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Aliphatic long chain quaternary ammonium compounds analysis by ion-pair chromatography coupled with suppressed conductivity and UV detection in lysing reagents for blood cell analysers

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Abstract

A method has been developed which allows simultaneous determination of three linear alkyl trimethylammonium salts. Dodecyltrimethylammonium chloride, tetradecyltrimethylammonium bromide and hexadecyltrimethylammonium chloride are widely used as main active ingredients of lysing reagents for blood cell analyzers which perform white blood cells differential determination into two or more subpopulations by impedance analysis. The ion-pair on styrene–divinyl benzene chromatographic phase looks like a suitable, reliable and long term stable tool for separation of such quaternary compounds. The detection based on suppressed conductivity was chosen because of the lack of significance chromophores. A micromembrane suppressor device compatible with high solvent concentration (up to 80%) was used in order to minimize the conductivity background before the detection. In the present work we show how the chemical post column derivatization makes the alkyl chain detectable also by UV direct detection at 210 nm.

Keywords: Quaternary ammonium compounds; Surfactants; Alkyltrimethylammonium; Suppressed conductivity; UV; Lysing reagents

1. Introduction

Alkyltrimethylammonium salts are cationic surfactants widely used in different fields (cosmetic, pharmaceutical, diagnostic, metal working and paper industry), as biocides, corrosion inhibitors, antistatic agents, conditioners, catalysts, emulsifiers, preservatives and also as active ingredients for diagnostic reagents.

Trimethyl long-chain quaternary ammonium salts (C_{12} or dodecyl, C_{14} or tetradecyl and C_{16} or hexadecyl) are widely used as main active ingredients of lysing reagents for blood cell analyzers which perform white blood cells differential determination into two or more sub-populations by impedance analysis [1–9].

The lytic effect of these reagents must be balanced in order to lyse red blood cells and to reduce platelets to volumes below the threshold of detectability by the analyzer's counting and sizing measurement, and to produce a total white blood cell count and two- or three-part white blood cell differential determination.

These reagents must be strong enough in order to adequately stromatolyse all red blood cells (which will contaminate or even entirely obliterate the white cell distributions), and, at the same time, to produce the differential volume-shrinkage lysis of the white blood cell subtypes, without collapsing the cellular volume distribution to a single population.

Lymphocytes are the most sensitive to lysing reagents containing quaternary ammonium salts, while granulocytes are the least sensitive to these types of reagents. Monocytes, eosinophils and basophils are intermediate in sensitivity to these types of quaternary ammonium salt lysing reagents.

The lytic effect of these compounds on all of the white cell subtypes increases in lytic strength with increasing carbon chain length and decreasing in water (polar) solubility. All these compounds affect lymphocytes strongly, and the other subpopulations moderately to strongly, increasing in

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strength with carbon chain length. The dodecyl salt has the most moderate effect on monocytes, eosinophils and granulocytes of any of these three compounds.

Lysing reagents for the volumetric differentiation of two or three leukocytes sub-population by impedance analysis are mainly formulated as mixture of aqueous solutions containing active lytic agents consisting of long chain quaternary ammonium salts, especially trimethyl long-chain quaternary ammonium salts (C_{12} or dodecyl, C_{14} or tetradecyl and C_{16} or hexadecyl). In order to achieve the optimum separation between subpopulations of white blood cells, it is important to find a suitable ratio of the individual quaternary ammonium salts.

This is because is extremely important not only to design a suitable lysing formulation, with a balanced concentration of various chain lengths of quaternary ammonium salts, but also to provide a suitable quality control method, in order to guarantee batch to batch reproducibility and product performance.

In this work a method has been developed for the separation and simultaneous determination by reverse-phase ion pair chromatography of three linear alkyltrimethylammonium salts: dodecyltrimethylammonium chloride (DTAC), tetradecyltrimethylammonium bromide (TTAB) and hexadecyltrimethylammonium chloride (HTAC).

