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Headspace Gas Chromatography-Mass Spectroscopy Analysis and Antibacterial Tests of Commercial Hand Sanitizers

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ABSTRACT

As of late 2019, with the rapid and alarming spread of the SARS-Cov-2 (Covid-19) virus from the coronavirus family, serious measures had to be taken all over the world. The efforts to prevent this global epidemic have started with the legal measures taken by the countries in this regard and the warnings of the World Health Organization (WHO) that the epidemic should be taken seriously. In this process, the success of the use of masks and the use of alcohol-based hand sanitizer in preventing the disease has been evaluated and approved by scientists. In terms of the effectiveness of hand sanitizers, it is seen that the main components are ethyl alcohol and isopropyl alcohol, which are alcohol derivatives and they are considered as active ingredients due to their antibacterial and antiseptic effect . In this study, 11 commercially purchased hand sanitizer active and additional ingredients were identified and listed by headspace gas chromatography-mass spectroscopy (HS/GC-MS) and their antibacterial activities were studied. Hand sanitizers containing alcohol derivatives were used in the study. As a result of this study, it was observed that 4 out of 11 commercial hand sanitizers were not suitable for the final concentration values of hand sanitizer determined by the World Health Organization (accepted as 80%(v/v) for alcohol derivatives). Apart from this, hand sanitizers numbered 5 and 9 did not show antibacterial properties against *Escherichia coli* and hand sanitizers numbered 1 and 10 did not show antibacterial properties against *Staphylococcus aureus*. This situation shows that the standards of hand sanitizers should be controlled with much more stringent rules.

Keywords:
Alcohol; Antibacterial; GC-MS; Hand sanitizer; Headspace

INTRODUCTION

Hand hygiene is very important in reducing the incidence of infectious diseases. Considering that both respiratory and digestive system infections are transmitted through the mouth, it is inevitable that the microorganisms taken from the contact areas reach our respiratory and digestive systems with our hands. Studies have proven the effectiveness of hand sanitizers on microorganisms [1].

As of late 2019, it was necessary to take serious measures all over the world, as a result of the rapid spread of the SARS-Cov-2 (Covid-19) virus from the Coronavirus family. Within the scope of preventive measures, the World Health Organization (WHO) has reported that alcohol-based hand sanitizers have a very important role in preventing surface contamination. In addition to the antibacterial properties of alcohol-based hand sanitizers, its success in antiviral effectiveness has been evaluated and approved by scientists. In this sense, WHO has set some standards in 2010 in order to direct the production of commercial hand sanitizers around the world. These standards were revised and reorganized in 2020. Accordingly, the formulas containing ethanol and isopropyl alcohol after the preparation stages are shown in Table 1. It has been determined that hand sanitizers have an optimum effect at the specified concentrations [2].

Hand sanitizers containing ethanol and/or isopropanol cause less irritation and skin dryness compared to disinfection with soap and water, in addition to effectiveness of hand sanitizers on live pathogens. It is seen that the increase in the use of hand sanitizers, especially by healthcare workers, prevents the transmission of pathogens [3].

An alcohol-based hand sanitizer may contain one or more types of alcohol, with or without other excipients and moisturizers, to be applied to the hands to destroy microorganisms and temporarily stop their growth [4]

Table 1. Hand sanitizer final concentration formulas determined by WHO

FORMULA 1	FORMULA 2
Final concentration	**Final concentration**
Ethanol (80%)(v/v)	Isopropyl alcohol (75%)(v/v)
Glycerol (1.45%) (v/v)	Glycerol (1.45%) (v/v)
Hydrogen peroxide (0.125%) (v/v)	Hydrogen peroxide (0.125%) (v/v)

Globally, governments determine their own regulations regarding the volume fraction of alcohol in hand sanitizers, other ingredients to be added, and packaging and handling. The concentration of alcohol-containing hand sanitizers determined by WHO [5] has been accepted by many countries with legal regulations (Table 1). While ethanol or isopropyl alcohol, one of the active ingredients in the formulation, can be 75% or more by volume, an average of 80% is targeted. In addition, the US Food and Drug Administration (FDA) and the Canadian Ministry of Health made it necessary to add denaturants such as denatonium benzoate, sucrose octaacetate and isopropanol to the formulation in order for alcohol to lose its drinkable character [6].

