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Original article

QUANTIFICATION OF SODIUM DEOXYCHOLATE IN PROTEOMICS SAMPLE PREPARATION USING METHYLENE BLUE

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ABSTRACT

Anionic detergents like Sodium Deoxycholate (SDC) are commonly used in biochemical and molecular biology research for solubilizing and denaturing proteins and nucleic acids. In the presence of anionic detergents, the electrostatic interaction between positively charged methylene blue (MB) and the anions forms a complex that can be extracted into chloroform, allowing for quantification. Without formation of this complex, water-soluble MB remains immiscible in chloroform. Thus, the spectrophotometric detection method of MB-detergent complexes can be employed to determine the concentration of SDC during LC-MS/MS sample preparations. The quantification protocol for SDC was optimized, with the consideration of factors such as pH, buffer solutions and the spectrophotometer wavelength. Several extraction methods were employed to remove detergent from the sample, namely, ethyl acetate, mineral oil extraction and acid precipitation. All of the methods have about the same efficiency. The MB method was used as a standard technique for the precise quantification of detergent amounts which allowed as achieving an approximate concentration range between the lower limit of *ca.* 0.025% and 0.1% of the upper limit.

Key words: Calibration curves, liquid chromatography tandem mass spectrometry (LC-MS/MS), methylene blue (MB), methylene blue active substances (MBAS), sodium deoxycholate (SDC), sodium dodecyl sulfate (SDS), spectrophotometry, serial dilution.

INTRODUCTION

In recent years, the quantification of detergents in various samples has gained significant importance, primarily due to concerns regarding environmental pollution [1]. This is due not only to the widespread use of this class of substances in industry, but also to the increase in their production and consumption during the recent COVID-19 pandemic. Many surfactants are resistant to natural degradation, resulting in irreversible changes in biogeochemical processes in soil and water reservoirs [2]. In addition to environmental issues, the analysis of detergent content in samples is also important for other areas. For instance, in medicine and tissue engineering, detergents such as Sodium Dodecyl Sulfate SDS [3] and sodium deoxycholate (SDC) [4] are used for decellularization from the scaffold of transplanted organs. Since these substances possess cytotoxic properties, it is crucial to determine the residual amount of SDS and SDC, which should remain below the threshold to ensure the viability of newly implanted cells on the matrix. Detergents are also often used as components of buffers for cell lysis during RNA and DNA isolation, as well as protein denaturation for subsequent SDS-PAGE electrophoresis [5]. In the latter case, detergents bind to denatured proteins, making them negatively charged. In addition, the total charge of the resulting complex depends on the number of amino acids, thereby the electrophoretic mobility of the ions proportional to the molecular weight of the protein. Detergents are also widely used in the proteomics sample preparation process, but must be eliminated in the last steps of protocols due to their incompatibility with liquid chromatography system [6] and electrospray ionization (ESI) source [7, 8]. SDS, SDC, and other surfactants also tend to accumulate as deposits on ESI emitters, chromatography columns, and the front end of the mass spectrometer, which incurs additional equipment maintenance costs. Therefore, it is of utmost importance to ascertain the detergent content in a sample intended for LC-MS/MS analysis.

The simplest method for the quantification of detergent is based on the spectrophotometric measurement of the concentration of their complexes with methylene blue which is a standard method being used to determine the surfactants in tap-water samples (ISO 7875-1, 1996). Despite the fact that this method was developed in the 1940s of the last century [9, 10], today there are various modifications of protocols with optimized parameters of ratios and volumes of reagents [11-13].

As an object for quantitative analysis, we chose the detergent SDC, which is not inferior to SDS and other detergents in the ability to solubilize and denature proteins, as well as enhance trypsin activity. Moreover, as shown by Masuda et al.[14], this detergent can be easily removed by extraction with ethyl acetate.

The aim of this study was to use this method for the quantitative analysis of SDC in solutions during sample preparation for LC-MS/MS analysis.

