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Alcohol-based hand rubs against SARS-CoV-2: Analysis of ninety commercial samples by HS-GC/MS and electrochemical biosensor

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Abstract:	Alcohol-based hand rubs (ABHRs) have found large diffusion during the Severe Acute Respiratory Syndrome Coronavirus 2, SARS-CoV-2, becoming the most widespread means for hand hygiene. For this reason, it is fundamental to assess the alignment of commercial ABHRs to the indications provided by the principal health agencies regarding alcohol content and possible impurities. In this study, a survey was conducted on ninety ABHRs, purchased on the Italian market, with the aim of obtaining an overview of products available to the public during the first period of the pandemic. Samples were firstly analysed in terms of pH and by Scanning Electron Microscopy and optical microscopy, in order to investigate their morphology and the effect of polymer crosslinks on alcohol release. Alcohols in samples were determined by static Headspace Gas Chromatography Coupled with Mass Spectrometry (HS-GC/MS) and by an electrochemical biosensor, based on the immobilisation of Alcohol oxidase on screen printed electrodes (SPEs). The analytical approaches were compared through a correlation study, showing a screening method by biosensors and a quantification one by HS-GC/MS. The survey has evidenced that 26% of the tested cosmetic products had the recommended average alcohol content, confirming the importance of analytical controls on this type of products.
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Dear Editor,

We would be very grateful if you kindly consider the original article entitled "**Alcohol-based hand rubs against SARS-CoV-2: Analysis of ninety commercial samples by HS-GC/MS and electrochemical biosensor**" for publication in **International Journal of Pharmaceutics**.

The novelty of this paper is an analytical investigation of commercial alcohol based hand rubs using two different analytical approaches: electrochemical biosensor and Headspace Gas Chromatography Coupled with Mass Spectrometry. The first tool for fast, low-cost monitoring of hand rubs present on the market in order to avoid consumer fraud and preserve their health while the second technique confirms and identifies in analytical and quantitative way the alcohol content in the hand rubs. In details, results concerning the survey we conducted on ninety alcohol-based hand rubs (ABHRs), purchased on the Italian market are presented. During the Severe Acute Respiratory Syndrome Coronavirus 2, SARS-CoV-2, ABHRs have found large diffusion as means of obtaining rapid and effective hand hygiene. According to the principal health agencies, ABHRs must contain at least 60 %v/v alcohol to have effect on pathogens, including SARS-CoV-2. To this end, we conducted the survey with the aim of assessing the alignment to the indications of the sanitary authorities of products available to the public between April and November 2020. To the best of our knowledge, this is the first survey involving such a number of ABHRs. All samples were analysed for the simultaneous determination of ethyl alcohol, isopropyl alcohol, n-propyl alcohol and methyl alcohol, with a sensitive analytical technique based on static Headspace Gas Chromatography Coupled with Mass Spectrometry applying a specific method that was in-house developed. Samples were also analysed by an electrochemical biosensor and its application on ABHRs is described in this paper for the first time.

This study highlighted that the combination of the biosensor as screening approach and the HS-GC/MS analysis as sensitive and specific technique, would allow to have a powerful tool for the analysis of alcohol in gels. In fact, results obtained from the two different analytical approaches were compared and showed good correlation according to 99% in a prediction interval of 35% v/v and 85% v/v.

We believe that our paper will be of interest for the readership of International Journal of Pharmaceutics and we are confident in a positive response. This manuscript has not been published and is not under consideration for publication elsewhere. Thank you for your consideration.

