at high pH values (>10.0) due to the generally slower kinetics of the BP reactions (Fig. 3). It should be noted that in all cases, the temperature had a negligible effect on the blank signals.

3.2.3. *Effect of the amount concentration of the reagents*

The effect of the MP and BP amount concentrations on the reaction kinetics was investigated in the range of 5–20 × 10^{-3} mol L^{-1}. In order to have a more complete view of the phenomena, the effect of each concentration was examined at four pH values namely 8.0, 9.0, 10.0 and 11.0. In the case of MP were the reactions have faster kinetics compared to BP the effect of the amount concentration of the reagent was inversely proportional to the pH. For example for the MP–CAP system a ca. 238% increase was observed at pH 8.0 whereas the phenomenon was less profound at higher pH values (99.6% at pH 9.0 and 29.4% at pH 10.0). On the other hand, the amount concentration of BP had a considerable effect even at highly alkaline pH values. For example, variation of the amount concentration of BP in the range of 5–20 × 10^{-3} mol L^{-1} resulted in a ca. 80% increase in the signals at pH 8.0, 125% at pH 9.0 and ca. 150% at pH 10.0 and 11.0. The behavior was also confirmed by stopped-flow experiments for a time frame up to 300 s.

3.2.4. *Specificity of the reaction*

The selectivity of the derivatization reactions was investigated against amino-acids and disulfides. The latter group of compounds is of particular importance since they are oxidation by-products of thiols and are therefore potential impurities of pharmaceutical formulations. On this basis, glycine and cystine were selected as model compounds respectively, at an amount concentration of 2.5 × 10^{-4} mol L^{-1}. The experimental results of stopped-flow experiments showed that both glycine and cystine failed to differentiate from the kinetic profile of the blank.

3.3. *Analytical applications*

The potential applicability of the derivatizing reagents was demonstrated by the development of two SI methods for the dissolution studies of CAP tablets. Drug absorption after oral administration of a solid dosage form depends on the release of the active ingredient from the formulation, its dissolution under physiological

Table 1
Study of the SI variables for the determination of CAP.

SI variable	Studied range	Selected value
$L_{(RC)}$ (cm)/(0.5 mm i.d.)	0–100	60
$V(S)$ (μL)	25–300	200
$V(R)$ (μL)	25–100	50
$V(B)$ (μL)	15–75	25
q_v (mL min^{-1})	0.4–1.0	0.8

conditions, and the permeability across the gastrointestinal tract. Because of the critical nature of the first two of these steps, in vitro dissolution may be relevant to the prediction of in vivo performance [10]. It is therefore widely accepted that dissolution testing is a very important tool in the pharmaceutical industry for providing valuable information to both formulation teams that design new products and quality control scientists for ensuring lot-to-lot quality and consistency within pre-defined specification criteria [11].

3.3.1. Development of the SI methods

In order to proceed with the configuration of the final SI system it was necessary to examine the effects of the main instrumental variables on the determinations. These variables included the flow rate of the carrier stream towards the detector, the length of the reaction coil and the injection volumes of the thiol samples, the reagents (BP and MP) and the buffer. All studies were carried out by the univariate approach at an amount concentration of $c(CAP) = 1.0 \times 10^{-4}$ mol L^{-1}. The amount concentrations of the derivatization reagents and the reactions pH were selected based on the investigation of the reaction carried out in the previous sections. For MP the amount concentration was set at 10 mmol L^{-1} and pH 10.0 and for BP at 20 mmol L^{-1} and pH 11.0.

The variables studied, the examined range and the selected values are shown in Table 1. Similar behavior was observed for both MP and BP with the sample and reagent volumes and the flow rate having the most significant effect on the methods. In order to maintain the generic character of the procedures the same values of the SI variables were selected for both reagents making compromises in terms of sensitivity, sampling frequency and reagents consumption. Using the conditions shown in Table 1, a sampling rate of 40 h^{-1} was achieved.

3.3.2. Validation of the SI methods

The developed SI methods were validated in terms of linearity, limits of detection and quantitation, precision, selectivity and accuracy using – when applicable – pre-defined acceptance criteria. The experimental results are summarized in Table 2.