Because of the lack of suitable chromophores, linear alkyltrimethyl ammonium surfactants cannot be easily detected by direct UV; alternative detection systems as suppressed conductivity [10,11], mass spectrometry [12–14], indirect UV [15–19] or ion-selective electrode [20], have been proposed for the analysis of these cationic surfactants. In our method, detection was performed by two different detectors, an electrochemical detector and a UV detector: a post column micromembrane suppressor device, compatible with high solvent concentration (up to 80%), was also used, in order to minimize the conductivity background before the detection.

In this work we show how the chemical post column hydroxide derivatization makes the alkyl chain of this cationic surfactants detectable by UV detection at 210 nm, allowing a reliable and rapid method for the routine analysis of these compounds in commercial lysing reagents.

2. Experimental

2.1. Equipment and analytical conditions

A HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) equipped with a HP 1100 series quaternary pump, a HP 1100 series vacuum degasser, a HP 1100 thermostatted column compartment, a Rheodyne model 7725i injector having a 20 μl loop, a HP 1100 diode array detector, a HP 35900E A/D converter, was used combined with a Dionex electrochemical detector ED40, equipped with a conductivity cell (Dionex, Sunnyvale, CA, USA).

The chromatographic column used was a polymeric reversed-phase Dionex IonPac NS1 analytical column, 250 mm × 4 mm, particle size 10 μm, equipped with the recommended guard column IonPac NG1, 35 mm × 4 mm, particle size 10 μm (Dionex, Sunnyvale, CA, USA).

A chemical post column derivatization was made by including a micromembrane suppressor between the column and the electrochemical detector, in order to minimize the conductivity background. The suppressor used was a solvent compatible CMMS-II Cation MicroMembrane Suppressor (Dionex, Sunnyvale, CA, USA). The regenerant solution used was a 50 mM KOH solution.

Detection was made using two detectors in series (see Fig. 1 for the system configuration), a Dionex ED40 electrochemical detector and a HP 1100 series diode array detector with acquisition wavelength at 210 nm.

The analog signal coming from the Dionex ED40 detector was first converted by a HP 35900E A/D converter before being processed by the Hewlett-Packard HP ChemStation software, rev. A.06.03 (Hewlett-Packard, Waldbronn, Germany). The acquisition settings of the Dionex ED40 detector were the following: 20 μS range, 80% full scale as zero position, 1 V full scale. Acquisition data coming from the HP 1100 series diode array detector were directly processed by the HP ChemStation software. The method setting conditions are shown in Fig. 2.

2.2. Standards, eluents and regenerant preparation

Standards were prepared by accurately weighting and dissolving the three surfactants in HPLC-grade water, and were than filtered at 0.2 μm. DTAC (>97%) was purchased from Tokyo Kasei (Tokyo, Japan), TTAB, also known as Cetrimide BP (≥99%) was purchased from FeF Chemicals A/S (Køge, Denmark), and HTAC (≥98%) was purchased from Fluka Chemie GmbH (Buchs, Switzerland).

Eluents were prepared by premixing the organic modifier, acetonitrile (ACN) of analytical grade purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA), and freshly prepared 0.2 μm filtered HPLC-grade water, than adding, as

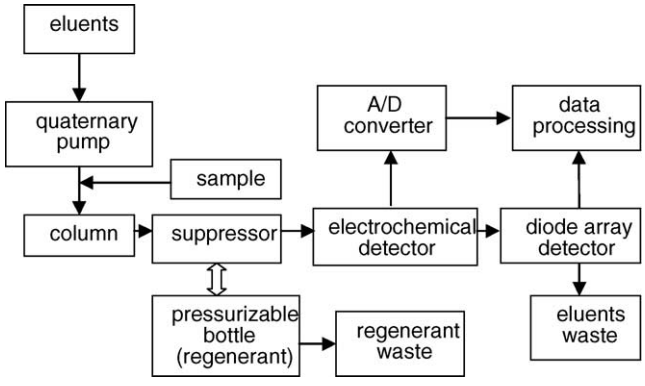

Fig. 1. Scheme of the system configuration.