Kind regards,

Costanza Majorani

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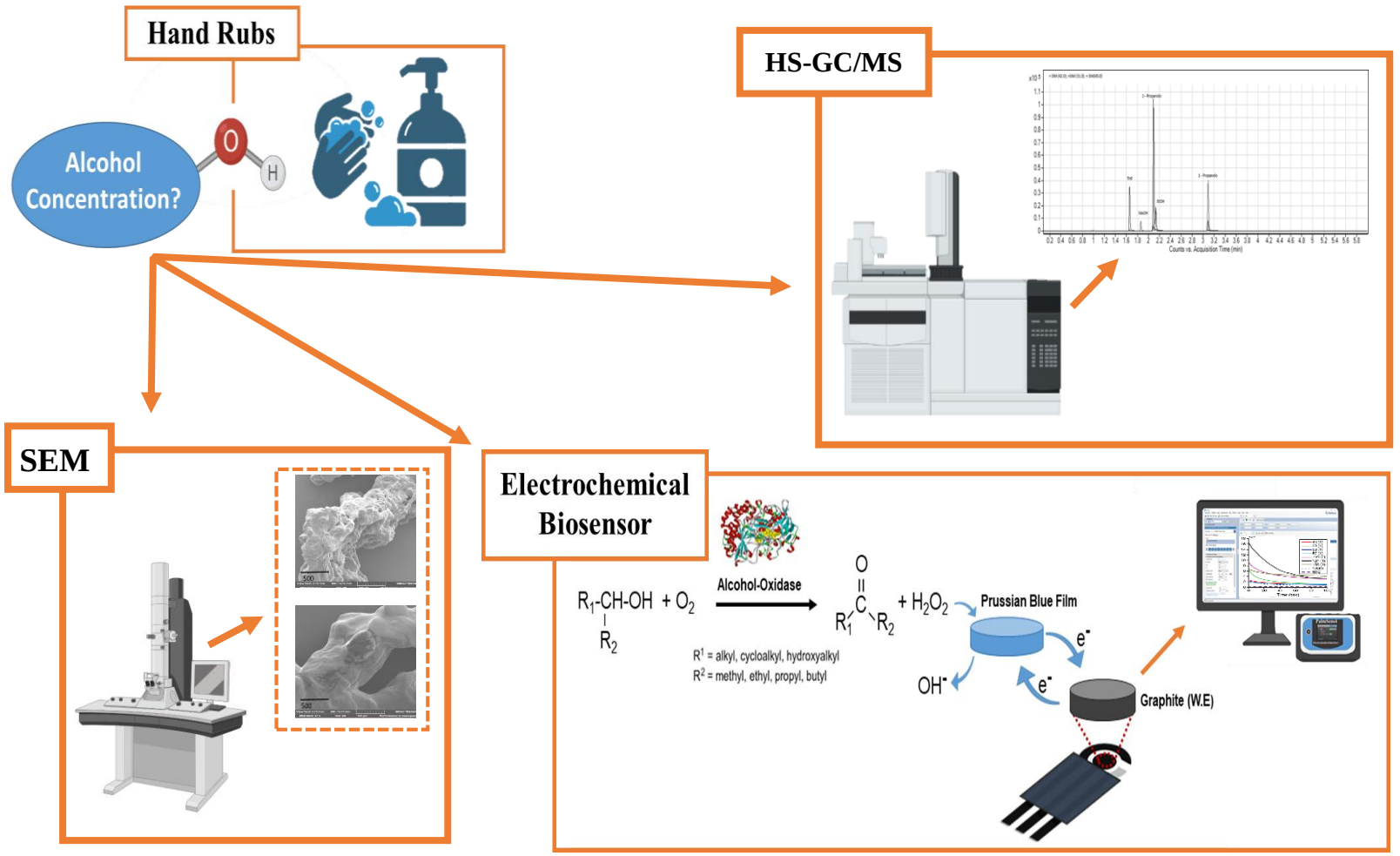
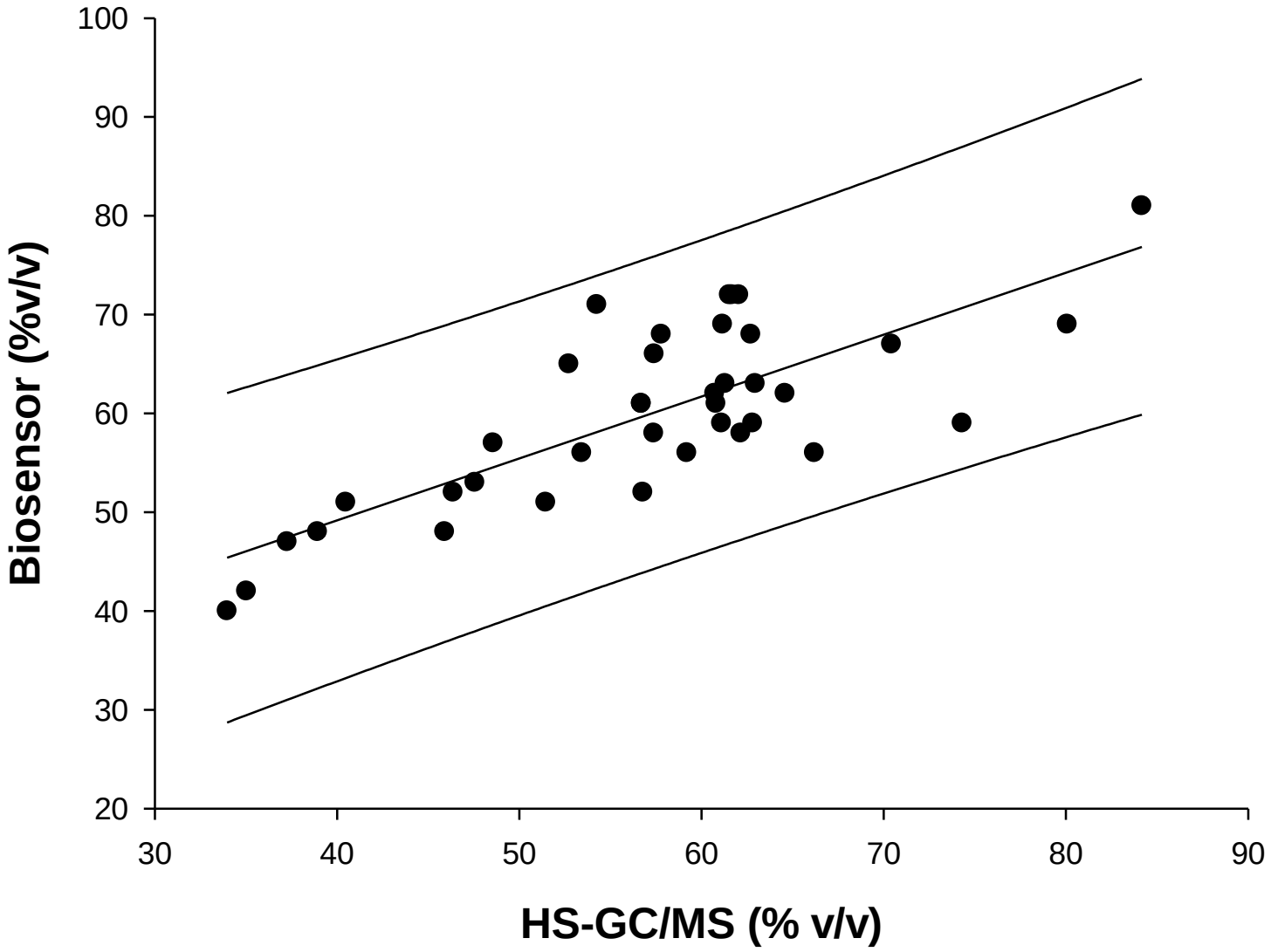
