Antibacterial Efficiency of Benzalkonium Chloride Base Disinfectant According to European Standard 13727, Chemical Analysis and Validation Studies

Çinel Köksal Karayıldırım[1*], Veysel Umut Çelenk[1,2]
[1]Center for Drug Research and Development and Pharmacokinetic Applications, Ege University, 35100, Izmir, Turkey, Tel: +90-232-339 21 60, Fax: +90-232-374 23 90
cinelbio@hotmail.com
[2]Department of Chemistry, Faculty of Science, Ege University, 35100, Izmir, Turkey
* Corresponding author

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Abstract

This study was aimed to provide principle of the chemical analyses, antibacterial efficiency test and validation procedures of the most commonly used benzalkonium chloride (BAC) base disinfectant as a biocide. Disinfectant which comprised 20 % BAC concentration was used as a prototype product and active substance was verified with chemical analysis. Additionally, BAC homologs (*n*-C12 and *n*-C14) in the product were identified by LC-MS/MS method. The validation results also confirmed the reliability of the chromatographic method. After active substance was confirmed, microbiological efficacy test was carried out against *E. coli* K12, *S. aureus*, *P. aerugionas* and *E. hirae*. Both bacterial strains were showed more than 5 logaritmic reduction after exposure for 1 min. to test product. The aim of this study was to evaluate the validation of chemical analysis of the test product by LC-MS/MS which is developed chromatographic method. Moreover the present study highlights that the antibacterial efficacy of BAC based disinfectant according to European National (EN) Standard. These results exhibited that chemical and microbiological analyses were significantly important before manufacturing and marketing processes of the biocidal products. This was the first study on biocides analysis which was conducted according to the European standard.

Keywords — Antibacterial activity, Benzalkonium chloride, Biocidal products, LC-MS/MS, Validation

Benzalkonyum Klorür İçerikli Dezenfektanın EN 13727 Standardına göre Antibakteriyel Etkinliği, Kimyasal Analizi ve Validasyon Çalışmaları

Özet

Bu çalışmada, biyosidal dezenfektan olarak yaygın bir şekilde kullanılan benzalkonyum kolürürün (BAK) kimyasal analizinin, antibakteriyel etkinlik testinin ve validasyon çalışmalarının prensiplerinin ortaya konması amaçlanmıştır. % 20 konsantrasyonda BAK içeren dezenfektan prototip olarak kullanılmıştır ve aktif madde içeriği kimyasal analiz ile doğrulanmıştır. Ayrıca LC-MS/MS metodu ile ürün içeriğinde yer alan BAK homologları (*n*-C12 and *n*-C14) tanımlanmıştır. Validasyon sonuçları ile kromotografik metodun güvenilirliği doğrulanmıştır. Aktif maddenin onaylanmısının ardından, mikrobiyolojik etkinlik testleri *E. coli* K12, *S. aureus*, *P. aeruginosa* ve *E. hirae* bakterilerine karşı

gerçekleştirilmiştir. Test ürünü ile 1 dakikalık temasın ardından bütün bakteri şuslarına karşılık 5 logartimik azalmadan fazla düşüş elde edilmiştir. Bu çalışmanın amacı, test ürününün kimyasal analizlerinin validasyonunu kromotografik bir metot olan LC-MS/MS ile değerlendirmektir. Ayrıca çalışma, BAC içerikli dezenfaktanların EN standartları ile antibakteriyal etkinliğinin ortaya konmasına ışık tutmaktadır. Bu sonuçlarla biyosidal ürünlerin üretim ve pazarlama öncesi gerçekleştirilen kimyasal ve mikrobiyobiyolojik analizlerinin önemi ortaya koymuştur. Bu çalışma biyosidal analizlerinin EU standartlarına göre gerçekleştirildiği ilk çalışmadır.
Anahtar Kelimeler — Antibakteriyal aktivite, benzalkonyum klorür, biyosidal ürünler, LC-MS/MS, validasyon

1 Introduction

Biocides are antimicrobial molecules which kill microorganisms and play an important and effective role in limiting the spread of infection moreover they destruction of microbial contamination [1-3]. Biocidal products are necessary for the control of organisms that are harmful to human/animal health and can cause damage to natural or manufactured materials (Regulation EU, No 528/2012 of the European Parliament and of the council of 22 May 2012). Cleaning and disinfection are essential to avoid microbial colonization and limit the risk of cross contamination [4, 5]. Overall the use of biocides is common and useful practice to control microbial growth.

