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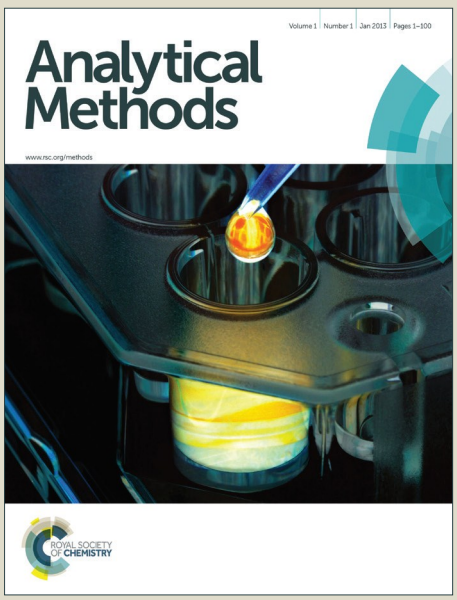

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Technical Note

Quantification of N-hydroxysuccinimide and N-hydroxysulfosuccinimide by hydrophilic interaction chromatography (HILIC)

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Abstract

N-Hydroxysuccinimide (NHS) esters are the most important activated esters used for many different bioconjugation techniques, such as protein labelling by fluorescent dyes and enzymes, surface activation of chromatographic supports, microbeads, nanoparticles, and microarray slides, and finally in the chemical synthesis of peptides. Usually, reactions with NHS esters are very reliable and of high yield, however, the compounds are sensitive to air moisture and water traces in solvents. Therefore, the quantification of NHS would be a very helpful approach to identify reagent impurities or degradation of stored NHS esters. No robust and sensitive method for the detection of NHS (or the more hydrophilic sulfo-NHS) was reported, yet. Here, a chromatographic method based on HILIC conditions and UV detection is presented, reaching a detection limit of about 1 mg/L, which should be sensitive enough for most of the applications mentioned above. Exemplarily, the hydrolytic degradation of a biotin-NHS ester and a purity check of a fluorescent dye NHS ester are shown. An important advantage of this approach is its universality, since not the structurally variable ester compound is monitored, but the constant degradation product NHS or sulfo-NHS, which avoids the necessity to optimize the separation conditions and facilitates calibration considerably.

Keywords

Hydrophilic interaction chromatography (HILIC); Bioconjugation;
N-Hydroxysuccinimide (NHS);
N-Hydroxysulfosuccinimide (Sulfo-NHS);
Active esters;
Quality control (QC)

1. Introduction

A multitude of NHS esters are commercially available, mainly for bioconjugation purposes. In relation to other active compounds, NHS esters are relatively stable and can be stored for months under dry conditions. However, in practice, hydrolysis of NHS esters is a frequent reason for suboptimal conjugation results or even a complete failure of the respective derivatization reactions. Therefore, a quality control of new batches of chemicals or the test of already opened vials of NHS esters is highly recommendable. This can be achieved by the quantitative determination of the NHS ester and its respective carboxylic acid.

More than 50 years ago, N-hydroxysuccinimide (NHS) was proposed by the group of ANDERSON for the preparation of active esters [1-3]. These compounds are extremely useful for peptide synthesis and the preparation of all kinds of bioconjugates. The favorable reactivity of NHS esters, their relative stability towards hydrolysis, good crystallisability and low toxicity made NHS esters to one of the most widely used chemical reagents. In Fig. 1 the structures of the compounds of interest are shown and in Table 1, some properties are collected.

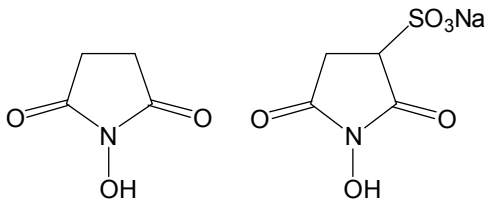


Fig. 1: Chemical structures of N-Hydroxysuccinimide (NHS) and N-Hydroxysulfosuccinimide (sulfo-NHS), sodium salt.

