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Short communication

# Separation and quantitative analysis of alkyl sulfobetaine-type detergents by high-performance liquid chromatography and light-scattering detection

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**Abstract**

An improved high-performance liquid chromatographic (HPLC) method for the separation of zwitterionic detergents is described. It is based on a reversed-phase liquid chromatography with evaporative light-scattering detection (ELSD). The method was shown to be highly specific, allowing the separation of three detergents of the alkyl sulfobetaine family: 3-(*N*-dodecyl-*N*,*N*-dimethyl-ammonio)-propane-1-sulfonate (SB12), 3-(*N*-tetradecyl-*N*,*N*-dimethyl-ammonio)-propane-1-sulfonate (SB14) and 3-(*N*-hexadecyl-*N*,*N*-dimethyl-ammonio)-propane-1-sulfonate (SB16). It was further used to develop a quantitation method for SB14, which was validated for linearity, precision, robustness, limits of detection and quantitation, specificity and accuracy. Linearity was found in the range of 50–500 μg/ml with a correlation coefficient of $0.9938 \pm 0.0029$. The mean value of slope and intercept were $1.567 \pm 0.06$ and $0.1541 \pm 0.0271$, respectively. The limits of detection (LOD) and quantitation (LOQ) were 2 and 10 μg/ml, respectively. The validated method was used to determine the concentration of SB14 in different biological samples, specially in bulks of a recombinant membrane protein, the *Klebsiella pneumoniae* outer membrane protein A, which is produced at the pilot scale for human clinical studies.


*Keywords:* High-performance liquid chromatographic (HPLC); Evaporative light-scattering detection (ELSD); Detergent; Zwitterionic; Alkyl sulfobetaine; Surfactant

## 1. Introduction

Alkyl sulfobetaines, also called Zwittergents®, are unique members of the class of zwitterionic detergents, mainly because they retain their zwitterionic character over a wide range of pH. This property is attributed to the presence of both a strongly basic quaternary ammonium ion and an acidic sulfonate ion of equal strength. Because of their relative pH-insensitivity, sulfobetaines appear to be more related to non ionic than to zwitterionic detergents. The general chemical name of alkyl sulfobetaines is 3-(*N*-alkyl-*N*,*N*-dimethyl-ammonio)-propane-1-sulfonate. They differ in the length of their linear hydrophobic tail which can contain 8–16 carbons (Fig. 1). Alkyl sulfobetaines, specially SB14, are widely used to extract proteins from membranes [1–4], as well as to purify and renature recombinant proteins [5–8]. During the extraction and purification of a protein, it is often necessary to change or remove the detergent, or to adjust its concentration to a certain level. Therefore, it is important to control the detergent concentration throughout a protein purification process. Various methods have been described in the literature for the quantitative determination of detergents, including spectrophotometric, potentiometric, titrimetric and labeling methods.

A colorimetric method has been described for the quantitative analysis of SB14 in protein mixtures [9]. This method was adapted from the classical Lowry protein determination technique and was based on the use of the Folin–Ciocalteu phenol reagent to measure the concentration of SB14 in the presence or absence of protein. A titrimetric method was also developed to determine the concentration of different zwitterionic detergents including SB12 in solution by titration with ammonium sulfate in the presence of Triton X-100 [10]. The sensitivity of these methods was relatively low, and they cannot allow the identification of detergents. A chromatographic method was recently described which combined the use of chemiluminescent nitrogen detection with an HPLC separation [11]. It allows the separation,

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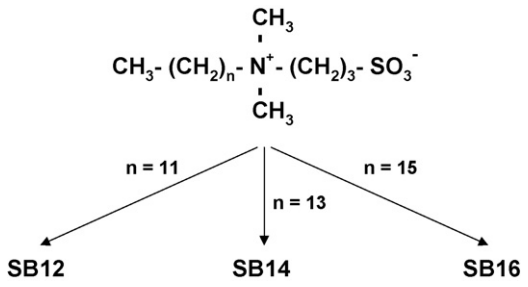


Fig. 1. Structure of the sulfobetaines used in the study: $n = 11$, 3-(*N*-dodecyl-*N*,*N*-dimethyl-ammonio)-propane-1-sulfonate or SB12; $n = 13$, 3-(*N*-tetradecyl-*N*,*N*-dimethyl-ammonio)-propane-1-sulfonate or SB14; $n = 15$, 3-(*N*-hexadecyl-*N*,*N*-dimethyl-ammonio)-propane-1-sulfonate or SB16.

identification and quantification of different cationic and zwitterionic surfactants. The LOD for SB12 was in the low micromolar range and a linear response was obtained between 50 μM and 5 mM. No other chromatographic method has been described so far.