Disinfectants with an alcohol concentration below 60% are not effective, but also pose a risk since they are not virulent [7]. The control of disinfectant contents in Turkey is within the scope of the "Biocidal Products Regulation" and is regulated by the "Regulation on the Usage Procedures and Principles of Biocidal Products" determined by the Ministry of Health. Biocidal products are defined as “active substances and preparations that contain one or more active ingredients, put on sale ready to use, have a controlling effect on any harmful organism chemically or biologically, or that limit its movement, remove, render it harmless, or destroy it”. Therefore, hand sanitizers are included in this group [8]. Moreover, within the scope of this regulation, the lists of approved chemicals that can be found in the content of the products have also been determined within this framework [8]

Headspace Gas Chromatography-Mass Spectroscopy

Pragst et al. (2001) published an article on the determination of ethyl esters in human hair due to chronic alcohol intake by GC-MS technique. Accordingly, it is possible to determine this ethyl ester and alcohol, since it can remain in the body for 24 hours after ingestion. In this way, alcoholic or social drinkers can be distinguished from each other and the causes of death can be clarified [9].

Bouche et al. (2002) studied the quantitative determination of n-propane, iso-butane and n-butane molecules taken into the body with lighter gas and passed into the blood by headspace GC-MS. Accordingly, they demonstrated that the headspace GC-MS technique is a sensitive, automated and fully validated technique [10]. Stambuli et al. (2004) developed a new analytical technique with headspace GC-MS (HS/GC-MS) for the determination of trace amounts of triacetone triperoxide (TATP) in post-explosion samples. According to this; have determined the optimum parameters and have determined TATP lower than 1 nanogram level [11]. Wang et al. (2008) published a review article on the advantages of HS/GC-MS gas chromatography. This article contains several examples of environmental, clinical, forensic, biological, food, powder and pharmaceutical analyzes between 2002 and 2008 [12]

Joshi et al. (2011) carried out the content determination of smokeless powder using HS/GC-MS. Nitroglycerin was determined as the main component, while 2,4-dinitrotoluene was detected at 44% [13]. Desharnais et al. (2012) used HS-GC-MS for cyanide detection in postmortem biological matrices. This method has been validated and used in forensic cases, burning victims and mass suicides [14]. Wu et al. (2012) conducted a study on the thermal decomposition of triacetone triperoxide (TATP), which is a potential explosive and can be easily synthesized under laboratory conditions, and used the GC-MS device in their study. As a result; They also proved from the GC-MS results that the synthesis of TATP with sulfuric acid would be more dangerous than the synthesis with hydrochloric acid, just as dangerous as TNT [15]. Almog et al. (2013) stated that this type of explosive, which is obtained from the combination of urea and nitric acid and looks like sugar, is used by terrorists. They used the GC-MS technique for the determination of trace amounts of urea nitrate from post-explosion debris during the attack. The aim of the study is to prevent the determination of urea and ammonium nitrate by acting as a nitronium cation when they come together, to prevent the formation of nitronium cation by creating a reaction step by including alko groups in the reaction and being able to easily determine urea nitrate from post-explosion debris with the help of GC-MS [16]. Lennert and Bridge (2018) carried out the determination and classification of smokeless gunpowder in their article using GC-MS technique. In this study, it is aimed to determine the smokeless gunpowder samples in the combustion or unburned residues after firing or explosion by GC-MS and DART-TOFMS techniques. According to this; Analysis of 34 smokeless gunpowder samples was made and it was stated that determination can be made with a fast visualization technique with GC-MS [17].