Table 1 Properties of NHS and sulfo-NHS.

	N-Hydroxysuccinimide (NHS)	**N-Hydroxysulfosuccinimide (sulfo-NHS)**
Molecular formula	$C_4H_5NO_3$	$C_4H_4NO_6SNa$ (sodium salt)
CAS No.	6066-82-6	106627-54-7
Molecular Mass	115.09 g/mol	217.13 g/mol
pK_a	6.0 [13]	-
Extinction coefficient	8200 mol/L*cm [14]	-
	8600 mol/L*cm [15]	
	9700 mol/L*cm [16]	

One of the most important NHS ester reagents are biotin-NHS and other esters of biotin derivatives. Furthermore, nearly all fluorescence dyes (fluoresceins, rhodamins, cyanins, bodipy dyes, coumarins, and others) used in bioanalysis can be purchased as NHS esters, often as a simple reagent kit. Another popular application is the use as an activated carrier material in affinity chromatography or affinity extraction. Cross-linked beaded agarose (known as Sepharose) is one of the most frequently used materials, which can be obtained as NHS preactivated beads or columns. In addition, NHS activated magnetic beads are commercially available. NHS esters are used for a multitude of scientific purposes in biochemistry, biotechnology and bioanalysis. A typical method is the activation of a hapten carboxylic acid for conjugation on a protein carrier to obtain a suitable immunogen for antibody generation [4,5]. Even complete virus particles have been selectively modified with NHS esters [6]. Cross-linking of proteins or biochemical complexes are other applications of NHS (esters). The selectivity of NHS esters towards different amino acids and the applicability of NHS cross-linkers to protein-protein interactions were examined by mass spectrometry [7-9].

Anderson et al. emphasized the excellent water solubility of NHS, which facilitates peptide synthesis under aqueous conditions. However, in most cases, the corresponding esters are by far less polar than NHS itself. Therefore, most NHS esters can be analyzed or purified on reversed phase columns without difficulty. For nonpolar carboxylic acids, the resulting NHS esters are nearly insoluble in water. Therefore, more hydrophilic alcohols were developed to improve the solubility of the respective esters. N-Hydroxysulfosuccinimide (sulfo-NHS) is the most popular one, which was introduced by STAROS [10]. It is often used for the catalysis of aqueous condensation reactions with water-soluble carbodiimides, such as EDC (1-Ethyl-3-(3-dimethylaminopopyl)carbodiimide) [11]. Another application was presented by GRUMBACH and VEH, who prepared aqueous stock solutions of a long-chain derivative of biotin, activated by sulfo-NHS [12]. Organic solvents are completely avoided with these reagents. Other valuable reagents based on sulfo-NHS are hydrophilic protein cross-linkers [10].

In most cases, analytical efforts are focused on the carboxylic part of the NHS ester or the ester itself. The released NHS is considered to be a by-product or impurity. In some studies, either the product (e.g. an amide) was determined or the reaction was quenched with slightly acidic buffers, which enables the determination of the residual NHS ester.

Also, some quite indirect methods were reported, as in a recent paper, in which the residual amine was determined by a spectrophotometric method based on 2,4,6-trinitrobenzenesulfonic acid (TNBS) derivatization [17]. Others used titration of carboxylate groups, elemental analysis and depletion methods for the monitoring of NHS ester kinetics [18]. Even biochemical assays (e.g. BSA conjugation) have been used to determine the hydrolysis rate of biotin sulfo-NHS esters [12]. In the paper of CUATRECASAS and PARKIHN [18] tritium-labelled alanine in combination with liquid scintillation was used to determine the

hydrolysis rate of NHS activated agarose. Alternatively, amino acid analyses were performed after hydrolysis with constant-boiling hydrochloric acid.

Only a few researchers realized that the determination of free NHS is a powerful and very general method to monitor any reaction based on NHS or sulfo-NHS esters.