MATERIALS AND METHODS

Materials and equipments

Sodium Deoxycholate (Sigma-Aldrich, Germany, #30970-500G), Methylene blue (Sigma-Aldrich, St. Louis, MO, USA, #M9140-25G), Chloroform (Sigma-Aldrich, St. Louis, MO, USA, #34854-1L), 0.1M Tris hydrochloride (Tris-HCl), 1X Phosphate Buffered Saline (PBS), 1% Trifluoroacetic acid (TFA) (Sigma-Aldrich, Germany, #T6508-100ML), Ethyl acetate (Sigma-Aldrich, Germany, #34858-1L), Mineral Oil (Amresco, USA, High purity grade, #J217-100ML), 1M Na_2HPO_4 (ThermoFisher, Germany, #44814), 0.1M KH_2PO_4 (BLD Pharmatech Ltd, China, #BD140341-500g), 0.1M K_2SO_4 (ThermoFisher, India, #FLP304500), 0.1M KNO_3 (ThermoFisher, India, #44637), 5M NaCl (Sigma-Aldrich, St. Louis, MO, USA, #31434-1KG-R), H_2O (Milli-Q grade), Universal indicator paper pH 0-12 (LACHEMA, Czech Re-

public).

Shimadzu UV-VIS Spectrophotometer UV-1900i, Bio-Rad Model 680 microplate reader.

Sample preparations for spectrophotometric measurements.

Detergent quantification method

A 1 mL stock solution of 0.5% methylene blue in water was prepared in a 1.5 ml tube, covered with foil to avoid light exposure and kept over 50 mL of $CHCl_3$. 5% stock solution of SDC was prepared in water.

To generate standard curves, 0.5% SDC solutions were diluted in water (PBS or Tris-HCl) over a range consistent with use in serial dilution. The samples were then mixed with 0,005% MB aqueous solution at a ratio of 1:2 (MB:sample, v/v). After vortexing samples with MB, chloroform was added at a ratio of 1:2 (sample:chloroform, v/v). Samples were then vortexed four to six times of intermittent touching. The aqueous and chloroform phases were then separated by centrifugation at 2,000 rpm for 3 min at low temperature (4 °C). Tubes were allowed to stand for about 10 min until they were warmed to room temperature. The chloroform layer (2 ml) was transferred to a glass cuvette with an optical pass length of 1.0 cm and absorbance at the wavelength 655nm (OD_{655}) was measured with Shimadzu UV-1900i UV-Vis spectrophotometer. Pure chloroform was used as blank.

Optimized detergent quantification protocol

For standard curves, 0.5% SDC solution was diluted in H_2O (Milli-Q grade) with the range of 0.05%, 0.025%, 0.012%, 0.003%, 0.0008% and 0% (control). To determine residual detergent concentration of SDC after ethyl acetate extraction we initially added 1% trifluoroacetic acid to a 0.5% SDC solution as described by Masuda et al [14]. Then an equal volume of ethyl acetate was added in a 1:1 ratio to the sample, and the mixture was centrifuged at 5,000 rpm for 5 minutes. The organic phase was discarded and aqueous phase alkalized by adding 1M Na_2HPO_4 to pH 7.5 (universal indicator paper). Next, the samples were combined with a 0.005% MB aqueous solution in a 1:2 ratio of sample solution to MB (for instance, for 1.5 mL tubes, 200 mL of MB was added to 100 mL of analyzed sample). After vortexing the samples, chloroform was added in a 1:2 ratio with the sample, and the tubes were once again vortexed and centrifuged at 2,000 rpm for 3 minutes at room temperature. 100 µl of the bottom chloroform layer was immediately transferred to the 96-well polystyrene plate and the absorbance at the wavelength 655nm (OD655) was measured with Bio-Rad Model 680 microplate reader. It is important to minimize the exposure time to chloroform and polystyrene (Figure 2E).

Detergent removal methods

- Ethyl acetate

Ethyl acetate is a common organic solvent that is effective at extracting organic compounds, including many types of detergents [14]. The extraction of detergents was performed using ethyl acetate (99.7% purity) as the organic solvent. In 1:1 ratio ethyl acetate solution was employed to detergent-containing aqueous solution to ensure efficient extraction. The mixture was vigorously vortexed and centrifuged at 10000 rpm for 5 minutes at RT. Subsequently, the solution was separated into two distinct phases based on their immiscibility. The upper phase was discarded.

- Mineral oil

Mineral oil is another type of organic solvent that can be used for extraction purposes, including the removal of certain organic compounds from aqueous solutions based its ability to interact with nonpolar detergent molecules. Mineral oil was added in 1:1 volume ratio to the sample and thoroughly vortexed. After centrifugation at 10000 rpm for 5 minutes at RT, the upper phase and interphase containing SDC was removed.

- Effect of pH on linearity of calibration curve

The calibration samples containing serial dilutions of SDC in water were measured on spectrophotometer before and after acidification. 1% TFA was added to the sample (pH 2, universal indicator paper) with MB and chloroform after centrifugation. Absorbance of chloroform phases were measured on spectrophotometer at 655nm.