Column:	Dionex IonPack NS1, 10 μm , 250 x 4 mm
Guard column:	Dionex IonPack NG1, 10 μm , 35 x 4 mm
Eluent:	A) 10 mM MSA in ACN/HPLC Grade Water (50:50 v/v) B) 10 mM MSA in ACN/HPLC Grade Water (80:20 v/v)
Flow rate:	1.0 mL/min
Injection volume:	20 μl sample loop
Temp.:	25 °C
Detection:	suppressed conductivity, UV (210 nm)
Suppressor:	Dionex CMMS-II, Cation MicroMembrane Suppressor
Regenerant:	50 mM KOH
Gradient:	

time (min)	0	15	17	20
% A	100	0	0	100
% B	0	100	100	0

Fig. 2. Method setting conditions.

ion pair reagent, methanesulphonic acid (99%), purchased from Acros Organics N.V. (Geel, Belgium), in order to obtain a final concentration of 10 mM, and were than filtered at 0.45 μm.

The suppressor regenerant was prepared by diluting KOH 45%, purchased from J.T. Baker (Phillipsburg, NJ, USA), with freshly prepared 0.2 μm filtered HPLC-grade water. This suppressor regenerant was less expensive and less toxic than the Dionex recommended 100 mM tetrabuthylammonioum hydroxide (TBAOH) [11], allowing, at the same time, a suitable background conductivity.

HPLC-grade water for standards, eluents and regenerant preparation was freshly produced by a water purification system Smeg WP4100 (Smeg, Italy).

2.3. Suppressor

Respect to the Dionex original method [11], which refers to a Dionex Cation Self-Regenerating Suppressor CSRS-II in external water mode, in our method we used a chemical suppressor, a Dionex CMMS-II Cation MicroMembrane Suppressor (Dionex, Sunnyvale, CA, USA), connecting the eluent in port to the end of the column, the eluent out port to the inlet of the conductivity cell, the regenerant in port to the pressurizable plastic regenerant reservoir and the regenerant out port to the waste. All the connections were made using peek tube and peek ferrule/bolt fittings. Chemical regeneration was made by a 50 mM KOH solution. The regenerant flow was controlled by a pressure gauge, using nitrogen as pressuring gas. Pressure was set around 5 psi, providing a good background and at the same time a limited regenerant consumption.

3. Results and discussion

A simple, rapid and specific quality control method has been developed for the determination of three linear alkyl trimethylammonium salts widely used as main active ingredients of lysing reagents for blood cell analyzers.

3.1. Dynamic range

The dynamic range was established considering these two factors: (1) the usual concentrations of these surfactants in the commercial lysing reagents, (2) the signal acquired by the

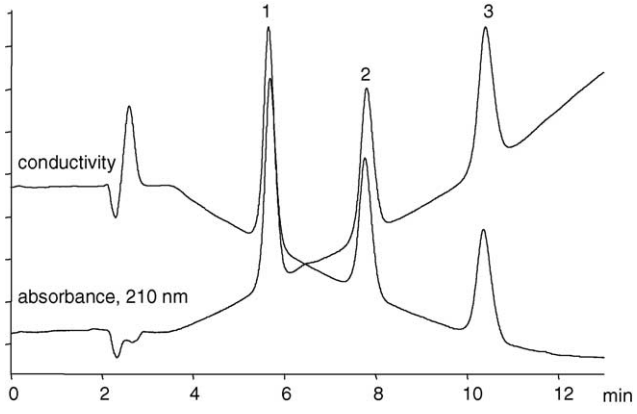

Fig. 3. Chromatogram for a standard solution containing approximately 100 μg/mL of the three alkyl trimethylammonium salts: (1) dodecyltrimethylammonium chloride (DTAC); (2) tetradecyltrimethylammonium bromide (TTAB); and (3) hexadecyltrimethylammonium chloride (HTAC).