Most of the published HPLC methods for chemical compounds used detection by UV absorbance and gradient elution with different solvents, such as acetonitrile, methanol or isopropanol. UV detection however has two essential disadvantages. First, the choice of mobile phase is limited to solvents with low UV absorbance. Secondly, the analysis of compounds exhibiting poor UV responses is impossible. For such compounds, refractive index detection can be used. However, it has many disadvantages, such as lacking sensitivity and incompatibility with gradient elution, and quantitative analysis is therefore extremely difficult. Some of these problems can be overcome with the evaporative light-scattering detection (ELSD). Indeed, it is especially attractive for analyzing compounds which do not absorb in the near UV (200–400 nm) or which have weak chromophores in this UV range, such as carbohydrates, steroids or surfactants. In the recent past, ELSD has become more popular as a universal detector, and applications are now widely reported for the determination of detergents, such as polysorbate 80 [12] and octylglucoside [13], and for the separation of surfactant mixtures [14,15].

We have recently described *Klebsiella pneumoniae* outer membrane protein A (OmpA), called P40, as a new carrier protein for vaccines. This protein was demonstrated to be as potent as the reference carrier tetanus toxoid already used in human conjugate vaccines since it was able to induce strong antibody responses against B cell epitopes, such as peptides [16,17] and bacterial polysaccharides [17–19] coupled to it. Moreover, the protein also works as a potent immunological adjuvant for specific cytotoxic T lymphocyte (CTL) response induction *in vivo* [20,21]. P40 is currently evaluated in clinical phase I in human in association with a CTL peptide in melanoma. It was produced at the pilot scale in *Escherichia coli*. It is overexpressed as an intracellular protein in inclusion bodies and thus needs to be renatured before purification. As P40 is a membrane protein, the presence of a detergent was essential for complete renaturation. The detergent SB14 was selected for its higher capacity to solubilize and renature P40 [16].

The goal of the present study was first to develop a liquid chromatography method for the separation of alkyl sulfobetaines. The basis of the method developed here consists of a solvent extraction of the detergent from the protein solution followed by its separation on a C-18 column. The detection is achieved using ELSD. This method has been further applied to quantify SB14 in bulks of the adjuvant protein P40 developed for human cancer vaccines.

## 2. Experimental

### 2.1. Reagents

SB12, SB14 and SB16 were purchased from Sigma (Saint Quentin Fallavier, France). Acetonitrile was HPLC grade (SDS, Peypin, France). Water was purified by means of a Milli-Q Plus Water system (Millipore, Saint-Quentin-en-Yvelines, France). Solvents were filtered through a 0.45 μm filter (Millipore) before use and degassed with Helium during the experiments. Y-PER reagent was purchased from PIERCE (Rockford, IL, USA). The protein P40 was produced in *Escherichia coli* as previously described [16]. Briefly, the inclusion bodies were collected after lysozyme treatment by centrifugation at $10{,}000 \times g$ for 10 min and solubilized in 7 M urea in the presence of 10 mM dithiothreitol for 2 h at 37 °C. Renaturation of the protein was achieved by diluting the mixture in the presence of 0.1% SB14 (mass/vol). The refolded protein was purified by a two-step ion exchange chromatography process including anion and cation exchange chromatography steps in the presence of 0.1% SB14. This intermediate product, also called P40Z, was further treated to generate a detergent-free protein. After precipitation of the protein by addition of cold ethanol, the pellet was collected by centrifugation at $10{,}000 \times g$ for 10 min and solubilized in 7 M urea. This solution was submitted to a gel filtration step on a Superdex G-25 column (Amersham Biosciences, Saclay, France). The detergent-free protein, called P40s, and the intermediate product P40Z were analyzed for protein by the bicinchoninic acid method and for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

### 2.2. Chromatographic equipment

The chromatographic system consisted of a Agilent (Massy, France) 1050 pump equipped with a model 1100 autosampler and an evaporative light-scattering detector Sedex 55 (Sedere, Alfortville, France). An interface module 35900 (Agilent) was used to convert the analogue signal from the light-scattering detector to digital data.