RESULTS AND DISCUSSION

As mentioned in introduction it is important to remove detergent from sample before LC-MS/MS analysis. Therefore residual amount of detergent should be determined on final steps of sample preparation. According to literature data spectrophotometric quantification of MB-detergent complexes [9, 10] is simple and sensitive method available to any laboratory. However, standard protocol is based on detergent quantification in water, PBS, Tris-HCl buffers at pH range 7-7.5. But in case of SDC, protocol is not adapted since acidic solution cause formation of MB aggregates (dimers, trimers, tetramers etc.) which soluble in chloroform phase [15] and also competes by absorbing light with MB-SDC complexes. This results in loss of linearity for calibration standards (Figure 1 B, D; Figure 2 A, B). MB exhibits higher solubility in water, remaining within the aqueous phase. Under low pH values, MB predominantly exists in its protonated form (MBH^{2+}), which exhibits reduced solubility in water compared to its unprotonated form (MB). Consequently, the protonated form of MB demonstrates an enhanced affinity for organic solvents, such as chloroform [16, 17].

In order to get rid of SDC Masuda et al. [14] acidified tryptic digest by TFA to precipitate or extract detergent. Acidification is also standard to inactivate trypsin during sample preparation for proteomics [18]. Furthermore, TFA is used in desalting step on C18 columns, since the greatest binding of peptides occurs only in an acidic environment [19]. However acidic solution is not acceptable for residual SDC quantification by spectrophotometry as mentioned above. This method proved to be flawed due to the TFA induced formation of $CHCl_3$ soluble MB aggregates, leading to inaccurate measurements (Figure 2). To address this issue, standard samples were prepared within the pH 7.0-7.5. By adding the 1M Na_2HPO_4, we optimized the protocol for SDC quantification. Regarding the work of Masuda et al. ethyl acetate extraction method was employed to remove the remaining SDC from the sample. Nonetheless, an alternative method of detergent removal that we have used is mineral oil extraction. It is a relatively simple and effective technique for extracting SDC from an aqueous solution (Figure 1C). The interphase is being formed due to its amphiphilic properties. All these methods turned out to be compatible in terms of results.