GC-MS device is a high selectivity, fast and sensitive device used to characterize alcohol and impure compo-

nents. It is a preferred method because it is quantitatively reproducible, provides a database, and is easy to analyze [18]. For this reason, a chromatography device was preferred in this study.

HS/GC-MS method, which is used to detect alcohol from hand sanitizer samples, is a widely used method in recent years because it is used to detect volatile compounds in solid and liquid samples and mostly does not require any preliminary preparation [19]. The reason why it was preferred in this study is that it is considered as a suitable method for alcohol determination, and it responds in a short time and with high accuracy in solid-liquid phase compounds.

MATERIAL AND METHODS

HS/GC-MS Analysis

A total of 11 commercial hand sanitizers were used in this study. Hand sanitizers were chosen on the basis of being easily accessible and widespread. No price criteria were specified in the selection . They were purchased from accessible markets, pharmacies and e-shopping platforms, and provided between September-October 2020. No dilution with MeOH was made, as it may cause incorrect assessments of the amount of isopropyl alcohol/ethyl alcohol and the area covered by the amount of alcohol in the hand sanitizer will be determined (percent-%-v/v). 12 vials were prepared by absorbing 10 µL sample into each 20 mL autosampler vial with cellulose paper (One of the vials was a control sample. It is not shown in the table).

In this study, Agilent 5977B GC/MSD and Agilent 7697A Headspace Sampler in Forensic Chemistry Laboratories established within the Directorate of Forensic Sciences Institute of the Turkish National Police Academy were used. After making general adjustments with MSD-Mass Hunter program, comparisons were made with MSD Data Analysis and Quantitative Data Analysis methods. The parameters determined as a method in the GC-MS device are given in Table 2, Table 3 and Table 4 below.

Table 2 shows the values selected for the inlet parameters. Accordingly, the heater was kept at 250 °C for complete evaporation, the pressure was set to 10.121 psi, and the septum purge flow was set to 3 mL/min. Inlet mode is set to "Split" and split ratio is set to 50:1. The column flow was determined as 1.3 mL/min. Column pressure and constant flow are created automatically. Temperature values and durations are given in the table (Table 3) created for temperature parameters. The initial temperature was determined as 40 °C. In the next stage, it was increased up to 100 °C at 5 °C intervals. (from the 1st minute to the 15th minute, for 2 min) In the 2nd stage, it was increased up to 200 °C at 20 °C intervals (from the 15th minute to the 22nd minute for 2 minutes).

In the HS/GC-MS parameters (Table 4), the oven temperature, loop temperature, and transfer line temperature were set in increments of 10 °C, respectively (100 °C, 110 °C, 120 °C, respectively).

Table 2. Inlet Parameter for GC-MS

Heater	250
Pressure	10.121 psi
Total Flow	69.23 mL/min
Septum Purge Flow	3 mL/min
Inlet Mode	
Split Ratio	50:1
Split Flow	65 mL/min
Columns	
Flow	1.3 mL/min
Pressure	10.121 psi
Constant Flow	1.322 mL /min

Table 3. Temperature Parameter for HS/GC-MS

	Rate °C/min	Value °C	Hold Time min	Run Time min
(Initial)		40	1	1
Ramp 1	5	100	2	15
Ramp 2	20	200	2	22

Table 4. Headspace Parameter for HS/GC-MS

HS Parameter		
Name	**Setpoint**	**Actual**
Oven Temperature	100 °C	100 °C
Loop Temperature	110 °C	110 °C
Transfer Line Temperature	120 °C	120 °C
Vial Pressure	--	1.390 psi
Vial Flow	--	-0.02 mL/min
Carrier Pressure	External Supply	13. 595 psi

Antibacterial Experiment

In this study, antibacterial experiments were carried out in Molecular Biology Laboratory, Department of Biology, Hacettepe University, Beytepe, Ankara. The antibacterial properties of 11 hand sanitizer were examined by agar disk diffusion assay.