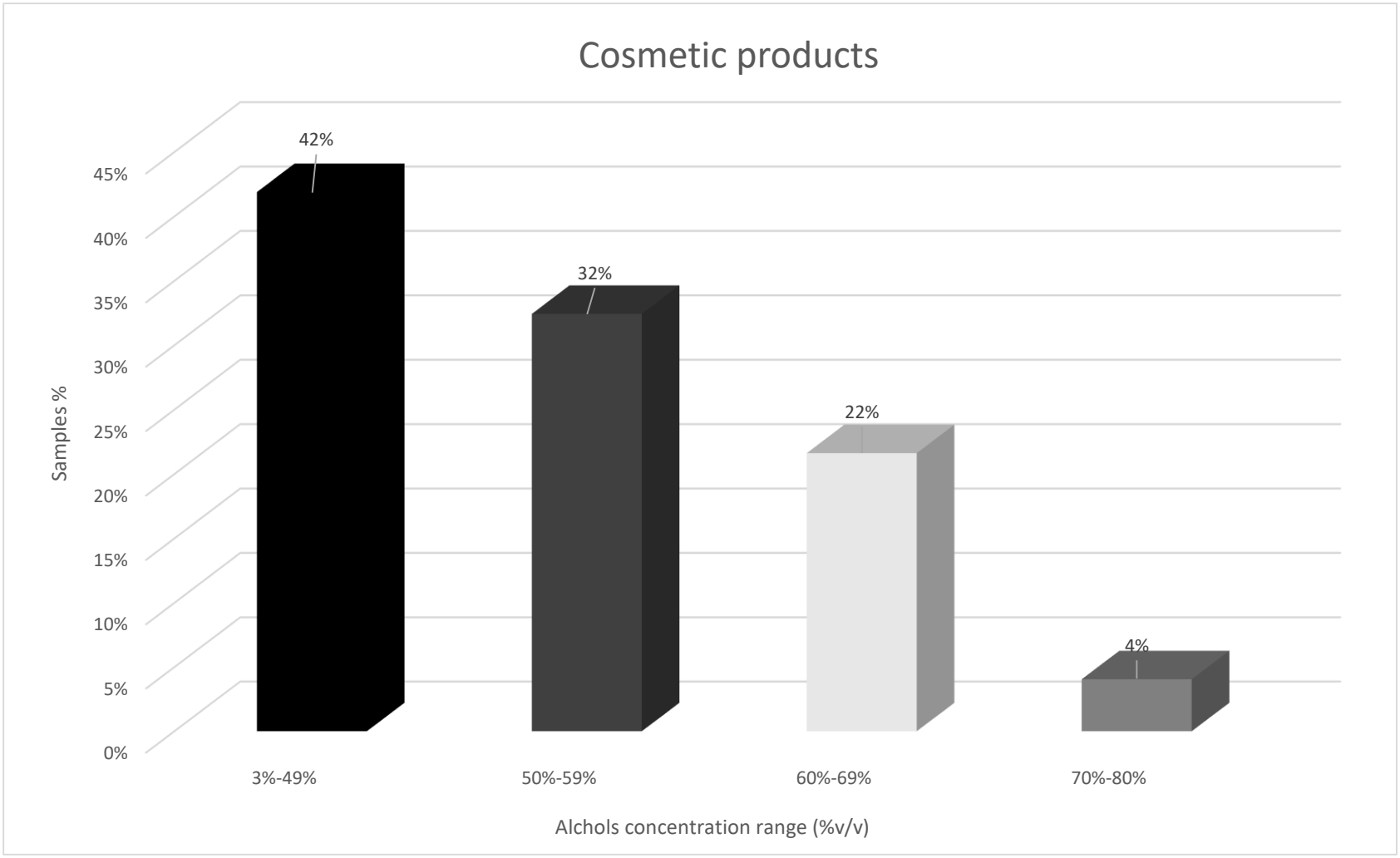
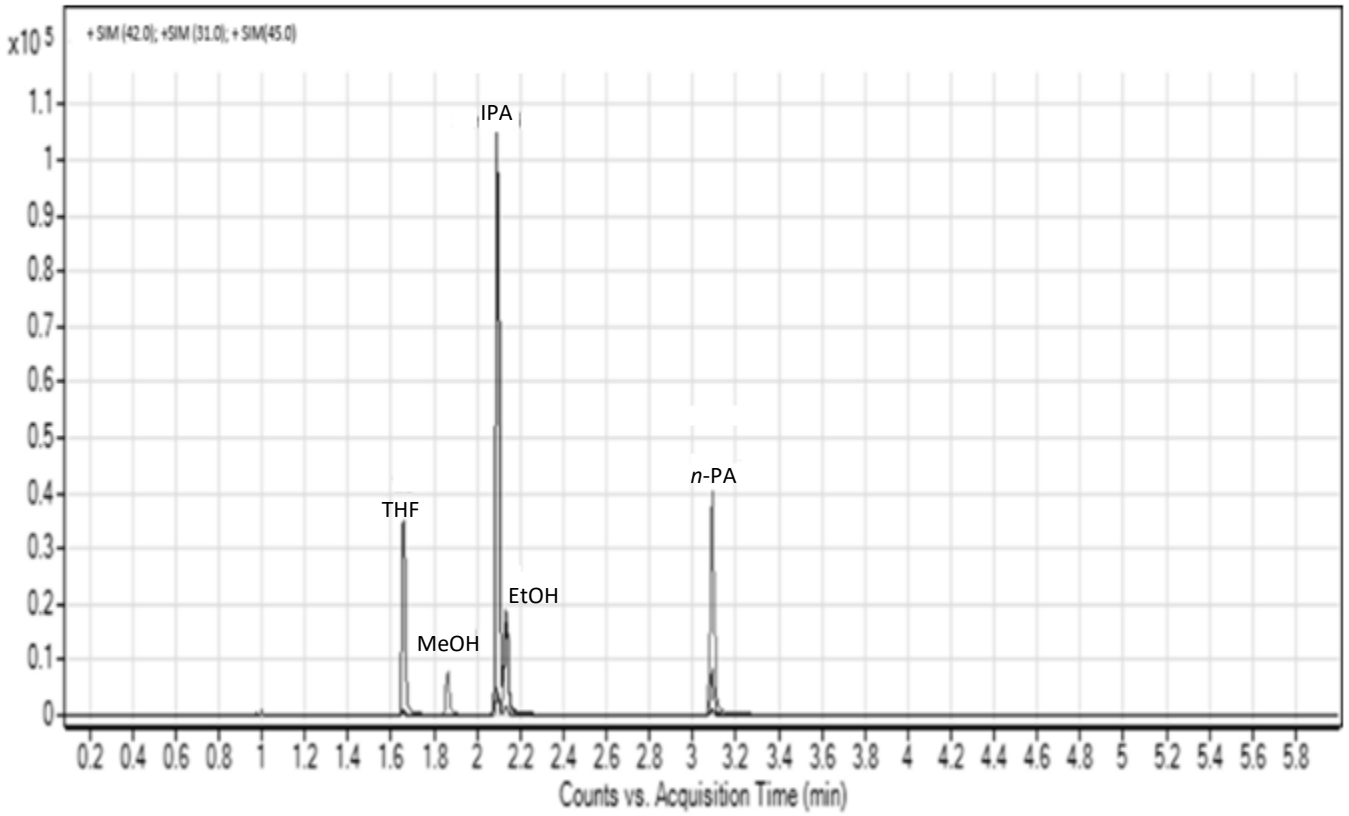