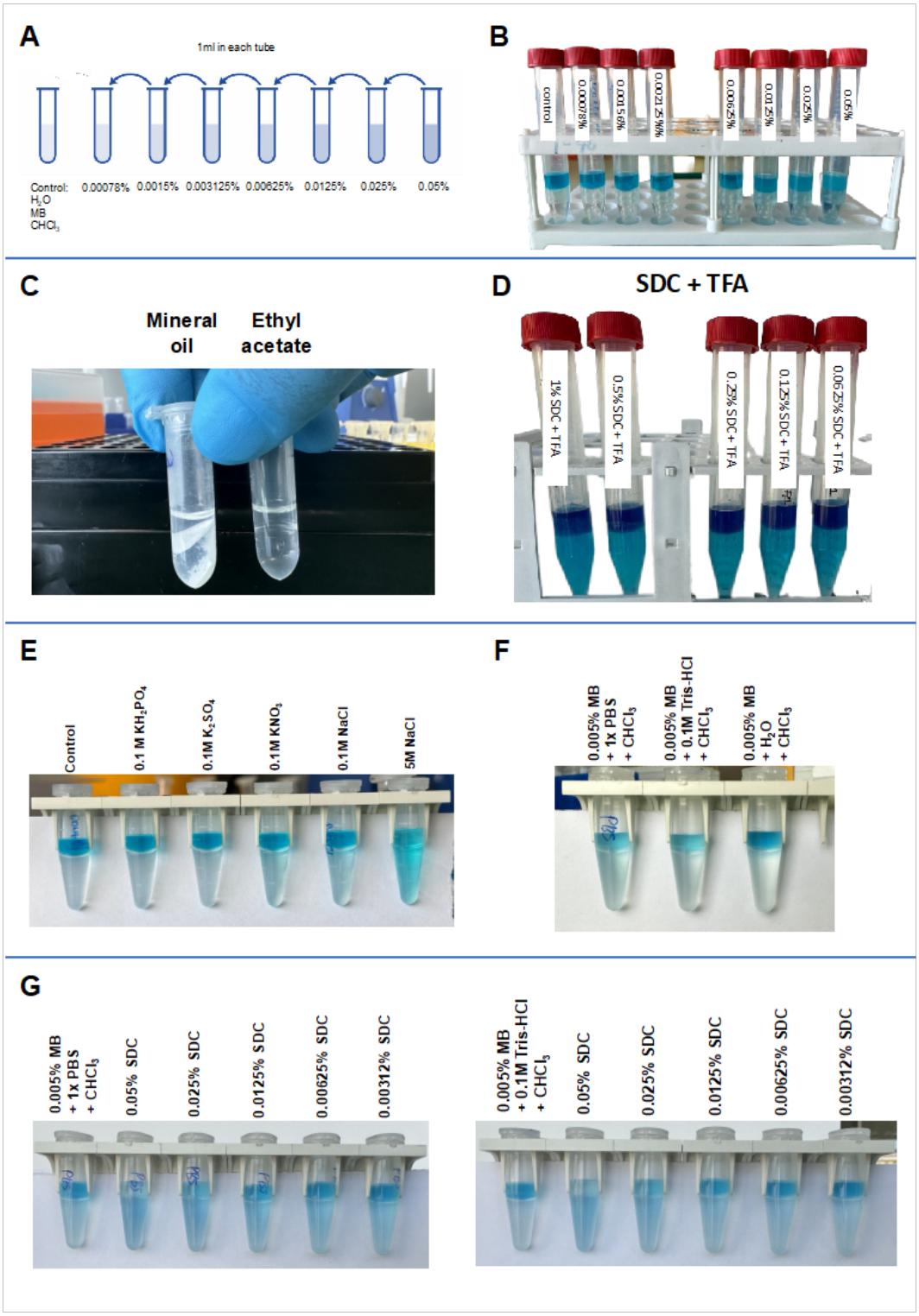

Figure 1. Detergent removal methods (upper phase is organic). Effects of pH and salts on formation MB aggregates and MB-SDC complexes (lower phase is organic). **(A)** Schematic diagram of twofold aqueous serial dilution. **(B)** The visual observation of serial dilution of SDC, where initial concentration (from right to left) is 0.05% and final is 0.00078%. The first sample is indicated as control (no detergent). **(C)** Detergent removal with mineral oil and ethyl acetate extraction. **(D)** Loss of linearity after addition of TFA to SDC samples from **(B)**. Effect of salts **(E)** and buffers **(F)** on aggregation of MB without SDC detergent. Aggregates of MB partition to organic phase. **(G)** Visual comparison of 1X PBS and 0.1M Tris-HCl buffers with serial dilutions of SDC.

We also employed commonly used buffer solutions in cellular biology, such as PBS and Tris-HCl in order to maintain a stable pH. Nonetheless, compared to PBS, in Tris-HCl with or without the detergent, less MB aggregates transit into the organic phase of the solution (Figure 1 F, G). Therefore, it was decided to use H_2O (Milli-Q grade) to mitigate the complex formation between MB and chloride ions. Thus, the coloration of the chloroform layer decreases in this order PBS>-Tris-HCl>H_2O (Figure 1F). In addition, we tested the effect of various buffer solutions on the formation of dimmers, trimmers, and other aggregates of MB without SDC. The results showed that the presence of NO_3^- and Cl^- in the buffer solutions and an acidic environment were found to lead to the formation of undesired complexes with MB, potentially interfering with our experimental measurements (Figure 1E). According to George *et al.* [12] occurrence of NaCl in a detergent sample does affect the methylene blue active substances (MBAS) assay, because MB-Cl complex partitions pass into the chloroform layer. As a result, the use of H_2O MQ provided a chloride-free environment (Figure 1 A, C), ensuring the accuracy and reliability of our data. The experiment was carried out at different wavelengths of the spectrophotometer. So based on the results obtained, at the OD630, OD655 and OD665 there is no obvious difference (Figure. 2 C, D).