Staphylococcus aureus (*S. aureus*) is among the bacteria that is the most common cause of infection in skin surface damage [20]. *Escherichia coli* (*E. coli*), on the other hand, has become the most widely understood and studied microorganism in microbiology in terms of use, ease of production and durability, as well as being a very common microorganism [21] Since *E. coli* is Gram (-) and *S.aureus* is Gram (+) bacteria, the antimicrobial activity of hand sanitizers will be

evaluated. Therefore, these two strains were considered sufficient in this study.

In this study *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as bacterial strains for the agar disk diffusion assay. First of all, *E. coli* and *S. aureus* were inoculated into 15mL LB (Luria Bertani) medium in order to obtain a bacterial cell suspension from bacterial strains and incubated for 24 hours at 37 °C. At the end of the incubation period, bacterial cell suspensions adjusted to 0.5 McFarland (1.5×10^8 CFU/mL) by diluted with PBS. 100 µL bacterial suspensions was taken and inoculated into LB agar plates. Whatman papers has been cut with the help of a 9 mm diameter cutter and sterilized under UV light. 100 µL commercial hand sanitizers were taken with sterile pipette tip and quickly soaked to sterile whatman papers and placed on LB agar plates. As the control, antibiotic discs containing 10 µg ampicillin were also placed on LB agar plates. Subsequently, LB agar plates were left to incubate for 24 hours at 37 °C and zone diameters were measured at the end of the incubation period [22].

RESULTS AND DISCUSSION

In this study, it was aimed to determine the active ingredients (ISA and/or ethanol as active ingredient) of 11 commercial hand sanitizers in terms of volume (% v/v) by HS/GC-MS; and aimed to investigate their antibacterial activities. The amounts of alcohol, which are the active ingredients of the samples, were verified and it was determined whether they reached the limits set by WHO (75% for isopropyl alcohol (ISA), 80% for ethyl alcohol according to the World Health Organization). In addition, its antibacterial properties have been demonstrated using two bacterial strains (Gram-negative *E. coli* and Gram-positive *S. aureus*, were selected) Since there are samples in which isopropyl alcohol and/or ethanol are used together in the hand sanitizer content, the total amount of alcohol was determined and the limit value was accepted as 80% alcohol by volume [6]. According to the analysis, it was seen that the amount of isopropyl alcohol/ethanol in the hand sanitizers differs and 7 of them are above the 80% (v/v) value determined by the World Health Organization (WHO).

