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ORIGINAL

Liquid Chromatography Analysis of Reactive Oxoammonium Cations

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Abstract
This study presents the first liquid chromatography method for the quantitative and qualitative analysis of highly reactive oxoammonium cations based on a simple derivatization reaction. Rapid 1,2-electrophilic addition reactions with olefins were used to transform these reactive species into analyzable derivates. Three model substances were chosen to represent each of the main application fields of oxoammonium cations and to demonstrate the versatility of the method. The measuring protocol was validated according to the ICH and USP guidelines. The method revealed an excellent linearity ($R^2 = 0.9980–0.9990$) with a low limit of detection (0.16–0.14 mmol L^{-1}) and a low limit of quantification (0.55–0.43 mmol L^{-1}). The protocol was finally used to determine the oxoammonium cations in the presence of their corresponding radical, showing a robustness against impurity concentration of up to approx. 30%.

Keywords LC · LC–MS · Highly reactive analytes · Oxoammonium cations · Derivatization protocol

Introduction

Despite the fact that 2,2,6,6-tetramethylpiperidinyloxyl (TEMPO)-based oxoammonium cations (OCs) play an important role in many current developments, like green oxidation chemistry or organic energy storage, they are fairly unheeded. Their most outstanding property is their stable and reversible redox behavior (Fig. 1) involving the oxoammonium cation **1** and the corresponding aminoxyl radical **2**. Another redox reaction, between the radical **2** and the hydroxylamine **3**, is possible but the pH-dependency of this step is limiting the application possibilities [1].

The reversible redox reactions of OCs are frequently used in organic synthesis to oxidize primary or secondary alcohols to the corresponding aldehydes/ketones or even carboxylic acids [2, 3]. The oxoammonium cation can act as the oxidant itself or as oxidation catalyst in connection with cost-efficient inorganic oxidants like sodium hypochlorite or oxygen, offering a sustainable and green way to produce aldehydes, ketones, and carboxylic acids from alcohols [4–6]. Further fields of application are electrochemical energy storage, with the oxoammonium–aminoxyl redox couple as one of the best investigated systems with regard to organic batteries [7–9], and biological and pharmaceutical applications of the oxoammonium cation, *e.g.*, as antihypertensives or pro-oxidants [10–12].

Despite the broad application possibilities of OCs, the analytical methods for this species are rather limited. While many of their corresponding hydroxylamines and radicals can be identified and quantified using standard methods, like ESR, NMR, titration, or MS, even the qualitative analysis of the oxidized state is difficult [8, 13–15]. On the one hand, oxoammonium cations, in general, are prone to decomposition reactions and possess only a limited lifetime [16]. On the other hand, there are also method-specific drawbacks. Small amounts of radical disturb NMR measurements and ESR is neither sensitive nor sufficient accurate to quantify radical impurities to draw conclusions on cation purity [17, 18]. Furthermore, the IR or UV/Vis spectra of the radical and the oxoammonium cation are in many cases too similar for proper analysis [19–22]. A simple evaluation utilizing the increase or decrease of specific bands is thus often not suitable. However, more advanced techniques like near-infrared

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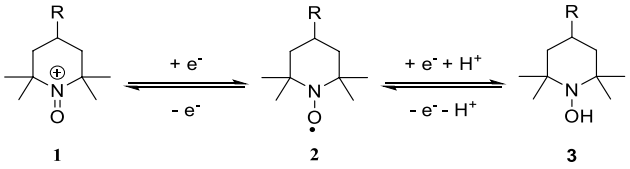

Fig. 1 Schematic representation of the redox chemistry of TEMPO-based molecules

spectroscopy in combination with chemometrics might overcome the problems [23–25]. MS techniques are suitable in terms of accuracy and sensitivity but the discrimination between the two redox species is not sufficient. Furthermore, the radical is oxidized to the corresponding oxoammonium cation under MS conditions[26] and the commonly used steel parts in HPLC and GC systems can reduce the oxoammonium cation to the radical form due to its high oxidation potential [8]. To circumvent the stated drawbacks, a derivatization reaction that transforms the oxoammonium cation into a less reactive and more distinguishable species is highly desirable.