Table 1
Correlation coefficients obtained with the diode array detector (r_{DAD}) and with the electrochemical detector (r_{ED40})

Compound	Correlation coefficients	
	r_{DAD}	r_{ED40}
DTAC	0.9995	0.9999
TTAB	0.9997	0.9998
HTAC	0.9989	0.9997

Table 2
Results obtained by UV detection at 210 nm

DTAC				TTAB				HTAC			
μg/mL	Peak area		Rf	μg/mL	Peak area		Rf	μg/mL	Peak area		Rf
	Average	RSD			Average	RSD			Average	RSD	
48.3	57.08	1.63	0.846	50.0	47.60	0.88	1.050	49.4	53.01	3.44	0.932
81.5	96.02	0.94	0.849	79.4	75.42	1.02	1.053	80.8	83.18	1.97	0.971
100.7	117.33	1.85	0.858	99.9	97.06	1.60	1.029	100.4	108.07	2.06	0.929
121.5	144.68	0.65	0.840	119.9	116.42	0.55	1.030	120.5	127.58	1.07	0.945
149.0	179.42	0.72	0.830	150.5	149.32	0.90	1.008	149.7	155.34	0.74	0.964

Table 3
Results obtained by suppressed conductivity detection

DTAC				TTAB				HTAC			
μg/mL	Peak area		Rf	μg/mL	Peak area		Rf	μg/mL	Peak area		Rf
	Average	RSD			Average	RSD			Average	RSD	
48.3	24.65	1.28	1.960	50.0	18.72	0.74	2.671	49.4	16.98	3.50	2.909
81.5	42.04	0.57	1.939	79.4	29.09	0.55	2.729	80.8	26.76	1.45	3.019
100.7	51.78	0.64	1.945	99.9	37.36	0.24	2.674	100.4	34.13	1.61	2.942
121.5	62.73	0.94	1.937	119.9	44.56	0.35	2.691	120.5	40.71	1.61	2.960
149.0	77.72	0.05	1.917	150.5	56.61	0.23	2.658	149.7	50.26	1.74	2.979

ED40 detector needs to be converted by the HP 35900E A/D converter before being processed by the HP ChemStation software. Many trials were performed in order to evaluate the best acquisition settings for the ED40 electrochemical detector. The aim was to obtain a good sensitivity and, at the same time, to contain the entire chromatogram inside the acquisition range of the detector, avoiding out of scale signals.

Fig. 3 shows the chromatogram obtained injecting a standard solution containing approximately 100 μg/mL of the three alkyl trimethylammonium salts.

3.2. Linearity

The linearity of the method was verified in the concentration range 50–150 μg/mL: according to the International Conference on Harmonization Guidelines five calibration standard solutions containing approximately 50, 80, 100, 120 and 150 μg/mL of each analyte (intended as salt) were prepared and an external calibration curve was generated for each analyte by injecting each level three times. The 15 samples were analysed in the same day. All the eluents and the regenerant were freshly prepared.

The correlation coefficient (r) obtained with the electrochemical detector exceeded 0.999 for all the analytes. The correlation coefficient obtained with the diode array detector (DAD) exceeded 0.999 for DTAC and TTAB and 0.998 for HTAC (see Table 1).

According to the International Conference on Harmonization Guidelines linearity was evaluated for each analyte also checking the response factor (Rf) stability in the investigated concentration range: all Rf values (intended as amount/peak area ratio) were inside ±3% of deviation from the mean Rf value. Tables 2 and 3 show the results obtained with the two detectors for each analyte.