MIRON and WILCHEK presented a spectrophotometric method for the determination of NHS at 260 nm [16]. In this protocol, ammonium hydroxide is used to adjust the optimal pH. However, a very basic pH (e.g. a NaOH solution) leads to the destruction of NHS. The authors already mentioned that UV absorbing substances, such as aromatic amino acids might cause some interference. Nevertheless, under favorable conditions, this method can be useful [17,20].

In the textbook *Bioconjugate Techniques*, the author Greg T. HERMANSON states: "Unfortunately, the sensitivity of assays using absorption usually does not allow for measuring the rate of reaction in an actual cross-linking procedure" [21]. In addition, the strong UV absorbance of NHS at 260 nm may also lead to severe interferences to any protein or DNA determination based on UV. Furthermore, it was reported that sulfo-NHS even interferes with the popular and usually robust BCA protein test [22].

The literature overview shows that NHS and sulfo-NHS is determined only rarely and with complicated and mostly indirect means. The major reason for this surprising situation might be the difficulty to analyze NHS or sulfo-NHS by conventional chromatographic techniques. Therefore, we wanted to develop a robust and convenient method to determine these analytes by HPLC. Due to the extremely high polarity of both compounds, there is essentially no retention on reversed-phase columns. In contrast, the use of hydrophilic interaction liquid chromatography (HILIC) [23], introduced by ALPERT around 1990 [24], seems to be a good choice to resolve this separation problem. Our aim was to develop a chromatographic method for the determination of NHS or sulfo-NHS compatible with UV detection.

2. Materials and methods.

2.1. Chemicals

NHS (98%, # 130672, Sigma-Aldrich) and sulfo-NHS, sodium salt (≥98%, # 56485, Sigma-Aldrich) have been used. Ultrapure water, which was used for samples and mobile phases, had been purified by a stationary MilliQ system (Merck-Millipore). For the analysis of the real samples, biotin-NHS (Thermo Scientific) and Cyanine5 NHS esters (95%, Lumiprobe Life Science Solutions, # 13020; similar to the dye Cy5) have been used. For the preparation of mobile phases for chromatographic analysis, acetonitrile (Fischer Chemicals, LC-MS grade), MilliQ water, ammonium acetate (for HPLC, ≥99%, Fluka, # 17836) and 10% ammonium

hydroxide in water (HPLC grade, Fluka, # 17837) were used. Molecular sieves (4 Å, # 208604, beads, 8-12 mesh) were obtained by Sigma-Aldrich.

2.2. Columns and Chromatography

A silica-based Thermo Synchronis HILIC column (150 x 3 mm) with a particle size of 3 µm was used for experiments at a pH up to 8. A polymer-based SeQuant Zic-pHILIC column from Merck (150 x 2.1 mm, 5 µm) was used for experiments at a pH range between 10 and 11 (Results see Supplement). All HPLC measurements were performed on an Agilent 1200 HPLC system with UV detection at 220 nm and 260 nm.

2.3. Chromatographic method

A zwitterionic silica-based HILIC column (Thermo Synchronis HILIC, 150 mm x 3 mm, 3 µm) was used for the isocratic separation of NHS and sulfo-NHS samples in a 10 mM ammonium acetate buffer (pH 7.5). The mobile phase consisted of 90% acetonitrile and 10% of 10 mM aqueous ammonium acetate (pH 7.5 before mixing). The column temperature was set to 30°C. The system was equilibrated for 10 minutes before each run. The flow rate was 0.4 mL/min and the injection volume was 1 µL. Detection was performed by UV at 220 and 260 nm; the total run time was 20 min. The detection limit was defined as a signal exceeding 3 times the noise of the baseline and the limit of quantitation as 6 times the noise (rounded values).

2.4 Analysis of NHS impurities in biotin NHS ester and Cyanine5 NHS ester

For the analysis of real samples, NHS esters of biotin and Cyanine5 were chosen. The silica-based column (Thermo Synchronis HILIC, 150 x 3 mm, 3µm) was used with the protocol given above. The samples have been dissolved in 10 mM ammonium acetate buffer, pH 7.5. Injection volume was 1 µL. Detection was performed at 220 nm.