In 1999, Takata and co-workers showed that oxoammonium cations can undergo fast 1,2-electrophilic additions with olefins under excellent yields [27]. But the derivates were not stable, even at − 20 °C. We used the reaction introduced by Takata et al. as the starting point for our derivatization method (Fig. 2). We could improve the reaction conditions by optimization and could apply the new protocol to three different oxoammonium cations. Namely, the Bobbitt salt (*N*-(2,2,6,6-tetramethyl-1-oxopiperidin-1-ium-4-yl)acetamide tetrafluoroborate) (**4**), the TMA-TEMPO (2,2,6,6-tetramethyl-1-oxo-4-(trimethylammonium)piperidin-1-ium dichloride) OC (**5**), and the TEMPOL (4-hydroxy-2,2,6,6-tetramethyl-1-oxopiperidin-1-ium chloride) OC (**6**) were chosen as model substances for oxidation catalysts, organic radical batteries, and pharmaceutical applications, respectively, due to their wide spread in their respective fields (Fig. 2). We report that the standard solutions of the derivatives lose only approx. 0.5% d^{-1} over 12 days at 5 °C. We then demonstrated a valid derivatization-based liquid-chromatography method for separation, identification, and quantification of the oxoammonium cations, even in presence of the corresponding radical species.

Materials, Methods and Instruments

Chemicals and Reagents

Sodium chloride (≥ 99.5%, Fisher Scientific Ltd., United Kingdom), TEMPOL (ABCR GmbH, Germany), 4-acetylamino-2,2,6,6-tetramethyl-1-piperidinoxy (TCI, Japan), (*N*-(2,2,6,6-tetramethyl-1-oxopiperidin-1-ium-4-yl)acetamide tetrafluoroborate (TCI, Japan), tetramethyl urea (TMU) (TCI Chemicals, Japan), mesitylene (VWR International GmbH, Germany), acetonitrile (HiPerSolv CHROMANORM®, VWR International GmbH, Germany), water (HiPerSolv CHROMANORM®, VWR International GmbH, Germany), formic acid (HPLC LiChropur, Sigma Aldrich, USA), *N*-vinylcarbazole (Sigma Aldrich, USA) and ESI-L Low Concentration Tuning Mix (Agilent Technologies, USA) were purchased and used without further purification. TMA-TEMPO and methyl viologen were synthesized according to literature procedure [8].

Instrumentation

An UltiMate 3000 HPLC system equipped with a DAD (Thermo Scientific, USA) was used for separation, identification and quantification of the three derivatization products as well as the internal standard. For data processing the Chromeleon 7.2 software was used.

For assignment of the DAD signals, LC–MS measurements were performed utilizing a Series 1200 HPLC (Agilent Technologies, USA) equipped with a micrOTOF QII mass spectrometer (Bruker, Germany) and a DAD. Ions were generated using the electrospray ionization source (ESI) and a measurement range between *m/z* 280 and 3000 was used. The MS-data were evaluated with the Bruker Data Analysis software version 4.2 and the HPLC data were analyzed with the HyStar 3.2 software.

Weighing was done on an XS205 Dual Range balance (Mettler Toledo, USA).

Chromatographic Conditions

The HPLC separation was done with a C18 column (Kinetex EVO C18, 150 mm × 4.6 mm, 5 µm, Phenomenex, Germany) using a gradient program with two mobile phases A and B. The mobile phase A is acetonitrile with 0.1 vol.% formic acid and the mobile phase B is pure water with 0.1 vol.% formic acid. The flow rate was set to 1.5 mL min^{-1} and the gradient was programmed as: Time (min)/B (%): 0.0/85.0, 11.0/40.0, 12.0/40.0, 14.0/5.0, 16.0/5.0, 17.0/85.0 and 22.0/85.0. The column temperature was maintained at 40 °C and the auto-sampler temperature was set to 5 °C. The injected volume of the sample solutions was 10 µL. The absorbance was monitored using a DAD-detector.