Table 4
Correlation coefficient (r), slope coefficient (b) and relative t values coming from the comparison between the peak areas obtained with the two detectors

Compound	Regression parameters			
	r	$t(r)$	b	$t(b)$
DTAC	0.9998	81.6	2.31	46.3
TTAB	1.000	225.4	2.68	141.3
HTAC	0.9996	64.2	3.09	43.4

Considering that we were working in the same chromatographic conditions, we decided to make a comparison between these two detection techniques by plotting the peak areas obtained by UV detection at 210 nm, versus the peak areas obtained by conductivity detection. For each compound the t values for the correlation coefficients (r) and for the slope coefficient (b), were also evaluated: all t values coming from the regression analysis were greater than the upper critical value tabulated for a level of significance of 0.05 and $n-2$ degrees of freedom (3.182), indicating the statistical significativity of the correlation between these two detection techniques. Table 4 shows the regression analysis results coming from the comparison between the two detectors.

4. Conclusion

Trimethyl long-chain quaternary ammonium salts dodecyltrimethylammonium, tetradecyltrimethylammonium and hexadecyltrimethylammonium halides, may be considered as the main active ingredients of commercial lysing reagents for blood cell analyzers which perform white blood cells differential determination into two or more sub-populations by impedance analysis.

In the present work we show how a simple HPLC system equipped with a UV detector may be suitable for the analysis of these compounds, just employing a chemical suppressor for post column derivatization, allowing a simple, rapid and reliable method for the routine analysis of this kind of cationic surfactants as main active ingredients of commercial lysing reagents for hematology.

References

[1] S.L. Ledis, T. Sena, H.R. Crews, J.H. Carter, II, US Patent 4,485,175 (1984).
[2] J.H. Carter, S.L. Ledis, H.R. Crews, T. Sena, F.L. Larsen, US Patent 4,528,274 (1985).
[3] F.L. Larsen, US Patent 4,529,705 (1985).
[4] J.D. Lapicola, S.M. Edmondson, Jr., US Patent 4,745,071 (1988).
[5] W.L. Ryan, US Patent 5,250,438 (1993).
[6] S.-C. Wong, US Patent 5,227,304 (1993).
[7] E.L. Carver, Jr., D.D. Carver, M.P. Skiptunas, US Patent 5,316,951 (1994).
[8] E.L. Carver, Jr., US Patent 5,486,477 (1996).
[9] H.R. Crews, J.H. Carter, II, T. Sena, US Patent 6,632,676 (2003).
[10] J. Weiss, Ion Chromatography, second. ed., VCH, Weinheim, 1995, pp. 279–280.
[11] Technical Note 12, Method development using ion-pair chromatography with suppressed conductivity detection, Dionex, Sunnyvale, CA.
[12] S.A. Coran, M. Bambagiotti-Alberti, V. Giannellini, G. Moneti, G. Pieraccini, A. Raffaelli, Rapid Commun. Mass Spectrom. 12 (1998) 1.
[13] S.A. Coran, V. Giannellini, M. Bambagiotti-Alberti, S. Catinella, P. Traldi, J. Mass Spectrom. 31 (1996) 819.
[14] M. Bambagiotti-Alberti, S.A. Coran, V. Giannellini, D. Favretto, P. Traldi, Rapid Commun. Mass Spectrom. 8 (1994) 439.
[15] P. Helboe, J. Chromatogr. 261 (1983) 117.
[16] K. Heinig, C. Vogt, G. Werner, J. Chromatogr. A 781 (1997) 17.
[17] C.S. Weiss, J.S. Hazlett, M.H. Datta, M.H. Danzer, J. Chromatogr. 608 (1992) 325.
[18] H.-Y. Liu, W.-H. Ding, J. Chromatogr. A 1025 (2004) 303.
[19] E. Piera, P. Erra, M.R. Infante, J. Chromatogr. A 757 (1997) 275.
[20] E.M. Basova, N.V. Shvedene, T.V. Shishkanova, O.A. Shpigun, J. Anal. Chem. (1995) 450.