According to the European Union Biocides Directive (98/8/EC), biocides are classified in four main categories and twenty-three product types [6]. The main group which disinfectants are used for human hygiene purposes, medical environments, swimming pools, (PT1 and 2), veterinary sanitize (PT3), food industry-feed area (PT4) and drinking water (PT5) (EU Regulation 1 September 2013). All these products can be bactericidal, fungicidal, sporicidal, virucidal and anti-fouling agents. In such a manner, antimicrobial active substances which are used in biocidal products are approved by Biocide Products Committee of The European Chemicals Agency (ECHA).

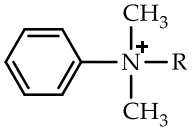
One of these confirmed active substance is benzalkonium chloride (BAC) which is classed as a Category III antiseptic active ingredient by the US FDA [7]. The *n*-C12 and *n*-C14 homologs in BAC products comprise a major portion of the alkyl group [8]. In general, the *n*-C12 homolog is most effective against yeast and fungi, and the *n*-C14 homolog against gram-positive bacteria [9]. BAC is the member of quaternary ammonium compounds (QACs) which is a mixture of alkylbenzyl dimethyl ammonium chlorides of many alkyl chain lengths and it is commonly used as disinfectants in food, industrial and domestic areas [1]. The mode of action of quaternary ammonium compounds appears to be associated with their effect on the cytoplasmic membrane, which controls cell permeability [9]. The hydrophilic region of BAC interacts with the bacterial cell membrane and the hydrophobic region causes the cell death [10]. Therefore it is a powerful disinfectant and has been shown good activity against the highly resistant microorganism [11, 12] Numbers of studies were published on anti-microbial activity of BAC against many gram positive bacteria even gram negatives [12, 13].

The purposes of this study are to investigate the efficacy of 20 % BAC based disinfectant as biocides and accomplish pre-validation of BAC's antibacterial efficacy test according to EN 13727 standard [14]. Biocide susceptibility was tested against *E. coli* K12, *P. aeruginosa, S. aureus* and *E. hirae.* Liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used to separate and quantify BAC homologs also verification of active substance in this disinfectant. Additionally, validation studies have been reported for method reliability. According to results it allowed to acquire data about this product's disinfectant potential before manufacturing and marketing. This is the first study on chemical and microbiological analyses including verification and validation methods before manufacturing and marketing processes of the biocidal products.

2 Materials and Methods

2.1 Chemicals and Reagents

TSA (Tryptic Soy Agar, Merck, Germany), TSB-ST (Tryptone soya broth, Biomerieux®, France), Diluent (MRTD, Biomerieux®, France), Polysorbate 80 (CAS: 9005-65-6, Sigma Aldrich, Germany), Lecithin (CAS: 8002-43-5 Alfa Aesar), Bovine serum albumin (CAS: 9048-46-8, Sigma Aldrich), Sodium thiosulfate (CAS: 7772-98-7, Merck) were used. *N*-Benzyl-*N*-dodecyl-*N*,*N*-dimethylammonium chloride (BAC-C12) and *N*-benzyl-*N*-tetradecyl-*N*,*N*-dimethylammonium chloride (BAC-C14) were supplied by Sigma–Aldrich (Steinheim, Germany). Water (LC-MS/MS grade), acetonitrile (LC-MS/MS grade) and acetic acid (≥ %99.7) were obtained from Sigma–Aldrich (Steinheim, Germany). All sample solutions were filtered through 0.22 μm PTFE filters (Millex-HV, Germany) before injection.



R	Name
$C_{12}H_{25}$	BAC-C12
$C_{14}H_{29}$	BAC-C14

2.2 Chemical Analysis

Instrumentation and Chromatographic Conditions: An Waters Acquity series LC system (Waters, USA) equipped with binary solvent manager (D07UPB225M), sample manager (E07UPS996M) and tandem quadrupole detector (QBA157) were used to inject 5 μL aliquots of the processed samples on a Acquity UPLC HSS T3 (1.8μm, 2.1×50 mm) (Waters, USA) which was kept at (40 ± 2 °C) in a column oven.