The same conditions, mobile phase, injected volume and column was used for LC analytics with subsequent MS. The gradient program was extended to 27 min and programmed as: time (min)/B (%): 0.0/85.0, 11.0/40.0, 12.0/40.0, 14.0/5.0, 16.0/5.0, 17.0/85.0 and 27.0/85.0 and

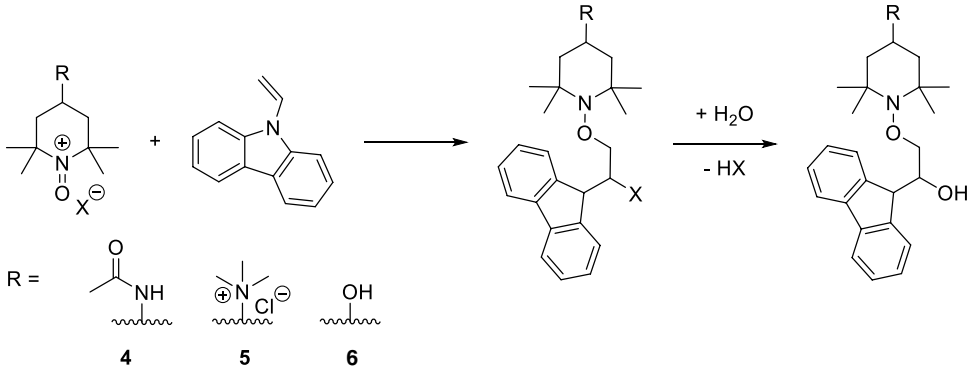

Fig. 2 Schematic representation of the derivatization reaction of the Bobbitt salt (**4**), TMA-TEMPO OC (**5**) and TEMPOL OC (**6**) to the corresponding analyzable derivatives

the auto-sampler temperature was room temperature. The split to the MS was measured as 1/3.

Derivatization Method and Sample Preparation

Stock Solution

Stock solutions of 0.1 M oxoammonium cations with 0.1 M sodium chloride in water were prepared. While the Bobbitt salt was used directly, for TEMPOL OC and TMA-TEMPO OC, the radicals were used and, subsequently, electrochemically oxidized in a redox flow battery (details in the supporting information). The stock solutions were stored at 4 °C until usage.

Sample Preparation for Stability Test

Samples of 1 mL for the stability tests were prepared from the oxoammonium stock solutions and a 0.1 M *N*-vinylcarbazole TMU solution, as well as 100 µL of a 83 mmol L^{-1} (10 mg mL^{-1}) mesitylene solution in TMU as internal standard. The amounts of the components were chosen to fit the respective targeted OC concentration (*cf.* "Results and Discussion"), with a ratio of the derivatization reagent *N*-vinylcarbazole to the analyte of 2:1. The resulting mixture was diluted with TMU to 1 mL and injected into the chromatographic system.

Standard Sample Preparation

The samples were prepared as described for the stability tests, but they were homogenized for 3 min with a vortexer (Vortex Genie 2, Scientific Industries, USA) prior to injection into the chromatographic system.

Sample Preparation for LC–MS Measurements

The samples were prepared as described for the standard sample preparation. But before injection into the chromatographic system, the probes were diluted with TMU to 0.1 mg mL^{-1} for the Bobbitt salt and TEMPOL OC and to 0.01 mg mL^{-1} for the TMA-TEMPO OC sample.

Results and Discussion

Aminoxyl radicals can be oxidized to their corresponding oxoammonium cations either by utilizing oxidizing mineral acids, like perchloric acid [28] and sulfuric acid [29], or other strong oxidants like hypochlorites [30, 31]. But these procedures require additional purification steps to remove potentially disturbing side products. Furthermore, some of these techniques do not tolerate specific organic functional moieties, *e.g.*, the hydroxyl group of TEMPOL can react with sulfuric acid to form an ester [9]. Thus, we decided to use the mild electrochemical oxidation as analyte solution preparation procedure since the received oxoammonium cation solution can be directly used without further purification.