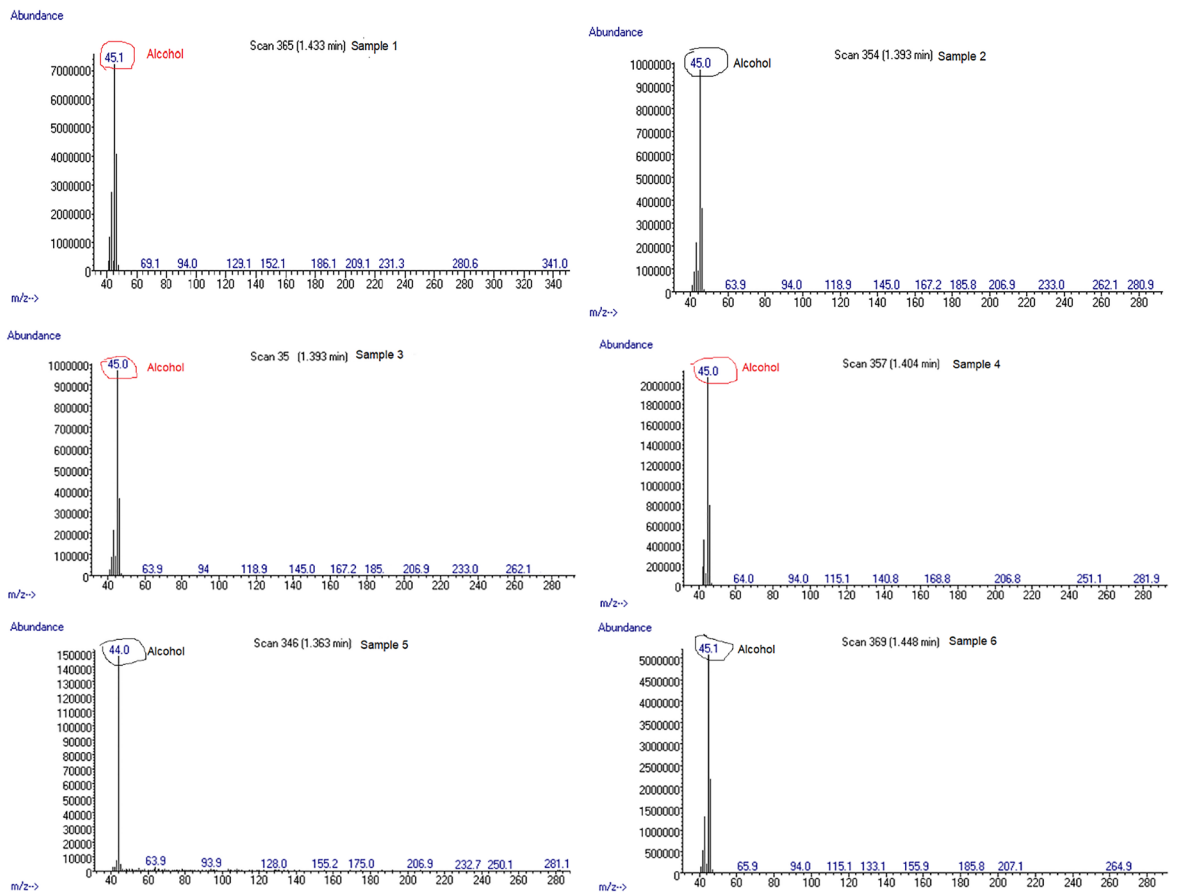

Figure 1. HS/GC-MS chromatograms and alcohol (ISA/ethanol) peaks of samples (S1-11)