Quantitation was achieved by MS–MS detection in positive ion mode for both homolog, equipped with a electrospray ionization (ESI) positive polarity. Source temperature and desolvation temperature were set at 120 °C and 350 °C respectively. The common parameters viz., cone gas and collision gas were set at 100 L/h and 20 L/h respectively. The compound parameters viz., cone energy and collision energy were 15 V and 65 V. The detector was linked to a data system (Waters, MassLynx) for data acquisition and calculation.

Mobile phase A consisted 0.1 % of acetic acid in acetonitrile, mobile phase B was 0.1 % of acetic acid in water. A gradient program was used starting with a flow rate of 0.4 mL/min and 80 % of mobile phase A (20 % B) at injection time for 1 min, and gradually changing to 20 % A (80 % B) over 0.5 min and to 15 % A (85 % B) until 3 min after injection. Then, mobile phase composition was shifted to 80 % A (20 % B) over 0.5 min. This mobile phase composition was kept for 0.5 min. The conditions were maintained for 4 min for column equilibration. Injection volumes of 5 μL were employed and column temperature was adjusted at 40 °C.

The protonated precursor ions $[M + H]^+$ of BAC-C12 and BAC-C14 were detected in the full scan mass spectra at m/z 304 and 332, respectively. The collision energy was optimized, and the following daughter ions were selected, m/z 304 → 91, m/z 304 → 212 m/z 332 → 91 and m/z 332 → 240 for the determination of BAC-C12 and BAC-C14, as summarized in Table 1.

Table 1. Ion transitations of BAC compounds

BAC Homologs	Precursor Ion (m/z)	Daughter Ion (m/z)	Dwell (s)	Cone (v)	Collision (V)
C12	304	91	0.15	41	30
	304	212	0.15	41	21
C14	332	91	0.15	41	31
	332	240	0.15	41	22

Standard solutions

Primary stock solutions of standard were prepared by weighing separately. The primary stock solution (1.00 mg/mL) of BAC-C12 and BAC-C14 were prepared in acetonitrile. Appropriate dilutions were made in acetonitrile to produce working solutions of 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL and 250 ng/mL before the analysis and these standards were used to prepare calibration curve.

Sample Preparation

Sample solutions were prepared as follows;

Sample Solution **1**: 100 μL 20 % BAC consisted formulation was measured and diluted to 10 mL in a

volumetric flask with acetonitrile (included 0.1 % acetic acid). Then, the solution was agitated for a few minutes.

Sample Solution **2**: 100 µL Sample Solution **1** was measured and diluted to 10 mL in a volumetric flask with acetonitrile (included 0.1 % acetic acid). Then, the solution was agitated for a few minutes.

Sample Solution **3**: 100 µL Sample Solution **2** was measured and diluted to 10 mL in a volumetric flask with acetonitrile (included 0.1 % acetic acid). Then, the solution was agitated for a few minutes.

Total dilution factor was calculated as 1×10^6. Prior to injection, sample solution **3** was filtered through a 0.22 µm PTFE filter and then injected.

Assay Validation

The method was validated in-house in terms of selectivity, sensitivity, linearity and repeatability for the 20 % BAC consisted formulation.

Selectivity

Selectivity was assessed using acetonitrile as blank and primary standards (conc. 100.0 ng/mL). Acetonitrile and primary standards were prepared and analysed as described, and the peak chromathograms were compared.

Sensitivity

Sensitivity was assessed by determining the limit of detection (LOD) and limit of quantification (LOQ) for the analyte. The detection limits for each of the BAC homologs were calculated accordingly S/N ratio. LOD was defined as the lowest concentration of analyte that could be detected, the presence of quantifier ions each with a signal-to-noise ratio of at least 3. LOQ was the lowest concentration that met the LOD criteria and a signal-to-noise ratio of 10.

Linearity

Linearity for BAC was investigated by making three replicate injections of each of the five standard concentrations covering a range of 10.0-250.0 ng/mL. The external standard method was used to establish the calibration curve and to quantify BAC in samples.