(i) Linearity was evaluated by both the regression coefficient (r^2) and the residuals approaches. The acceptance criteria were set at an $r^2 > 0.999$ and residuals in the range of ±3%. (ii) The limits of detection (LOD) and quantitation (LOQ) were calculated based on the signal-to-noise ratio criterion (LOD = 3 × S/N and LOQ = 10 × S/N). (iii) The precision of the methods was validated by replicate injections ($n = 12$) of standard solutions of the analytes at the 1.0×10^{-4} mol L^{-1} level. The relative standard deviation (R.S.D.) should be less than 2.0% in all cases. (iv) The selectivity of the SI methods was evaluated against common pharmaceutical excipients (colloidal silicon dioxide, pre-gelatinized starch, talc, lactose monohydrate, magnesium stearate, citric acid, titanium dioxide, gelatin and sodium saccharin). The placebo mixture was prepared by mixing 1.0 g of each excipient in a mortar until a homogenous fine powder was obtained. An accurately weighed amount of the resulting placebo was dispersed in water, ultrasonicated for 30 min, centrifuged at 3000 rpm for 15 min and filtered through 0.45 μm syringe filters. The resulting stock solution had a nominal placebo concentration of 10 mg mL^{-1}. Standard solutions of the analytes ($c = 1.0 \times 10^{-4}$ mol L^{-1}) were spiked with elevated placebo concentrations in the range of 100–1000 mg L^{-1} and analyzed. The criterion for interference was set at a relative error of less than ±5% versus an equivalent aqueous standard. (v) The accuracy was validated by analyzing synthetic samples of the analytes ($0.5–1.5 \times 10^{-4}$ mol L^{-1}) containing 1000 mg L^{-1} placebo. The acceptance criterion was set at percent recoveries in the range of 97.0–103.0%.

As can be seen from the values of Table 2, the pre-defined validation criteria were met in all cases.

Table 3
Dissolution data of CAP tablets.

Time (min)	Percent dissolution (±S.D.)			
	SI-MP	SI-BP	HPLC	SI-EP
5	29.2 (±3.2)	32.8 (±3.6)	28.1 (±3.3)	31.8 (±3.4)
10	61.4 (±2.4)	59.5 (±3.8)	61.8 (±3.2)	58.7 (±3.6)
15	84.5 (±2.3)	85.7 (±2.7)	83.7 (±2.4)	82.9 (±3.0)
20	92.3 (±3.2)	92.6 (±1.9)	90.5 (±3.1)	91.5 (±3.8)
30	101.3 (±2.1)	99.5 (±2.5)	98.6 (±.4)	98.4 (±2.1)
60	100.8 (±2.3)	98.6 (±3.3)	102.7 (±2.7)	101.8 (±2.0)

S.D.: standard deviation ($n = 6$).

3.3.3. Dissolution studies of CAP tablets

The dissolution tests for validation batches of CAP-containing tablets (50 mg/tab) were carried out in a type I dissolution apparatus (Distek Premier 5100) as described in the US Pharmacopoeia [12]. According to the specifications, not less than 80% of the labeled amount of the active pharmaceutical ingredient (API) should be liberated within 20 min. The data from the dissolution experiments using the proposed SI methods based on reaction of the thiol with MP and BP are shown in Table 3. Table 3 also includes comparative data using the HPLC procedure recommended by the USP [12] and SI derivatization by EP [6]. The obtained dissolution data were compared statistically by the similarity factor f_2 [11]. In all cases the

Table 2
Validation parameters of the SI methods.

Validation parameter	MP–CAP	BP–CAP
Linearity (mol L^{-1})	1.0×10^{-5} to 1.8×10^{-4}	1.0×10^{-5} to 2.2×10^{-4}
Regression equation	$A = (508.4 \pm 14.7) \times 10^4\ c(CAP) + (0.67 \pm 1.73)$	$A = (430.9 \pm 14.7) \times 10^4\ c(CAP) + (1.09 \pm 2.41)$
Regression coefficient (r^2)	0.9999	0.9997
Residuals (%)	−1.3 to +1.9	−2.1 to +1.4
LOD (mol L^{-1})	1.84×10^{-7}	2.08×10^{-7}
LOQ (mol L^{-1})	6.07×10^{-7}	6.86×10^{-7}
Precision ($n = 12$)[a]	1.2%	0.8%
Accuracy (%)[b]	98.3–102.7	98.5–102.6
Selectivity (%)[c]	97.5–101.1	99.5–102.6

[a] R.S.D. at 1.0×10^{-4} mol L^{-1} CAP ($n = 12$).
[b] Percent recoveries of synthetic samples at 0.5, 1.0 and 1.5×10^{-4} mol L^{-1} levels containing 1000 mg L^{-1} placebo.
[c] Percent recoveries in the presence of 0–1000 mg L^{-1} placebo.

values of the similarity factor were in the range of 68–80 proving the validity of the proposed methods for the intended application.

4. Conclusions

Two commercially available propiolate esters – methyl-propiolate and butyl-propiolate – were found to be suitable derivatizing reagents for thiols under flow conditions. Both reagents reacted with the model compounds adequately fast and under mild reaction conditions. The behavior of MP was proved to be quite similar to the previous reported EP in terms of both reaction kinetics and sensitivity. On the other hand, BP showed slower kinetics and sensitivity. Additionally, the cost of BP (0.139 €/mg) is considerably higher compared to MP (0.020 €/mg) and EP (0.008 €/mg). However, the enhanced hydrophobic character of the BP-thiols derivatives could be expected to offer advantages in terms of chromatographic separation and sample pretreatment/preconcentration using solid or liquid phase extraction techniques. In-depth investigation towards this direction is on-going by our group.

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