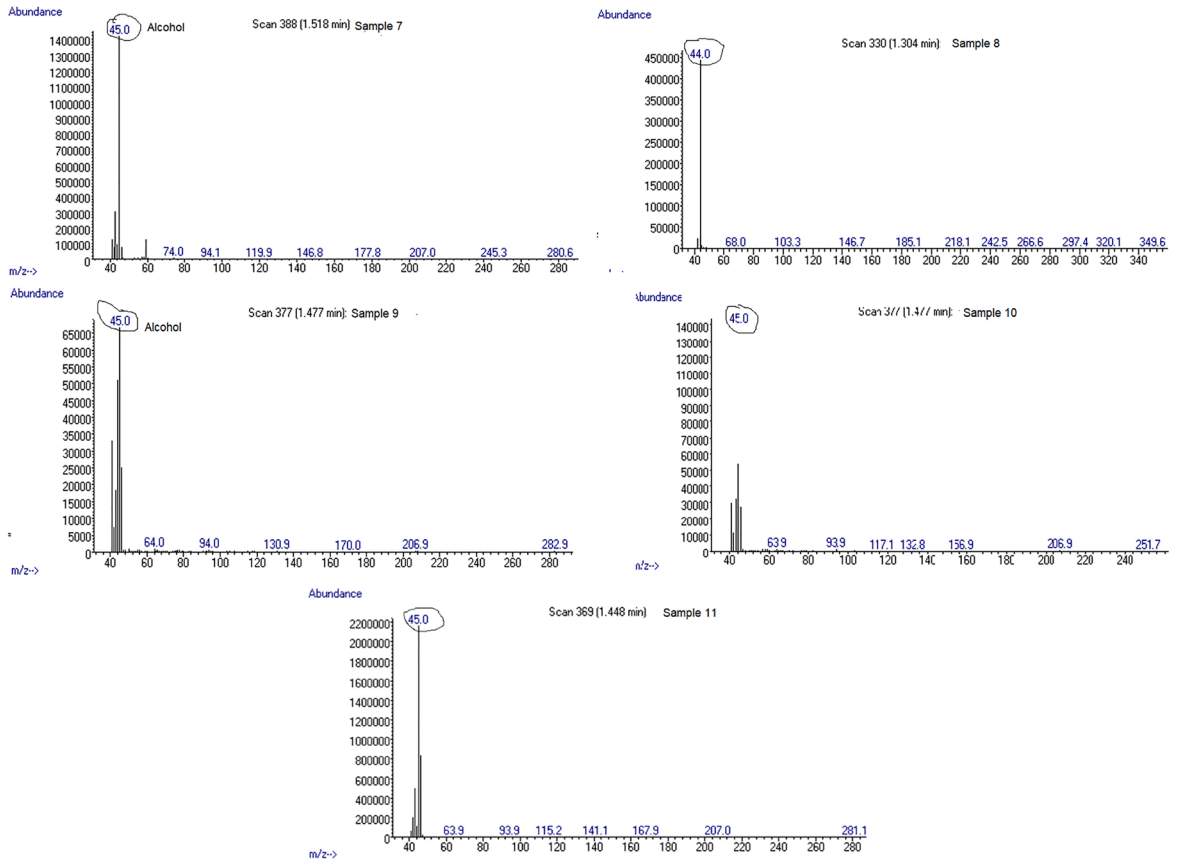

Figure 1. Continued.

The active ingredients in hand sanitizers usually destroy microorganisms. Also, inactive ingredients are added to disinfectants for reasons such as adding fragrance, protecting the health of the applied skin, and adjusting the disinfectant viscosity. Table 5 contains information on the active ingredients (alcohol derivatives) and additional ingredients found in commercial hand sanitizers. While ethanol and isopropyl alcohol are used as active ingredients in the samples, water, carbomer, glycerin, fragrance agent etc. are frequently used as additional ingredients [8].

Also, HS/GC-MS chromatograms and active ingredient (ISA-ethanol) peaks of 11 hand sanitizer samples are shown (Figure 1). Quantifer ion (m/z) of ethanol and isopropyl alcohol has a peak value of 45 [23]. In this context, it was shown that each example has had the active compound.

It has shown the retention time, percentage volume and match factor values of the total alcohol (isopropyl alcohol and/or ethanol) amounts obtained as a result of GC-MS/HS analyzes of commercial hand sanitizers for 11 samples (control group is not reflected in the table) in Table 5. The shown variables represent the ethyl alcohol/isopropyl alcohol ratio component (% v/v) based on data scanned in the SWGDRUG 3.5.L Library. Retention time (Rt, min) has shown the retention time of the substance, match factor (MF) shows the match rate in the library. Measurements were made 3 times and the average of the volumetric area and Match Factor (MF) values were reflected in the table (Table 6).

Based on the data in the Table 6, a graph was created including the percentage amounts of alcohol in the total mixture and standard deviation values. SSR and SSReg values were obtained by calculating the ANOVA (One-way analysis of variance, $P < 0.05$) (Figure 2). According to the value, difference between the observations and the predicted values are small and unbiased.

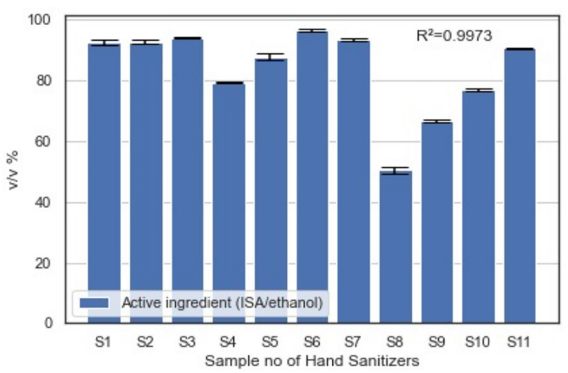

Figure 2. Alcohol amounts obtained as a result of HS/GC-MS Analysis of commercial hand sanitizers (ISA and ethanol, v/v%)