### 2.3. Sample preparation

A 1 mg/ml SB14 stock solution was prepared by dissolving 100 mg in 100 ml of water in a volumetric flask. For calibration curves, a series of standard solutions were prepared by diluting various amounts of the stock solution with water to give concentrations ranging from 50 to 500 μg/ml. For the determination of

the linearity range, dilutions were made from a 10 mg/ml stock solution in water.

For P40 samples, SB14 was quantified after solvent extraction. Briefly, NaCl was first added to the final concentration of 150 mM. After dilution (1:1.4) in acetonitrile, samples were shaken on a vortex mixer for 20 s for complete precipitation of the protein. The supernatant containing the detergent was collected by centrifugation at 10,000 × *g* for 5 min. No preliminary treatment was needed for Y-PER reagent as it does not contain proteins. Three dilutions of each unknown sample, ranging from 1:5 to 1:100, were performed in water to determine the SB14 concentration. Triplicate HPLC analyses of each dilution were conducted on the same day.

### 2.4. HPLC analysis

A Discovery C-18 (5 μm, 150 mm × 2.1 mm i.d.) column (Supelco, Saint Quentin Fallavier, France) was used at an ambient temperature. The injector valve of the autosampler was fitted with a 100 μl loop. The analysis was performed by gradient elution using solvent systems (A) water and (B) acetonitrile. Eluents were continuously flushed with helium. Gradient started with 30% B for 5 min, followed by a linear gradient to 100% B in 22 min. B was held at 100% during 4 min and then decreased linearly to 30% in 2 min. The time required to reequilibrate the column in a sequence of runs was 15 min. The mobile phase flow rate was 0.2 ml/min and the injection volume was 40 μl. All the samples were filtered through 0.45 μm filter before injection. The evaporation temperature of the light-scattering detector was maintained constant at 40 °C. The nebulization gas was air with a pressure of 2.2 bar. The gain (sensitivity) of the detector was 7.

### 2.5. Data analysis

Calibration curves were obtained with different injected quantities of SB14 between 2 and 20 μg. Each quantity was injected three times. Logarithm of mean peak area was plotted as a function of logarithm of the quantity injected. The linearity of the method was statistically tested. The equation of the regression line was used to quantify recovery and unknown samples.

## 3. Results and discussion

### 3.1. Separation of alkyl sulfobetaines

The HPLC procedure was optimized first to separate three zwitterionic detergents of the sulfobetaine class which differ from their alkyl part including 12, 14 and 16 carbons for SB12, SB14 and SB16, respectively (Fig. 1). Optimized chromatographic and detection conditions are described in Section 2. Acetonitrile–water was chosen as the mobile phase as the solubility of alkyl sulfobetaines was maintained at elevated concentrations of acetonitrile which are required not only for the elution but also for the precipitation of proteins. Optimum nebulizer gas pressure and evaporating temperature, which are important adjustable parameters significantly affecting the signal response in ELSD, were determined to be 2.2 bar and 40 °C, respectively. Fig. 2A shows a typical chromatogram for the separation of the alkyl sulfobetaines studied here. The three detergents were eluted as single symmetric peaks which were highly resolved by gradient elution, each compound being separated from its higher molecular weight form by a 4 min time. The retention times were also highly reproducible. As expected, the lower hydrophobic detergent, SB12, was eluted first from the column and the most hydrophobic one, SB16, needed a higher acetonitrile concentration to be eluted.