Repeatability

The method repeatability was estimated by making five consecutive injections of a 100.0 ng/mL BAC standards

2.3 Antibacterial Activity Test

Test Organisms

The antibacterial activity test was evaluated using the following four test organisms. *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus auerus* ATCC 6538 were obtained from Biomerieux® (France) as a Bioball which contains a precise number of cells in a water soluble ball delivering unprecedented accuracy for quantitative microbiological quality control. BioBall strains are an accredited reference material under the ISO Guide 34 standard. *Escherichia coli* K12 ATCC 10538 and *Enterecoccus hirae* ATCC 10541 were obtained from Microbiologics Inc. (USA).

Preparation of Test Organism Suspensions (N)

The bacterial cultures resuscitated in TSB-ST which were prepared from a first subculture from the stock culture by streaking onto TSA plates and incubated for 24 h at 36 ± 1 °C. All the number of cells in the suspension were adjusted 1.5×10^8 to 5×10^8 cfu/mL using diluent.

Preparation of Validation Suspensions (Nv)

Test suspensions were diluted to prepare the validation suspensions by serial dilution. The concentration of validation suspensions were 3.0×10^2 to 1.6×10^3 cfu/mL.

Experimental Conditions

The test and validation procedures were carried out at the same time. All studies were conducted duplicate and inoculated using the spread plate technique. According to standard, the obligatory test temperature was 20 °C, contact time was 1 minute for bacteria. Interfering substance was tested 0.3 g/L bovine albumin for clean conditions. Polysorbate 80 30 g/L, sodium thiosulphate 10 g/L, lecithin 3 g/L were used as a neutralizer to determined antibacterial activity by dilution-neutralization method.

Determination of bactericidal concentration (Na) according to EN 13727 started with 1 mL interfering

substance, 1 mL test suspension (N) in a water bath at 0 °C, 2 minutes then 8 mL 20 % BAC based disinfectant as a product was added. After contact time, 1 mL sample of the test mixture (Na) was taken and transfered into a tube containing 8 mL neutralizer and 1 mL water. The procedure was performed in a water bath at 20 °C, 5 minutes. 1 mL sample was separated into Petri dishes and added 20 mL of melted TSA for bacteria and they were incubated 36 ± 1 °C, 24 h. Antibacterial experimental conditions control (A), Neutralizer control (B) and Method validation (C) were carried out to determine the validation of the selected experimental conditions, verification of the absence of any toxic effect in the test conditions.

3 Results

3.1 Assay Validation

Selectivity

Results obtained from the analysis of acetonitrile and comparison to primary standards BAC-C12 and BAC-C14. No interferences with the determination of BAC-C12 or BAC-C14 were detected in the analysis.

Sensitivity

LOD value for homolog BAC-C12 and BAC-C14 were 0.42 ng/mL and 0.17 ng/mL respectively. And LOQ value for homolog BAC-C12 and BAC-C14 were 1.39 and 0.56 ng/mL respectively.

Linearity

As shown in Figure 1, excellent linearity was observed from 10.0 to 250.0 ng/mL on plotting the concentration versus the sum of peak areas for homologs BAC-C12 and BAC-C14. In all instances the coefficient of determination (R2) for BAC-C12 and BAC-C14 were 0.9998 and 0.9954 respectively.

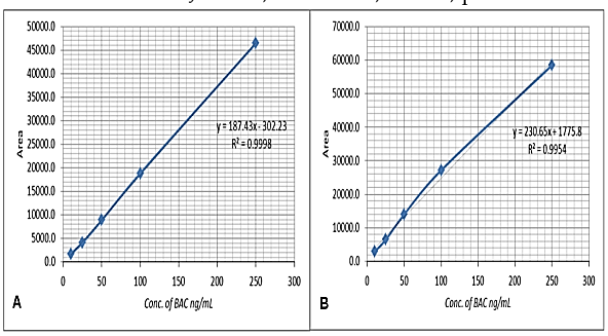


Figure 1. Calibration curve for (**A**) BAC-C12 and (**B**) BAC-C14

Repeatability

RSD percentage of the retention time for both homologs, BAC-C12 and BAC-C14 was 0.00 % . RSD percentage of the peak area for BAC-C12 and BAC-C14 homologs were 0.76 % and 0.62 %, respectively.