Table 5. Active ingredient (alcohol derivative) and additional ingredient information of hand sanitizer samples

Sample No	Active Ingredient (alcohol derivative)	Additional Ingredient
S1	Ethanol	water, carbomer, glyserin, triethanolamine
S2	Ethanol	water, carbomer, glyserin, fragrance agent
S3	Isopropyl Alcohol	water, butane-1.3-diol, lanolinpoly (oxyethlyene)-75, fragnance
S4	Isopropyl Alcohol	water, butane, isobutane, propane, tocopheryl acetate, benzalkonium chloride, panthenol, glyserin
S5	Isopropyl Alcohol	water, lanolinpoly (oxyethlyene)-75, fragnance
S6	Isopropyl Alcohol	water, carbomer, chlorhexidine digluconate, fragnance, glycerin, *Aloe barbadensis*, clay, sodium hydroxide
S7	Isopropyl Alcohol	water, glycerin, purified water, fragnance
S8	Isopropyl Alcohol	water, ,glycerin, polysobate 20,carbomer,triethanolamine, fragnance
S9	Isopropyl Alcohol	water, butylene glycol, carbomer, triethanolamine, perfume, polysorbate 60, DL-Panthenol
S10	Isopropyl Alcohol	purified water, fragnance
S11	Isopropyl Alcohol	glycerin, hydrogen peroxide, purified water USP

Table 6. Total alcohol (v/v%) obtained as a result of GC-MS/HS Analysis of commercial hand sanitizers (isopropyl alcohol and/or ethyl alcohol)

Sample	Rt (min)	Area% (v/v)	MF (Match Factor)
S1	1.444	92.51	84,6
S2	1.428	92.63	81.5
S3	1.428	94.01	80.3
S4	1.421	79.26	82.5
S5	1.421	87.70	82.3
S6	1.428	96.28	85.7
S7	1.419	93.36	81.0
S8	1.426	50,36	94.3
S9	1.430	66.48	83.0
S10	1.426	76.70	83.1
S11	1.428	90.50	82.2

sample both in the label information and as a result of the analysis (Table 5). In a study on the antibacterial effect of *Aloe barbadensis*, it has been shown that the antibacterial effect was stronger against both gram-positive and gram-negative bacteria at different concentration levels in samples using *Aloe barbadensis*, compared to those not used [24]. In this context, it can be said that plant agent (*Aloe barbadensis*) increases the antibacterial activity in S6. Likewise, it can be said that the fragrance agent found in S2 (fragrance could not be detected in HS/GC-MS) may be a herbal ingredient that increases antibacterial activity.

Table 7. Agar disc diffusion assay results for antibacterial properties of hand sanitizers (zone diameters, mm)

Bacterial strain	Ampicillindisc (10µg)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
S. aureus	19	0	15	14	11	12.5	17	13	14	15	0	12
E. coli	19	12	19	11	15	0	13	15	11	0	14	15

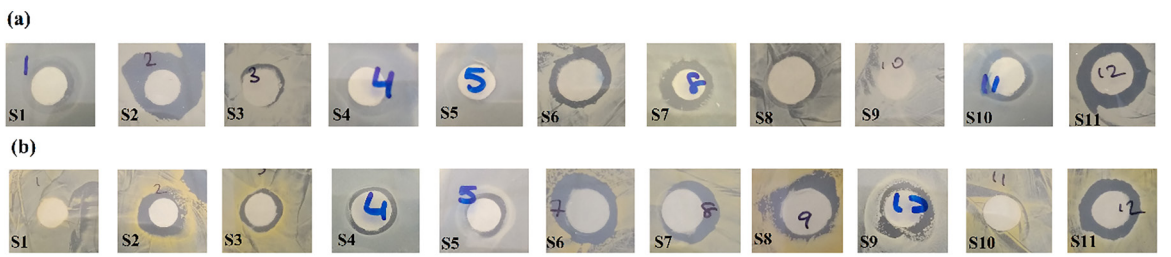

Figure 3. Inhibition zone of sample 1 to 11 for (a) *E. coli* and (b) *S. aureus.* (For all samples, sample numbers of were given at the bottom of left corner. Apart from this, the numbers written with acetate pen on the figures do not reflect the figure numbers).