2.5 Analysis of biotin NHS ester hydrolysis

The biotin NHS ester samples were dissolved in 10 mM ammonium acetate buffer adjusted to pH 7.0 with acetic acid to control the hydrolysis rate. At pH 6, NHS esters can be considered to be stable for some hours (not shown). Injection volume was also 1 µL.

2.6 Analysis of biotin NHS ester spiked with additional NHS

For spiking experiments solutions of NHS and biotin-NHS esters with the same concentration (1 g/L) have been prepared in dried ACN. For drying, molecular sieves with a pore size of 4 Å have been employed. Samples, containing from 0% to 10% of NHS solution and from 100% to 90% of Biotin-NHS ester solution respectively, have been prepared. Thermo Synchronis HILIC (15 cm x 3 mm, 2.7µm) has been employed with a mobile phase consisting of 90% acetonitrile and 10% of 10mM aqueous ammonium acetate solution with a pH of 7.5. A

column temperature of 30°C, a flow rate of 0.4 mL/min, an injection volume at 1 µl and detection by UV absorbance at 220nm were used.

3. Results and discussion

3.1. Determination of NHS and sulfo-NHS

Based on standard conditions used in HILIC separations, useful isocratic conditions were found to lead to convenient retention times. A neutral ammonium acetate buffer with a high concentration of acetonitrile proved to be suitable. Due to isocratic conditions and low injection volumes, equilibration issues, which are occasionally observed in HILIC separations, are avoided. NHS and sulfo-NHS could be completely separated with retention times of about 5.3 min and 12 min. Based on the new method, calibration lines for the concentration range between 0.5 and 100 mg/L for NHS and 1 and 100 mg/L for sulfo-NHS have been determined. In addition, a limit of detection (LOD) of 1 mg/L and 0.5 mg/L, respectively, and a limit of quantification (LOQ) of 3 mg/L and 1.5 mg/L, respectively, have been calculated.

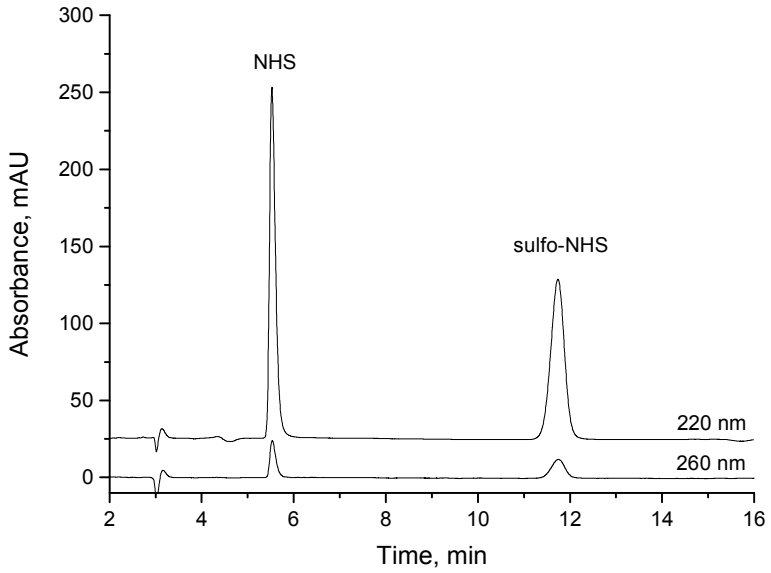
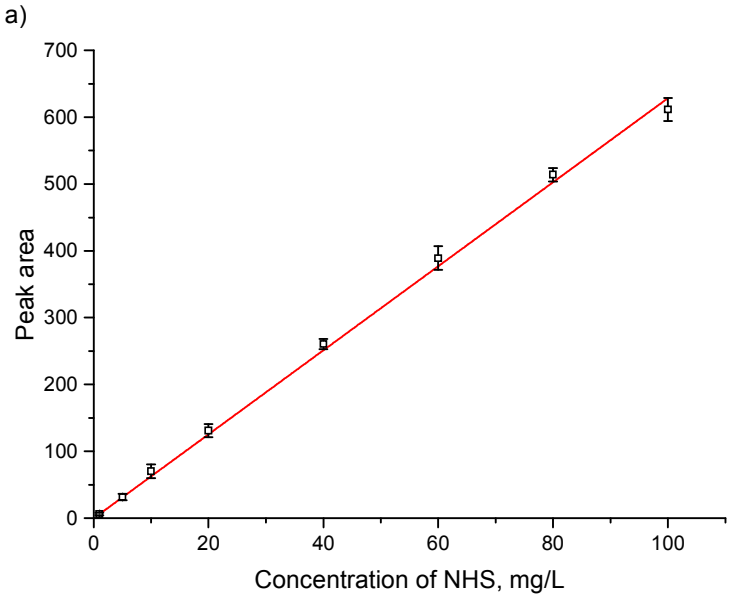