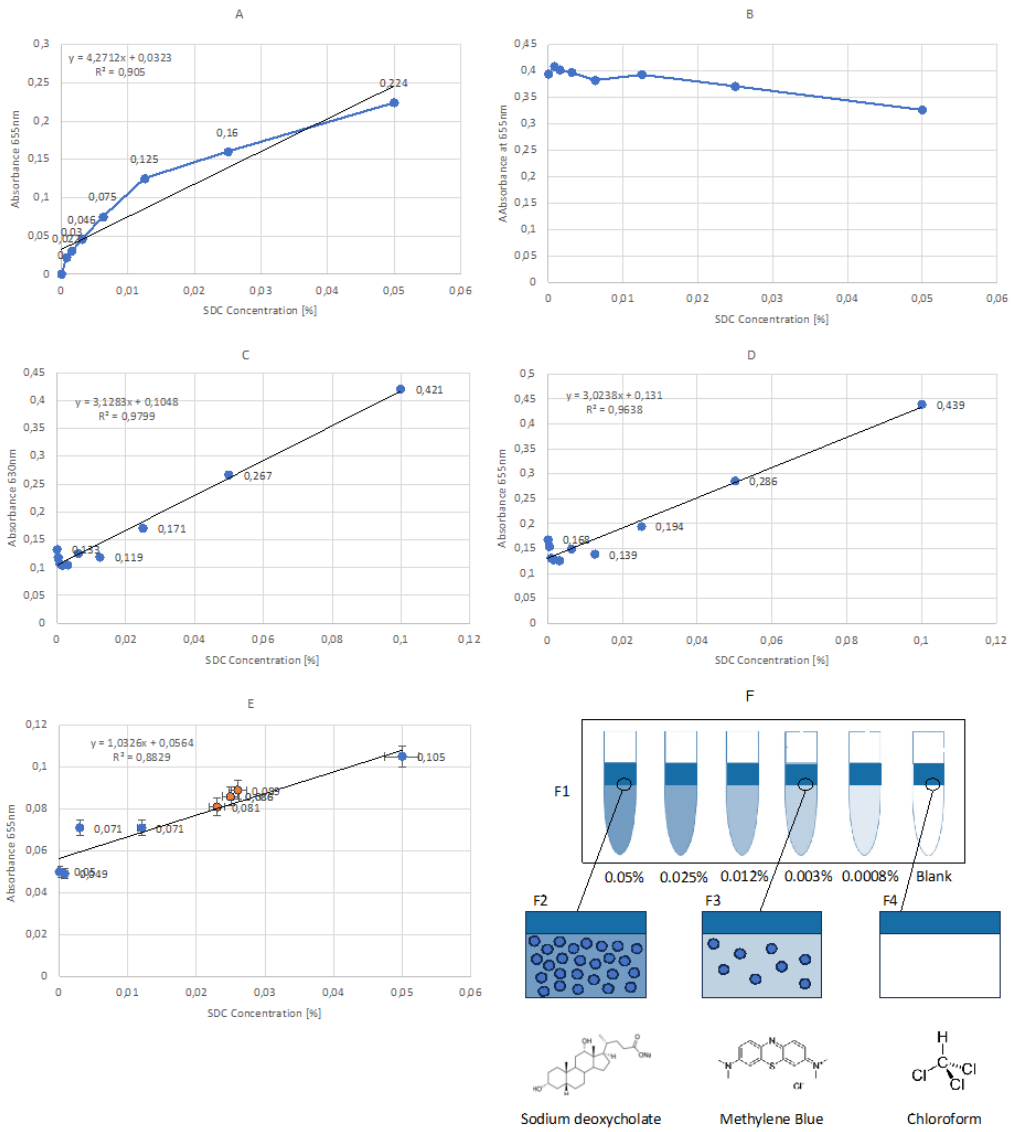

Figure 2. Spectrophotometric measurements of MB aggregates and MB-SDC complexes in $CHCl_3$ under various conditions. **(A)** $CHCl_3$ phase measurements of samples before adding TFA. **(B)** $CHCl_3$ phase measurements of samples after adding TFA. The effect of SDC absorption wavelengths at 630nm **(C)** and 655nm **(D)** on data linearity. **(E)** Determination of the residual SDC concentration after ethyl acetate extraction method (indicated with orange color). The experiment was conducted in triplicate at absorbance of 655 nm. The error bars indicate the standard deviation of concentration **(F)** Schematic illustration of transfer of MB-SDC complexes (blue circles) into organic phase. **F1**: Various concentrations of SDC (ranging from 0.05% to 0.0008% and pure water at 0%) were prepared to plot a calibration curve. **F2** and **F3**: When there is an abundance of SDC present, the interaction between MB and SDC results in the formation of a complex with a distinct spectral color. In cases of higher SDC concentrations, it becomes visually evident that the methylene blue-detergent complex passes into the chloroform phase. **F4**: In contrast, samples containing no detergent, only pure water, do not exhibit this phenomenon. In blank (control), the chloroform phase remains colorless.