Stability of the Analyte

Takata et al. stated that the addition products of oxoammonium cations and olefins are rather unstable [27]. We thus wanted to investigate if the derivatization protocol of Takata et al. can be used for the named oxoammonium cations. For proper solubility, the utilized chloroform must be exchanged with a 1:3 (v:v) mixture of water and acetonitrile. The stability of the TMA-TEMPO derivative was subsequently investigated using ^{1}H NMR spectroscopy. Herein, freshly prepared analyte samples were placed inside the NMR spectrometer with a pre-tempered probe head (5, 10, 15, 25, 40 °C) for 480 min and every 5 min a spectrum was recorded (*cf.* SI for detailed analysis procedure and all data). The results revealed that the decomposition rates of

17.1% d^{-1} at 5 °C and 99.9% d^{-1} at 40 °C are much too high for HPLC analysis.

Consequently, we exchanged the used solvent mixture with tetramethyl urea (TMU). On the one hand, it is capable of dissolving even double-charged oxoammonium cations as well as the nonpolar internal standard mesitylene. On the other hand, TMU is not nucleophilic and can act as base and binds (possibly) formed acids, which would lead to analyte decomposition via carbazole cleavage. To investigate the impact of the solvent on the stability of the addition products, we prepared three different samples for each of three different concentrations (35, 28, 21 mmol L^{-1}) for each cation. The samples were kept in the auto-sampler at 5 °C for 12 days, and one measurement per day was carried out for each sample.

We wanted to eliminate variations due to sample preparation using the internal standard calibration method. While the ratios of the analyte and internal standard signals differ by up to 40% among the three samples for each concentration, they stay nearly constant over the time of the experiment showing a small trend of decrease. The high differences among the samples with the same concentration might be linked to difficulties during the preparation of the standard solution and possible problems during homogenization of the mixture. The challenge of changing concentrations because of diffusion and osmosis in the RFB is discussed below. The slow decrease of the analyte signal over time can be correlated to the decomposition of the analyte. However, with approx. 0.5–1.0% d^{-1} for the three different concentrations for the different oxoammonium cations, the decay is reduced to approx. one twentieth in comparison to initial derivatization procedure.

Analyte and Side Product Identification

The assignment of the DAD signals (Fig. 3) was done by mass spectrometry (see supporting information Figure S8–S10).

Method Transfer

For method transfer, the retention time of the analyte and the internal standard were determined on the LC–MS as well as on the LC system. The differences of around 0.6 min between the LC–MS and the LC are due to the increased path length in the chromatographic system of the LC–MS.

Method Validation

The developed method was validated according to ICH and USP Guide lines [32, 33]. This includes a system suitability test and the determination of validation parameters like specificity, linearity, accuracy, and precision.