Fig. 2: Separation of NHS and sulfo-NHS on a HILIC column (isocratic, 10% 10 mM ammonium acetate, pH 7.5, 90% acetonitrile, 30°C), sample was 1 µL of 0.1 g/L NHS and 0.1 g/L sulfo-NHS.

3.2. Calibration lines of NHS and sulfo-NHS and validation



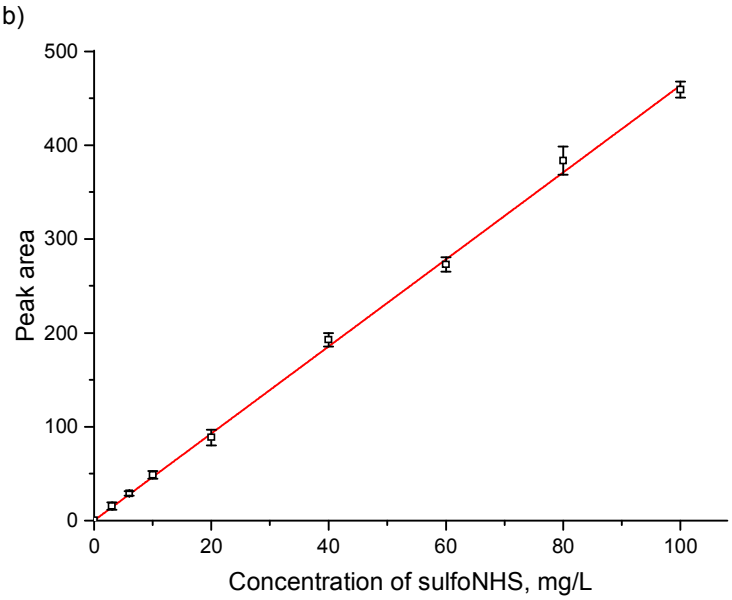


Fig. 3: Calibration lines for NHS (a) and sulfo-NHS (b) obtained on silica-based HILIC column. Limit of detection 1 mg/L (NHS) and 0.5 mg/L (sulfo-NHS); Limit of quantification: 3 mg/L and 1.5 mg/L, respectively.

A calibration for NHS and sulfo-NHS was performed on the silica-based HILIC column, Figs. 4 a) and 4 b). By preliminary experiments it was found that a linear range between about 1-100 mg/L could be expected. Concentrations above 100 mg/L lead to non-linear calibration

curves (not shown), which in some cases might be still acceptable. The detection limit of 1 or 0.5 mg/L is in the range, which can be expected with a standard UV-detector and a compound with extinction coefficients as given in Table 1.

Reproducibility of the method was tested in terms of intra-assay precision (Table 2) and day-to-day precision (Table 3) at three different concentration levels. The latter is based on three replicates per day and a series of five consecutive days.