The antibacterial properties of hand sanitizers were evaluated with the agar disc diffusion assay. Agar disc diffusion assay results were given in Table 7 also zone inhibition of hand sanitizers was given in Figure 3. Based on the results, it was seen that sample 5(S5) and sample 9(S9) were not shown antibacterial properties against *E. coli.* It was seen that S1 and S10 were not shown antibacterial properties against *S. aureus.* By looking at the formed zone diameters, it was concluded that the antibacterial properties of S6 and S2 were high for both *S. aureus* and *E. coli.* It was determined that there was plant content (*Aloe barbadensis*) in the S6

It has been stated from company that S5 was effective for *E. coli* (NCTC 1038) strain and *S. aureus* (ATCC6538) strain. However, the zone diameter was formed only for S. *aureus.* In our study, non-resistant bacterial strain *E. coli* (ATCC 25922) was used however, in all repeated agar disc diffusion assays, no zone diameters were formed for S5 in *E. coli.*

It was stated that S11 had antibacterial properties for *E. coli* (ATCC10536) and *S. aureus* (ATCC6538). From the results of the agar disc diffusion assay S11 has created zone

against both *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) strains. It was expected from hand sanitizers to show their antibacterial properties the shortest possible time and in the most effective way [25]. Based on this information, it is expected that the effects of sanitizers against bacteria strains in agar plates have been observed before the alcohol has evaporated.

Also, According to Table 5 about contents of hand sanitizers, it was seen that samples had contained ethanol or isopropyl alcohol as the main antibacterial agent. As a result of the repeated zone inhibition experiments, the inhibition zone has not been seen in S1-S10 for *S. aureus*, and S5-S9 for *E. coli.* It was possible that the bacterial concentration was intense, so these hand sanitizers could not show the antibacterial effect. Since the same protocol was applied for all samples in the experiment, equal bacterial concentration was cultivated and zone inhibition experiments with diluted bacterial concentration were not repeated for these samples.

CONCLUSION

The use of hand sanitizers, which became widespread with the Covid pandemic, has aroused our curiosity about how much antibacterial effect they have shown. First of all, the alcohol content of hand sanitizers was investigated by HS/GC-MS analysis and their antibacterial activity were investigated on *S. aureus*, one of the most common disease-causing bacteria, and *E. coli*, one of the most frequently studied strains. Then, by these strains the zone inhibition experiments were performed.

As a result of this study, it was seen that S4 out of S11 commercial hand sanitizers were not suitable for the final concentration values of hand sanitizers determined by the World Health Organization (accepted as 80%(v/v) for alcohol derivatives). Apart from this, it has been seen that S5 and S9 did not show antibacterial properties against *E. coli*, S1 and also, sample S10 did not show antibacterial properties against *S.aureus*. This results have showed that the control of the standards of hand sanitizers should be controlled with much more stringent rules. The present study needs to be supported by more samples of hand sanitizers.

CONFLICT OF INTEREST

Authors approve that to the best of their knowledge, there is not any conflict of interest or common interest with an institution/organization or a person that may affect the the review process of the paper.

AUTHOR CONTRIBUTION

Aybuke A. Isbir Turan and Simge Varlik worked with GC-MS analysis data. Isik Percin Demircelik and Gulsen Bayrak performed antibacterial analyzes. Gulsen Bayrak and Simge Varlik revised the article. All authors contributed to the finalization of the article.

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