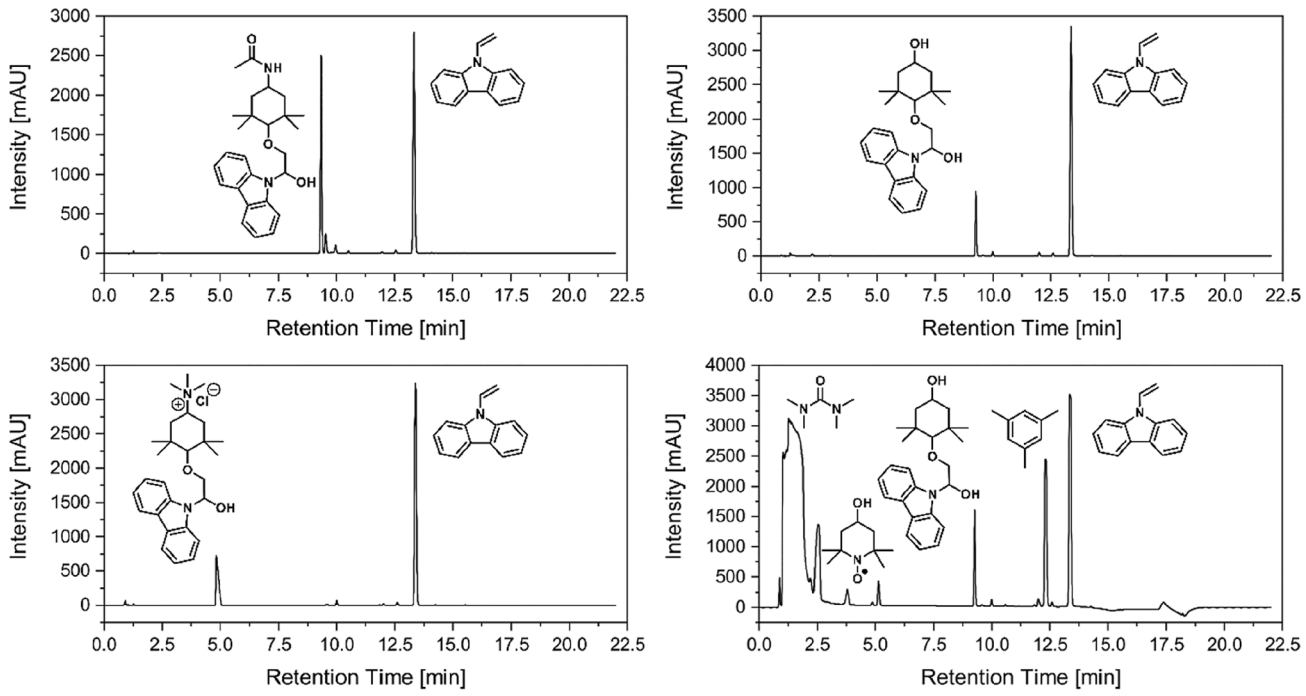

Fig. 3 Typical UV-chromatogram at 291 nm of the *N*-vinylcarbazole adducts of the Bobbitt salt (upper left), TEMPOL OC (upper right), TMA-TEMPO OC (lower left) and the UV-chromatogram at 209 nm of the adduct of the TEMPOL-OC (lower right)

System Suitability Test

After optimizing the sample preparation process (longer homogenization process), system suitability parameters were determined and compared to USP guidelines [34]. The parameters include resolution, plate count, capacity factor, tailing factor, and relative standard deviation (RSD) of the peak areas. To determine the parameters, the data from six replicate injections of solutions with a concentration of 30 mmol L^{-1} were used. The parameters are summarized in Table 1. In general, the parameters fulfill the requirements of the guidelines. An exception is the resolution of the Bobbitt salt, which is a little bit too low with 1.87 (required to be higher than 2) but the peak overlap is so narrow that it is considered as not crucial. Furthermore, the tailing factor of the TMA-TEMPO OC is too high (should be ≤ 2), but it was considered acceptable because of the high resolution. Overall, the system suitability test was considered as successful.

Specificity

To determine the specificity of the method, the resolutions of the OC signals in a mixture with typical synthesis side products and decomposition products need to be investigated. For this purpose, the studied compounds were either commercially available synthesis-grade or self-synthesized chemicals. In both cases, the main side products and impurities are inherently included in the samples, rendering them suitable for specificity tests. The thus performed studies showed no interferences of the OC signals and resolutions higher than 1.5 for all chromatograms (see Supporting Information).