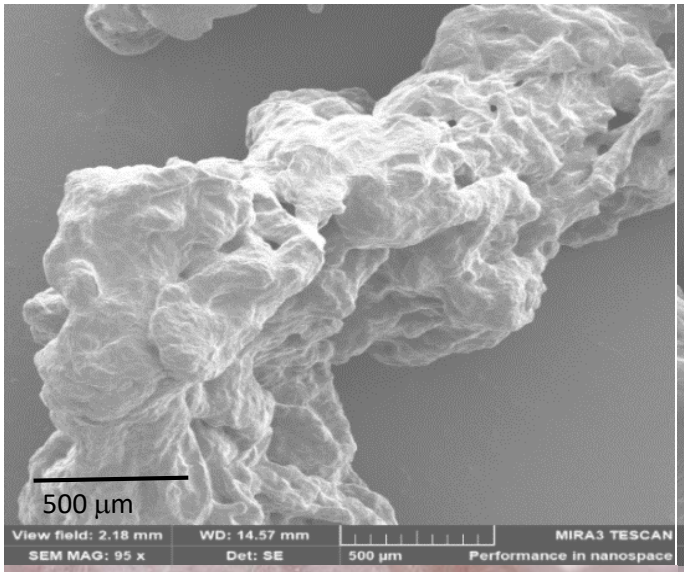
Graphical Abstract (for review)