Table 2: Intra-assay reproducibility (NHS)

Concentration [mg/L]	**Replicates** n	**Mean** [Peak area]	**SD (s)** [Peak area]	**Relative SD** [%]
20	10	130.0	1.9	1.5
100	10	655.5	1.6	0.24
400	10	2576.6	4.7	0.18

Table 3: Day-to-day reproducibility (NHS)

Concentration [mg/L]	**Replicates** n [days]	**Mean** [Peak area]	**SD (s)** [Peak area]	**Relative SD** [%]
20	5	124.7	5.41	4.3
100	5	651.5	14.3	2.2
400	5	2603.6	45.4	1.7

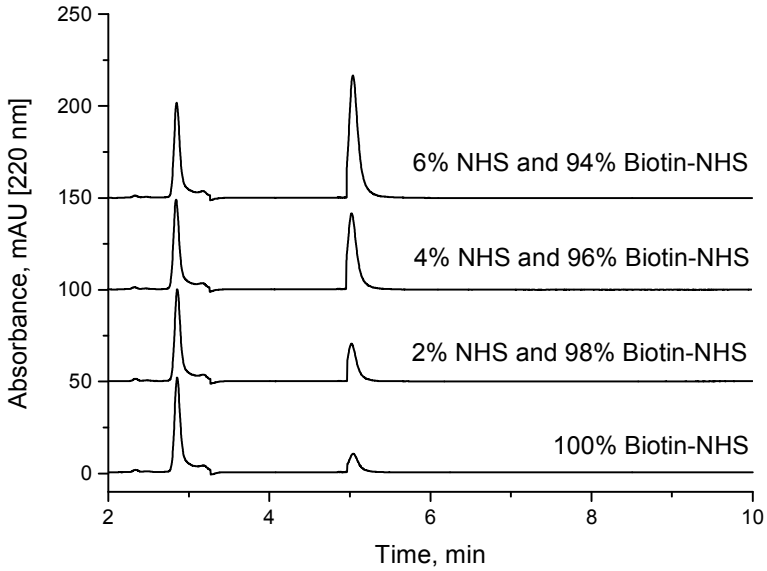


Fig. 4: Spiking experiments based on commercial biotin-NHS with added NHS in different ratios. The percentages are given as nominal values. This means that *100% Biotin-NHS* already contained some NHS as impurity. The biotin-NHS peak can be seen at 2.8 min.

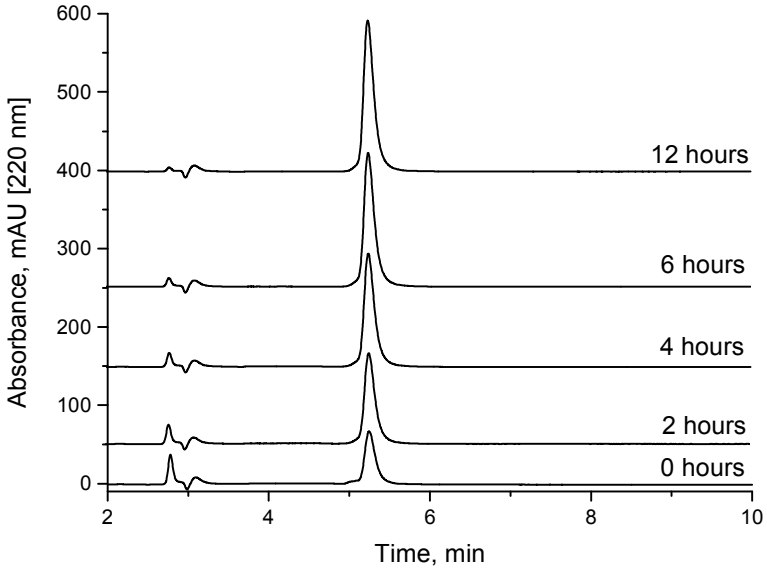


Fig. 5: Analysis of a freshly prepared (0 hours) and a partially hydrolyzed (lines above) biotin-NHS ester solution. The main peak shows the impurity or the degradation product N-hydroxysuccinimide (NHS). The sample was 1 µL of 0.1 g/L biotin-NHS in 10 mM ammonium acetate (pH 7.0), in which the hydrolysis was performed. It can be seen that

the biotin-NHS peak at 2.8 min is decreasing with time, whereas the NHS peak at 5.3 min is increasing.