Linearity and Range

The linearity was determined using calibration samples with analyte concentrations of 14, 21, 24, 28, and 35 mmol L^{-1}. For every concentration, six replicates were prepared and injected once per replicate. The received ratios of the peak areas of the analyte and the peak areas of the internal standard were plotted against the respective concentrations. The calibration curves were determined by the least-squares linear regression analysis and showed linear behavior in the whole studied range from 14 to 35 mmol L^{-1} with a determination coefficient (R^2) of 0.9980 to 0.9990. The limits of detection (LOD) and the limits of quantification (LOQ) were calculated using the following formula [33]:

$$\mathrm{LOD} = \frac{3.3\sigma}{S} \tag{1}$$

$$\mathrm{LOQ} = \frac{10\sigma}{S} \tag{2}$$

σ = standard deviation of y-intercept of regression lines, S = slope of the calibration curve.

The results of the linearity studies are summarized in Table 2.

Table 1 Results of the system suitability test ($n = 6$)

Parameter	Bobbitt salt	TMA-TEMPO OC	TEMPOL OC	Internal standard	Required according to USP
Resolution (R_s)	1.87	24.52	2.64	6.25	> 2
Plate count (N)	130,903	5773	127,494	64,575	> 2000
Capacity factor (k')	9.82	4.61	9.72	13.23	> 2
Tailing factor (T)	1.08	3.58	1.04	1.09	≤ 2
% RSD (peak area)	0.27	0.16	0.20	0.18	≤ 1

Table 2 Data of the linearity study

	Bobbitt salt	TMA-TEMPO OC	TEMPOL OC
Range (mmol L^{-1})	14–35	14–35	14–35
Determination coefficient (R^2)	0.9990	0.9985	0.9980
Slope of the calibration curve (S) $mmol^{-1}$ L	175.5695	142.7708	68.4183
Intercept	− 0.0068	− 0.0973	− 0.0299
Standard deviation of the slope (σ)	4.6347	3.1944	1.7834
Standard deviation of y-intercept (σ)	0.0075	0.0068	0.0037
LOD (mmol L^{-1})	0.13	0.14	0.16
LOQ (mmol L^{-1})	0.43	0.47	0.55

Accuracy and Precision

The accuracy and precision were evaluated using intraday and interday recovery experiments with three different samples with concentrations within the linearity range (18, 23 and 30 mmol L^{-1}). For each concentration, six replicates were prepared and injected, and the recovery and RSD were determined, representing the accuracy and precision, respectively. The procedure was repeated on two different days for interday testing (*cf.* SI for detailed analysis procedure and all data). The data are summarized in Table 3.

The precision tests of the method showed intraday RSD values of 6–7% for the Bobbitt salt and 6–8% for the TMA-TEMPO oxoammonium cation. Although the variations are rather high, they are acceptable for the analysis of highly reactive species with a completely newly developed method. We would link the high uncertainties to variations during the derivatization process and, although reduced, the still existing instability of the analytes. In comparison, the interday precision values of the Bobbitt salt (1–3%) and of the TMA-TEMPO oxoammonium cation (2–3%) are comparable to currently applied techniques for HPLC determination. The data for the TEMPOL oxoammonium cation revealed, in contrast, much higher variations of 16%, suggesting that the derivatization and analysis protocol is not suitable for this specific compound. A finding that is also confirmed by the recovery rates of 192–207% (intraday) and 165–175% (interday), with no clear correlation between concentration and deviation. This can be assigned to a possible oxidation of the hydroxy group in 4-position to the corresponding ketone, which can lead to the observed deviations [35].

The TMA-TEMPO oxoammonium cation also revealed high deviations of the recovery rates. In this case, an indirect linear dependency on the concentrations was found. Nevertheless, the received recovery values (121–142% intraday and 122–143% interday) are much too high. This might be caused by a demanding calibration because of the lack of commercially available reference substances. The consequent need of the preparation of the OC via electrochemical oxidation of the TMA-TEMPO radical utilizing a redox flow cell suffers from technical problems like variable concentrations due to osmotic pressure differences [36–40]. To further optimize the method for the TMA-TEMPO system, these problems have to be overcome during future studies. However, the validation parameters suggest the possibility for quantitative analysis of TMA-TEMPO oxoammonium cation with this protocol.