### 3.2. Validation of the SB14 quantitation method

The second scope of this study was to develop a method for the quantitation of SB14 in biological samples. Alkyl sulfobetaine peaks were highly symmetrical because of the chromatography conditions used and the homogeneity of the detergents studied. Consequently, the nearly Gaussian peaks obtained were thus estimated to be fully adequate for our purpose. The method was validated (linear range, LOD, LOQ, precision, accuracy) with SB14 samples prepared from stock solutions as described previously. To determine the linear range of the method, various quantities of SB14 ranging from 0.04 to 200 μg were injected three times and peak areas were determined for different detector sensitivities ranging from gain 3 to 9. As observed previously for other quantitative analyses with ELSD [13,22], there was no linear relationship between the peak area and the injected detergent mass. This problem, which was shown to be related to the droplet size inhomogeneity and to depend mainly on the nebulizer geometry and gas flow rate [23,24], can be overcome by transforming the mean peak areas and standard SB14 concentrations to logarithms and drawing a log–log plot. The largest linear range was obtained with a detector gain of 7. In this case, the ELSD response was found to be linear for concentrations of SB14 comprised between 5 and 750 μg/ml, corresponding to an injected quantity range of 0.2–300 μg.

In addition to these results and considering the samples to analyze, a detector gain of 7 and a calibration range of 50–500 μg/ml, within the linear range described above, were selected. Under these conditions, the log–log calibration plot obtained was linear over the entire range chosen (number of data points = 6) with a correlation coefficient ($R^2$) of 0.9938 ± 0.0029 (six determinations). The regression equation was found to be $\log y = 1.567 \pm 0.06\ (\log x) + 0.1541 \pm 0.0271$, where $y$ is peak area and $x$ is SB14 concentration. The detection and quantitation limit values were estimated as the concentrations providing a signal three and ten times higher than the standard deviation of the background noise, respectively. Thus, LOD and LOQ, calculated using the data from the standard curve with the lowest SB14 concentration, were 2 and 10 μg/ml, respectively, corresponding to molar concentrations of 5.5 and 27.5 μM, respectively (molecular weight = 363 Da). The intra-day precision was determined by injecting six different SB14 concentrations, selected in the linearity range of 50–500 μg/ml, 10 times in a single day. Precision of raw peak areas ranged from 4.4 to 13% R.S.D. (Table 1). Raw peak areas, rather than log(peak area), better represents precision because log values exhibited insignificant variations.