CONCLUSION

This study encountered limitations related to collapse of linearity of calibration curve for the quantification of residual detergents in case of low pH and presence of some ions in buffer solutions such as Cl^-, NO_3^- especially at higher concentrations of salts. The presence of chloride, nitrate ions and acids promote formation of MB aggregates to transit from the aqueous phase into the chloroform phase which also absorb at 655nm and cause competitive effect. By using optimized protocol we demonstrated that one extraction by ethyl acetate decreases the amount of SDC by the factor of 20 (from 0.5% to 0.025%). We also found usability of polystyrene 96 well plates for measurement of chloroform samples but for short period of time. This allows performing high-throughput experiments with fewer amounts of reagents and solvents or to get more measurements for statistical analysis.

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КОЛИЧЕСТВЕННАЯ ОЦЕНКА ДЕЗОКСИХОЛАТА НАТРИЯ С ИСПОЛЬЗОВАНИЕМ МЕТИЛЕНОВОГО СИНЕГО В ПРОБОПОДГОТОВКЕ К ПРОТЕОМНОМУ АНАЛИЗУ

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АННОТАЦИЯ

Анионные детергенты, такие как дезоксихолат натрия (SDC), широко используются в биохимических и молекулярных биологических исследованиях для растворения и денатурирования белков и нуклеиновых кислот. В присутствии анионных детергентов электростатическое взаимодействие между положительно заряженным метиленовым синим (МС) и анионами приводит к образованию комплекса, который растворим в хлороформе, что позволяет проводить количественную оценку детергента. Без образования этого комплекса растворимый в воде МС не смешивается с хлороформом. Таким образом, спектрофотометрический метод определения комплексов МС-детергент может быть использован для определения концентрации SDC в образцах для LC-MS/MS. Протокол количественной оценки SDC был оптимизирован с учетом таких факторов, как pH, состава буфера и длины волны спектрофотометра. Для удаления детергента из пробы использовались несколько методов экстракции, а именно: экстракция этилацетатом, минеральным маслом и осаждение с помощью кислоты. Все методы имеют примерно одинаковую эффективность. Метод метиленового синего был использован в качестве стандартного метода для точной количественной оценки детергента в образце, в диапазоне нижнего предела концентрации примерно 0.025% и 0.1% верхнего.

Ключевые слова: Liquid chromatography tandem mass spectrometry (LC-MS/MS), дезоксихолат натрия (SDC), додецилсульфат натрия (SDS), калибровочные кривые, метиленовый синий (MB), methylene blue active substances (MBAS), спектрофотометрия, последовательное разбавление.

ПРОТЕОМДЫҚ ТАЛДАУҒА ҮЛГІ ДАЙЫНДАУ БАРЫСЫНДА МЕТИЛЕН КӨКТІ ҚОЛДАНУ АРҚЫЛЫ НАТРИЙ ДЕЗОКСИХОЛАТЫН САНДЫҚ БАҒАЛАУ

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ТҮЙІН

Натрий дезоксихолат (SDC) сияқты аниондық детергенттер, ақуыздар мен нуклеин қышқылдарын еріту және денатурациялау үшін биохимиялық және молекулалық биология зерттеулерінде кеңінен қолданылады. Аниондық детергенттер қатысу барысында оң зарядталған метилен көк (МК) пен аниондар арасындағы электростатикалық әрекеттесу хлороформға экстракцияланатын комплекс түзе отырып сандық анықтауға мүмкіндік береді. Бұл комплекс түзілмесе, суда еритін МК хлороформмен араласпайды. Сонымен қатар, спектрофотометриялық МК-детергент комплексін анықтау әдісі, LC-MS/MS үлгілеріндегі SDC концентрациясын анықтау үшін қолдануға болады. SDC детергент мөлшерін анықтау хаттамасы pH, буфер құрамы және спектрофотометр толқын ұзындығы сияқты факторларды ескере отырып жетілдірілген. Үлгідегі детергенттен арылу үшін бірнеше экстракция әдістері қолданылды, атап айтқанда этилацетатты экстракция, минералды май экстракциясы және қышқылды тұндыру. Барлық әдістер шамамен бірдей тиімділікке ие. Метилен көк әдісі, төменгі шегі шамамен 0,025% және жоғарғы шегі 0.1% аралығында үлгідегі детергенттің мөлшерін сандық анықтаудың стандартты әдісі ретінде пайдаланылды.

Негізгі сөздер: Liquid chromatography tandem mass spectrometry (LC-MS/MS), калибрлеу қисық сызықтары, метиленді көк (МК), methylene blue active substances (MBAS), натрий дезоксихолаты (SDC), натрий додецилсульфаты (SDS), спектрофотометрия, тізбектік сұйылту.