In contrast, the results of the recovery study for the Bobbitt salt with 101–105% intraday- and 101–106% interday confirm the accuracy of the method for this analyte.

Sample Analysis

To further explore the applicability of the method, samples containing one type of OC and its corresponding radical were prepared and analyzed. For this purpose, 0.1 M solutions of the oxoammonium cation and 0.1 M solutions of the corresponding radical were used to produce samples with total concentrations of 35 mmol L^{-1} and OC ratios of 80, 65, and 50%, respectively. The samples were prepared like described above. For each mixture, six samples were prepared and injected once in the HPLC-System. The recovery

Table 4 Data of the recovery studies for samples of the three different oxoammonium cations mixed with their corresponding radicals

	Concentration OC/radical [mmol L^{-1}]	Recovery rate [%]	RSD [%]
Bobbitt salt	28/7	101.99	2.95
	22.75/12.25	109.50	2.54
	17.5/17.5	115.89	6.18
TMA-TEMPO OC	28/7	113.24	11.25
	22.75/12.25	140.82	4.83
	17.5/17.5	152.72	5.82
TEMPOL OC	28/7	202.93	2.97
	22.75/12.25	209.90	3.82
	17.5/17.5	225.15	1.79

Table 3 Data of the intraday (day one) and interday recovery experiments for accuracy and precision determination

	Concentration (mmol L^{-1})	Intraday		Interday	
		Recovery [%]	RSD [%]	Recovery [%]	RSD [%]
Bobbitt salt	30	101	6	101	3
	23	104	6	105	1
	18	105	7	106	2
TMA-TEMPO OC	30	121	8	122	2
	23	135	6	131	3
	18	142	8	143	3
TEMPOL OC	30	192	11	165	14
	23	206	4	175	16
	18	207	7	175	16

rates were determined using the calibration curve and are shown in Table 4.

The data revealed the same trend of deviations as described for the inter- and intraday recovery experiments but with slightly higher recovery rates for all three systems.

This suggests that an increasing amount of radicals interferes with the accuracy of the method, but the effect only becomes significant for radical concentrations higher than 30%. The problem of generally higher recovery rates of analyte in the presence of high radical amounts can be assigned to the possible reaction of the radical with allylic and benzylic substrates to alkoxyamines with similar structures to the analyte, in an NMP like manner [41, 42].

We concluded that the method can be in principle used for the quantitative analysis of oxoammonium cations even in the presence of a high amount of their corresponding radical, but the suitability for a certain substance has to been checked. The unsuitability of the method for the TEMPOL oxoammonium cation due to the possible oxidation of the 4-hydroxy group is confirmed [35].

Conclusion

We developed the first derivatization-based method for the HPLC determination of highly reactive oxoammonium cations. For this purpose, a 1,2-electropilic addition of oxoammonium cations to activated double bonds was used, as described by Takata et al. [27] Based on the latter, a determination protocol was developed and applied to three different cations, which act as model substances for oxidation catalysts, battery materials and pharmaceuticals. While the derivatized analytes are stable under auto-sampler conditions, with a very low decay rate of 0.5–1.0% d^{-1}, the method suffers from the lack of commercially available reference standards. Consequently, standards have to be self-synthesized utilizing electrochemical oxidation in redox flow cells. The consequent deviations in concentration due to osmotic processes makes the calibration error-prone. Nevertheless, the derivatization protocol and the HPLC determination method were validated according to the ICH and USP guidelines. Furthermore, we showed that the protocol can be used even for samples with high radical concentrations of up to 30%, rendering the method suitable for the qualitative and quantitative determination of a broad range of oxoammonium cations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10337-021-04084-1.

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Author contributions PR and RB designed the study. KS, RB, NF and PR performed the experiments. The first draft of the manuscript was written by PR. MDH and USS supervised and acquired the financial support for the project. All authors discussed the results and contributed to the final manuscript.

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Availability of data and materials During the experiments are no other data produced which are not included in the text or supplementary information.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

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