Determination of Total Benzalkonium Chloride in Samples

20 % BAC consisted formulation were tested by LC-MS/MS. Average concentrations of three samples which were injected for BAC-C12 and BAC-C14 were 100.9 ng/mL and 101.1 ng/mL according to chromatograms (Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6 and Fig. 7). These results were multiply by dilution factor (DF: 1×10^6) and results for BAC-C12 and BAC-C14 were 10.09 % and 10.11 % respectively. Total BAC content was calculated as 20.20 %.

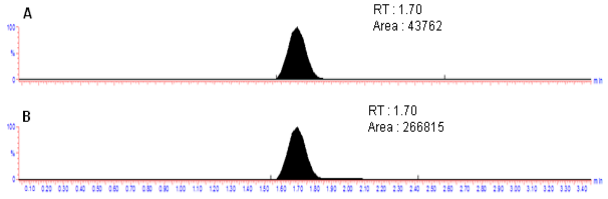


Figure 2. Sample 1 chromatograms for BAC-C12 (m/z 304 → 212 and m/z 304 → 91)

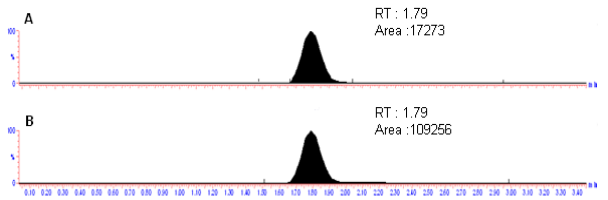


Figure 3. Sample 1 chromatograms for BAC-C14 (A: m/z 332 → 240 and B: m/z 332 → 91)

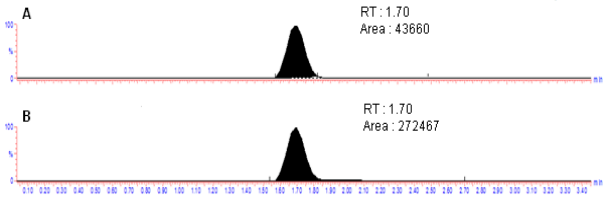


Figure 4. Sample 2 chromatograms for BAC-C12 (m/z 304 → 212 and m/z 304 → 91)

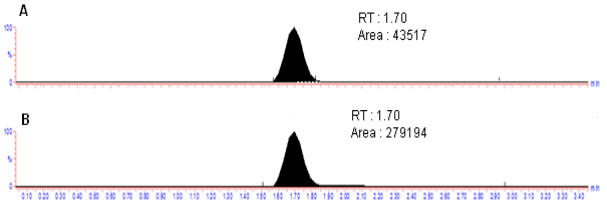


Figure 6. Sample 3 chromatograms for BAC-C12 (m/z 304 → 212 and m/z 304 → 91)

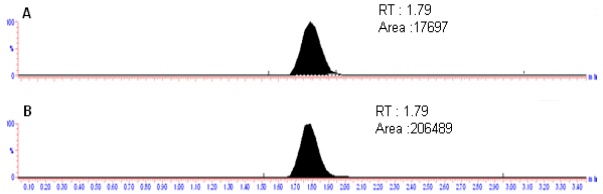


Figure 5. Sample 2 chromatograms for BAC-C14 (A: m/z 332 → 240 and B: m/z 332 → 91)

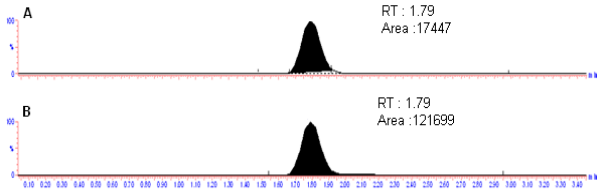


Figure 7. Sample 3 chromatograms for BAC-C14 (A: m/z 332 → 240 and B: m/z 332 → 91)

3.2 Antibacterial Activity Test

The results showed that the bactericidal concentration of the 20 % BAC based disinfectant as a product was determined according to the EN 13727 standard under clean conditions. The numbers of surviving bacteria were detected and the 5 decimal logaritmic reduction was calculated. According to dilution-neutralization method, the product demonstrated antibacterial activity as a chemical disinfectant against *E. coli K12, P. aeruginosa, E. hirae* and *S. aureus* (Table 2, 3, 4 and 5)