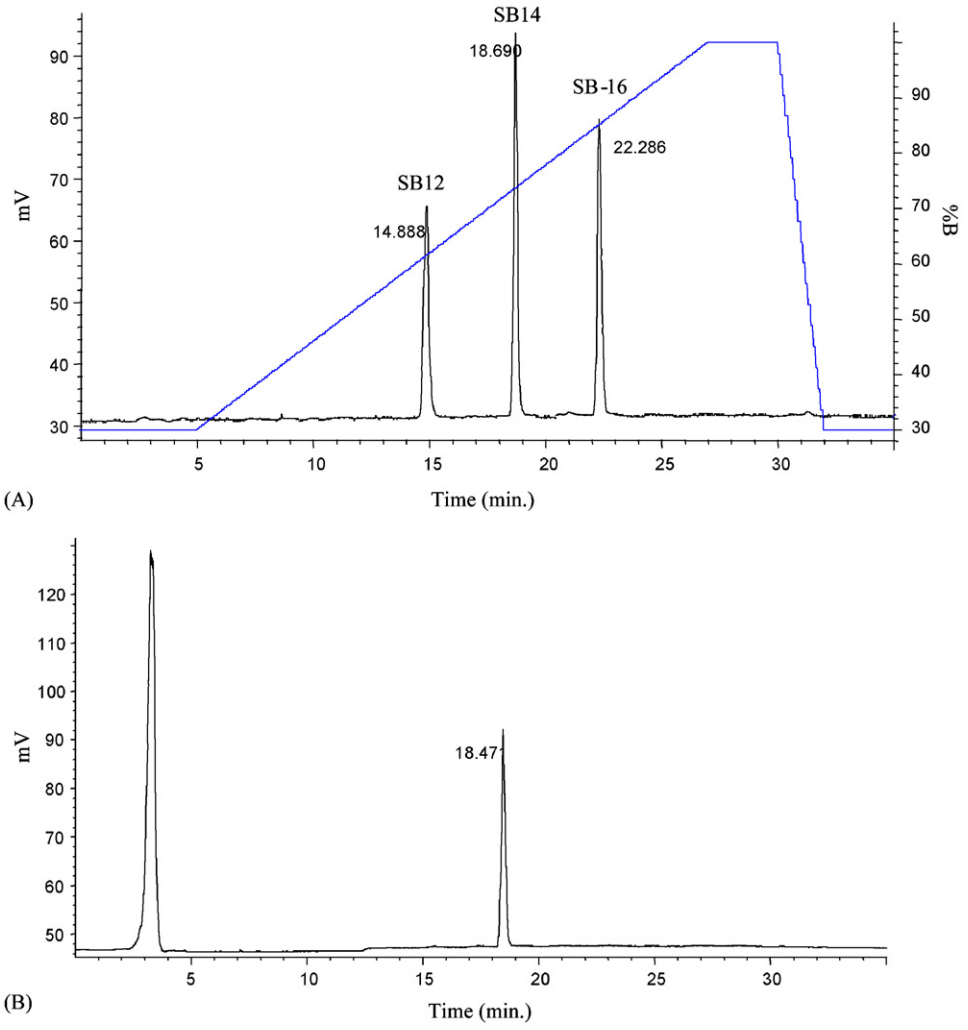


Fig. 2. Typical chromatograms for (A) the separation of three alkyl sulfobetaines: (1) SB12 ($t_R$ 14.88 min); (2) SB14 ($t_R$ 18.69 min); (3) SB16 ($t_R$ 22.28 min); and (B) for the analysis of SB14 after extraction from a P40Z sample.

The within-day precision was better for the lower concentrations in the range studied as the variability was relatively low, *i.e.* 4.4–6.4%, for concentrations comprised between 50 and 350 μg/ml, and the highest R.S.D. value of around 13% was obtained for the last concentration evaluated, 500 μg/ml. The inter-day precision was evaluated for the same standard concentrations. Three series of injection (triplicates) were performed for each standard solution freshly prepared on each day of the study. Precision of mean peak areas determined for each of the three days ranged from 2.8 to 7.3% R.S.D. (Table 1). These between-day R.S.D. values were not correlated with SB14 concentration. Spike studies were conducted for the determination of method accuracy (data not shown). Different amounts of SB14 were added to a known sample, whose concentration was determined before spiking. They were selected such that final concentrations were in the lower, middle and upper parts of the calibration

Table 1
Determination of intra-day and inter-day precision

| [SB14] (μg/ml) | 50 | 100 | 150 | 250 | 350 | 500 |
|---|---|---|---|---|---|---|
| Intra-day ($n = 10$) | | | | | | |
| Mean[a] | 922.74 | 2386.30 | 4208.40 | 8376.46 | 13702.00 | 29200.00 |
| S.D. | 59.40 | 105.58 | 230.58 | 398.63 | 720.66 | 3804.04 |
| R.S.D. (%) | 6.44 | 4.42 | 5.48 | 4.76 | 5.26 | 13.03 |
| Inter-day ($n = 3$) | | | | | | |
| Mean[a] | 796.96 | 2297.71 | 3958.88 | 8058.39 | 12362.22 | 27112.22 |
| S.D. | 22.79 | 108.60 | 168.92 | 405.71 | 903.58 | 1077.63 |
| R.S.D. (%) | 2.86 | 4.73 | 4.27 | 5.03 | 7.31 | 3.97 |

[a] Mean values of peak area.

range. Final concentrations were determined after plotting log-transformed peak area versus log-transformed concentration of the standards by using the equation of the regression line. Very high recoveries were obtained for the three spike concentrations selected, with values comprised between 102 and 107%.

### 3.3. Analysis of biological samples

The validated method was further used to determine the concentration of SB14 in different samples. A calibration was generated by plotting log-transformed peak area versus log-transformed concentration of the standards. The equation of the regression line was used to quantify SB14 in unknown samples. Two types of samples, containing or not containing proteins, were analyzed.