3.3. Determination of NHS as a degradation product in NHS esters

Under weakly acidic conditions, chromatographic analysis of many NHS esters can be performed on standard RP columns. However, some compounds, such as highly sulfonated fluorescence dyes and other hydrophilic compounds might be difficult to be analyzed this way. In addition, a new method might be required for each different NHS ester. A faster and more general method seems to be the determination of NHS in a NHS ester sample. Since most NHS esters are delivered as purified fine chemicals, initially, the content of free NHS should be very low. Degradation due to wrong or extended storage would be always accompanied by a corresponding increase of the content of free NHS. In addition, quantitation is facilitated, since only one cheap calibration substance (NHS or sulfo-NHS) is necessary, not the purified NHS esters or their corresponding carboxylic acids, which are not available in defined purities in most cases. The quantitative determination of NHS in NHS esters should be a quick and reliable method to verify the quality of the respective active ester. We have analyzed biotin-NHS and a NHS ester of a fluorescence dye (Cyanine5) with our method. The NHS content in spiked samples (Fig. 4) and partially hydrolyzed NHS esters (Fig. 5) could be quantified. Finally, the examination of a commercial fluorescence dye is shown in Fig. 6. All relevant compounds, the activated ester, the carboxylic acid and NHS (both degradation products) could be identified. The purity determination based on NHS and its external calibration leads to a value in good accordance with the declaration of the manufacturer.

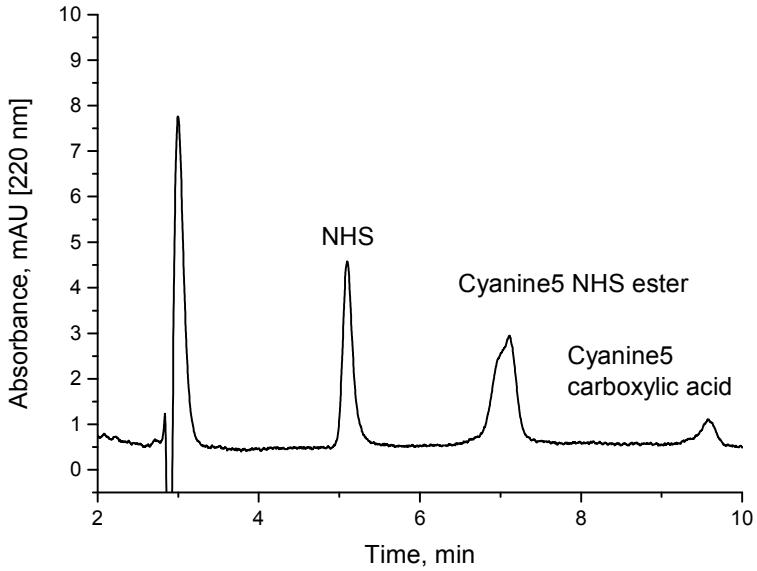


Fig. 6: Analysis of a freshly prepared cyanine5 NHS ester solution (activated fluorescent dye). The peak at about 5 minutes shows the impurity N-hydroxysuccinimide (NHS). The sample was 1 µL of 0.1 g/L cyanine5 NHS in 10 mM ammonium acetate (pH 7.5). Based on an external calibration (Fig. 3) a NHS content of 5.7% was calculated. The manufacturer claims a purity of 95%.

4. Conclusions

The chromatographic determination of the very hydrophilic compounds NHS and sulfo-NHS was considered to be difficult. It could be shown that the application of HILIC columns with mobile phases preferentially at neutral pH is a simple, robust and sensitive method for many experiments, in which (sulfo-)NHS esters are involved. A UV detection at 220 nm and 260 nm is possible, the latter being potentially less prone to spectral interference. The new method might be useful for the purity determinations of NHS esters, monitoring of homogeneous or heterogeneous bioconjugations, such as the biotinylation of proteins, the immobilization of biomolecules on polymeric carriers and the labeling of proteins with fluorescence dyes. An exceptional benefit of this approach is its universality. Since (sulfo-)NHS is the analyte of interest and not the variable part of the reaction, the separation conditions do not have to be optimized and a standard protocol might be applied routinely, particularly for quality control purposes.

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