Table 2. Validation and controls of *Escherichia coli* K12

Validation Suspension (*Nv0*)		Experimental Conditions Control (*A*)		Neutralizer Control (*B*)		Method Validation (*C*)	
V_{C1}	196	V_{C1}	201	V_{C1}	189	V_{C1}	176
V_{C2}	194	V_{C2}	180	V_{C2}	192	V_{C2}	203
$\overline{x}$ = 195.0		$\overline{x}$ = 190.5		$\overline{x}$ = 190.5		$\overline{x}$ = 189.5	
30 ≤ $\overline{x}$ of *Nv0* ≤ 160		$\overline{x}$ of A is ≥ 0.5 × $\overline{x}$ of *Nv0*		$\overline{x}$ of B is ≥ 0.5 ×$\overline{x}$ of *Nv0*		$\overline{x}$ of C is ≥ 0.5 ×$\overline{x}$ of *Nv0*	
Test suspension and Test (N and N_0)		N	V_C	$\overline{x}$ wm = ×10^6		$\log_N$ = 8.30	
		10^{-6}	190 198	N_0 = N/10		$\log_{No}$ = 7.30	
		10^{-7}	22 31	Limit: 7.17 ≤ log No ≤ 7.70			
Concentration of the Product (%)		V_{C1}	V_{C2}	Na = $\overline{x}$×10	lg Na	lg R	Contact time (min)
20		3	1	< 140	> 2.15	< 5.15	1

Table 3. Validation and controls of *Pseudomonas aeruginosa*

Validation Suspension (*Nv0*)		Experimental Conditions Control (*A*)		Neutralizer Control (*B*)		Method Validation (*C*)	
V_{C1}	216	V_{C1}	193	V_{C1}	205	V_{C1}	182
V_{C2}	205	V_{C2}	186	V_{C2}	190	V_{C2}	194
$\overline{x}$ = 210.5		$\overline{x}$ = 189.5		$\overline{x}$ = 197.5		$\overline{x}$ = 188.0	
30 ≤ $\overline{x}$ of *Nv0* ≤ 160		$\overline{x}$ of A is ≥ 0.5 × $\overline{x}$ of *Nv0*		$\overline{x}$ of B is ≥ 0.5 ×$\overline{x}$ of *Nv0*		$\overline{x}$ of C is ≥ 0.5 ×$\overline{x}$ of *Nv0*	
Test suspension and Test (N and N_0)		N	V_C	$\overline{x}$ wm = ×10^6		$\log_N$ = 8.31	
		10^{-6}	192 201	N_0 = N/10		$\log_{No}$ = 7.31	
		10^{-7}	33 25	Limit: 7.17 ≤ log No ≤ 7.70			
Concentration of the Product (%)		V_{C1}	V_{C2}	Na = $\overline{x}$×10	lg Na	lg R	Contact time (min)
20		2	1	< 140	> 2.15	< 5.16	1

Table 4. Validation and controls of *Staphylococcus auerus*

Validation Suspension (*Nv0*)		Experimental Conditions Control (*A*)		Neutralizer Control (*B*)		Method Validation (*C*)	
V_{C1}	221	V_{C1}	193	V_{C1}	214	V_{C1}	188
V_{C2}	204	V_{C2}	209	V_{C2}	193	V_{C2}	211
$\overline{x}$ = 212.5		$\overline{x}$ = 196.0		$\overline{x}$ = 203.5		$\overline{x}$ = 199.5	
30 ≤ $\overline{x}$ of *Nv0* ≤ 160		$\overline{x}$ of A is ≥ 0.5 × $\overline{x}$ of *Nv0*		$\overline{x}$ of B is ≥ 0.5 ×$\overline{x}$ of *Nv0*		$\overline{x}$ of C is ≥ 0.5 ×$\overline{x}$ of *Nv0*	
Test suspension and Test (N and N_0)		N	V_C	$\overline{x}$ wm = ×10^6		$\log_N$ = 8.33	
		10^{-6}	201 220	N_0 = N/10		$\log_{No}$ = 7.33	
		10^{-7}	31 20	Limit: 7.17 ≤ log No ≤ 7.70			
Concentration of the Product (%)		V_{C1}	V_{C2}	Na = $\overline{x}$×10	lg Na	lg R	Contact time (min)
20		2	2	< 140	> 2.15	< 5.18	1