Reversed-phase HPLC of low molecular weight analytes in biological samples containing excessive amounts of proteins usually requires removal of the protein species to eliminate irreversible protein interactions with the stationary phase, specially with C-18 phases which are the most hydrophobic phases, and thus minimize column degradation. For P40 samples, this was done by solvent precipitation with acetonitrile. Complete precipitation of P40 performed under the conditions described in Section 2 was assessed by using the bicinchoninic protein assay. Two different P40 samples were analyzed: SB14 containing samples (P40Z) were process intermediate samples with a detergent/protein ratio (w/w) of around 1 whereas detergent-free samples (P40s) were final products deriving from the former samples by detergent extraction. Four lots of P40Z produced at the pilot scale were analyzed. Fig. 2B shows a typical chromatogram for the analysis of the detergent extracted from a P40Z sample. In comparison with injection of the standard solutions, the peak eluted at 18.471 min was attributed to SB14. The concentration of SB14 for the four lots analyzed was comprised between 2.87 and 3.96 mg/ml with R.S.D. values of 4.35–9.4% (Table 2). On the basis of these results, the mean SB14 concentration of P40Z lots was $3.32 \pm 0.49$ mg/ml. This low batch-to-batch variability (R.S.D. = 14.76%) can be correlated with the fact that these lots were produced with the same process. A similar conclusion was obtained from a characterization study which we performed previously at the protein level by using mass spectrometry, analytical chromatography, electrophoresis and different immunoassays [25]. Together with the observations made after this extensive protein characterization study, the results of the present study showed that the P40 purification process is very reproducible and robust. Furthermore, the concentration of SB14 in different P40s lots was significantly lower than the limit of detection previously determined as no peak was detected, even when samples were analyzed without dilution. The concentration value for P40s is thus indicated to be 0 in Table 2. Consequently, this result validated the protocol of elimination of this detergent from the lots of P40Z by solvent precipitation with cold ethanol.

Table 2
Determination of the SB14 concentration in P40 samples and Y-PER reagent ($n = 3$)

| | [SB14] (mg/ml) | S.D. | R.S.D. (%) |
|---|---|---|---|
| P40Z | | | |
| Lot #1 | 2.87 | 0.13 | 4.35 |
| Lot #2 | 3.02 | 0.23 | 7.63 |
| Lot #3 | 3.46 | 0.33 | 9.40 |
| Lot #4 | 3.96 | 0.25 | 6.21 |
| P40s | | | |
| | 0 | – | – |
| Y-PER | | | |
| Lot #1 | 10.25 | 0.16 | 1.54 |
| Lot #2 | 9.8 | 0.43 | 4.42 |

The method was also used to determine the SB14 concentration of a commercially available reagent, Y-PER reagent, described by its manufacturer to use a mild detergent formulation for yeast membrane lysis and extraction of soluble proteins. The detergent of Y-PER was identified as SB14 by mass spectrometry analysis [26], but its concentration was not determined in this study. A series of analyses was conducted to determine this concentration. Contrary to P40 samples, no preliminary detergent extraction step was needed as Y-PER is a protein-free reagent. Our data confirmed first that Y-PER contains only one detergent which is SB14. Indeed, the chromatograms obtained showed only one peak with the same retention time as those of standard solutions. Concentrations of $10.25 \pm 0.16$ and $9.8 \pm 0.43$ mg/ml were determined for the two lots of Y-PER successively analyzed (Table 2). From these results, it can be concluded that the mean SB14 concentration of Y-PER reagent is 10 mg/ml.

## 4. Conclusion

An HPLC method has been developed for the separation of zwitterionic detergents of the alkyl sulfobetaine class. This method is based on a C-18 reversed-phase liquid chromatography with evaporative light-scattering detection. The three detergents studied were well separated and eluted as single symmetric peaks. This separation allowed the development of a quantitation method specific for SB14 which was validated. An excellent linearity was found in the range of 5–750 μg/ml. The method calibration range was selected within the linear range from 50 to 500 μg/ml. The sensitivity was higher than those of the colorimetric and titrimetric methods previously described [9,10]. LOD and LOQ were 2 and 10 μg/ml, respectively. LOD is comparable with the detection limit of 2.7 μg/ml previously reported for a SB12 assay based on a separation by HPLC using cyano-type columns and chemiluminescent nitrogen detection [11]. Validation of the method included also inter- and intra-day precision and recovery studies. As all the validation parameters studied gave acceptable results, the method was further used to determine the concentration of SB14 in different biological samples. It was successfully applied to bulks of a recombinant membrane protein, the *Klebsiella pneumoniae* outer membrane protein A also called P40, which is currently evaluated in clinical phase I in human in association with a CTL peptide in melanoma. This method was found to be rapid and highly reproducible, and there is no doubt that this HPLC–ELSD combination could be applied to the development of quantitative analyses of other

sulfobetaine-type detergents, and eventually of other zwitterionic detergents, in biological samples.

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