Table 5. Validation and controls of *Enterecoccus hirae*

Validation Suspension (*Nv0*)		Experimental Conditions Control (*A*)		Neutralizer Control (*B*)		Method Validation (*C*)	
V_{C1}	203	V_{C1}	195	V_{C1}	186	V_{C1}	190
V_{C2}	220	V_{C2}	206	V_{C2}	198	V_{C2}	203
$\overline{x}$ = 211.5		$\overline{x}$ = 200.5		$\overline{x}$ = 192.0		$\overline{x}$ = 196.5	
30 ≤ $\overline{x}$ of *Nv0* ≤ 160		$\overline{x}$ of A is ≥ 0.5 × $\overline{x}$ of *Nv0*		$\overline{x}$ of B is ≥ 0.5 ×$\overline{x}$ of *Nv0*		$\overline{x}$ of C is ≥ 0.5 ×$\overline{x}$ of *Nv0*	
Test suspension and Test (N and N_0)		N	V_C	$\overline{x}$ wm = ×10^6		$\log_N$ = 8.33	
		10^{-6}	210 214	N_0 = N/10		$\log_{No}$ = 7.33	
		10^{-7}	25 30	Limit: 7.17 ≤ log No ≤ 7.70			
Concentration of the Product (%)		V_{C1}	V_{C2}	Na = $\overline{x}$×10	lg Na	lg R	Contact time (min)
20		3	2	< 140	> 2.15	< 5.18	1

4 Discussion

Disinfection and hygiene are two of the oldest strategies for protective of disease. Disinfectants also known as biocides are used to protect human welfare which are formulation containing one or more active substance that control harmful organisms. [15]. Protection and prevention are the essential purposes of biocides. Therefore, it is very important to develop and manufacture new anti-microbial products to struggle with predominantly bacteria and yeasts. Currently a wide range of biocidal compounds showing different levels of antimicrobial efficacy [3, 16]. Due to this reason it is essential to determine antibacterial susceptibility profile and chemical analysis of active substance of these products.

The primary objective of this study was to verify the content of active substance by chromatographic method. Therefore it was confirmed the active substance of the 20 % BAC based disinfectant as a product test solution. Our study was also verified the separation of BAC homologs as BAC C12 and C14 by using LC-MS/MS. Benzalkonium ions made up of a long carbon chain. These are alkyl chain of 12 and 14 carbon atoms which comprise at least 70 % on the BAC [2, 17]. Another aim of this study was to develop the chemical method routinely determine the validation studies of BAC and its homologs in biocides. These findings indicated that this is the remarkable preliminary work for biocidal product development. Similarly HPLC method has been conducted and validated for chemical analysis of biocides by researchers [18-20]. However our LC-MS/MS method presented for the extraction and analysis of BAC proved to be highly reproducible, absolute and more effective. Thus, it was confirmed that the innovative biocidal product was developed and validated before microbiological test.

The biological activity of the test product was attributed to its quaternary ammonium group of BAC ions which are antibacterial properties [21, 22]. As a result of this it has been also shown that the exposure of microorganisms to 20 % BAC concentration was represented bactericidal efficacy. Quantitative suspension method and validation studies indicated that bactericidal efficacy for the evaluation of test product in the medical area. Because control of microorganism during cleaning and sanitization is important to the medical environments, food industry and household areas [23,19]. The experimental conditions (such as exposure time, hygienic conditions and strains) were chosen on the basis of the practical conditions of the test product. The test product showed strong antibacterial activity against *E. coli K12, P. aeruginosa, S. auerus* and *E. hirae.* Antibacterial efficiency of the novel product was emphasized that the importance of biocides especially in medicinal area. The results indicated that it was commended implementation for the use of the test product is within the responsibility of the manufacturer.

In conclusion, it was designed to reveal the appropriate researches and applicable methods for turning an active substance into a product which can be a biocide. The main goal of the present study was to characterize and quantify the level of the test product which was consisted of 20 % BAC concentration by LC-MS/MS method. Moreover, test product as a sanitizer treatment showed strong effect on bacteria when short exposure time and dirty conditions were utilized. Validation and verification studies were also advanced procedures that significantly supported the results. These overall findings demonstrated a remarkable correlation between the chemical and biological analyses. This is the first study to emphasize the importance of quantification and—efficiency tests before manufacturing and marketing processes of the biocidal products.

5. References

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