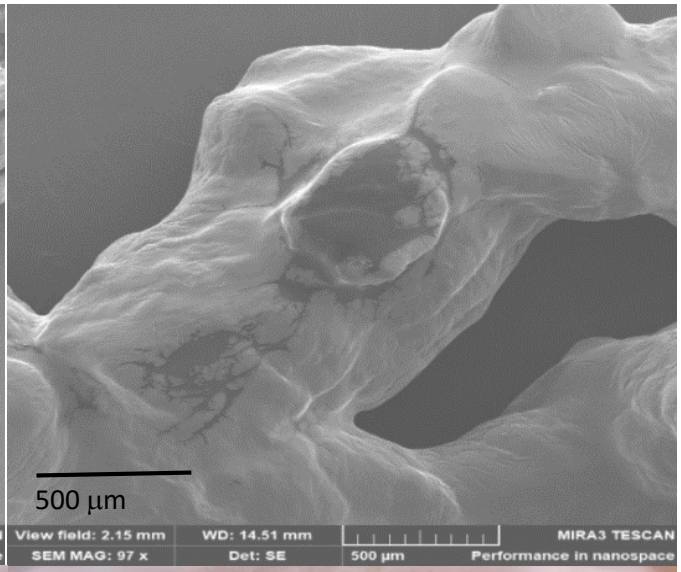


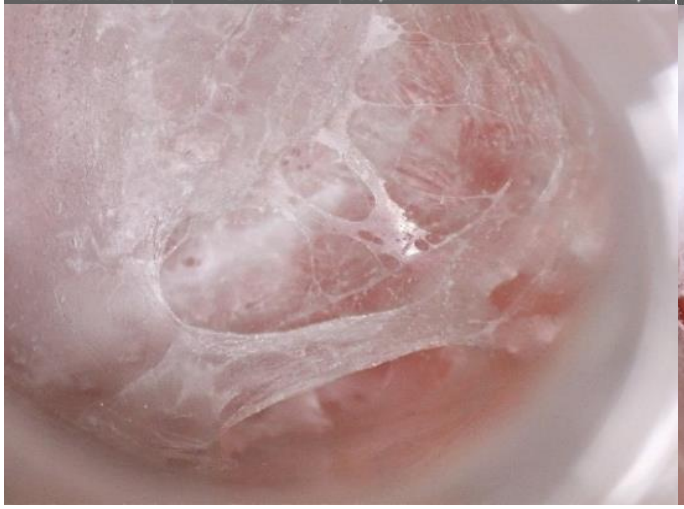

Biosensor (%v/v)
100
90
80
70
60
50
40
30
20
30
40
50
60
70
80
90
HS-GC/MS (% v/v)


Cosmetic products
42%
32%
22%
4%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Samples %
3%-49%
50%-59%
60%-69%
70%-80%
Alchols concentration range (%v/v)


+ SIM (42.0); +SIM (31.0); + SIM(45.0)
x10 5
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
IPA
THF
MeOH
EtOH
n-PA
0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4 2.6 2.8 3 3.2 3.4 3.6 3.8 4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6 5.8
Counts vs. Acquisition Time (min)



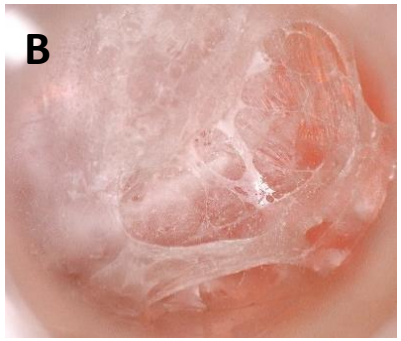




Hydroxyethylcellulose

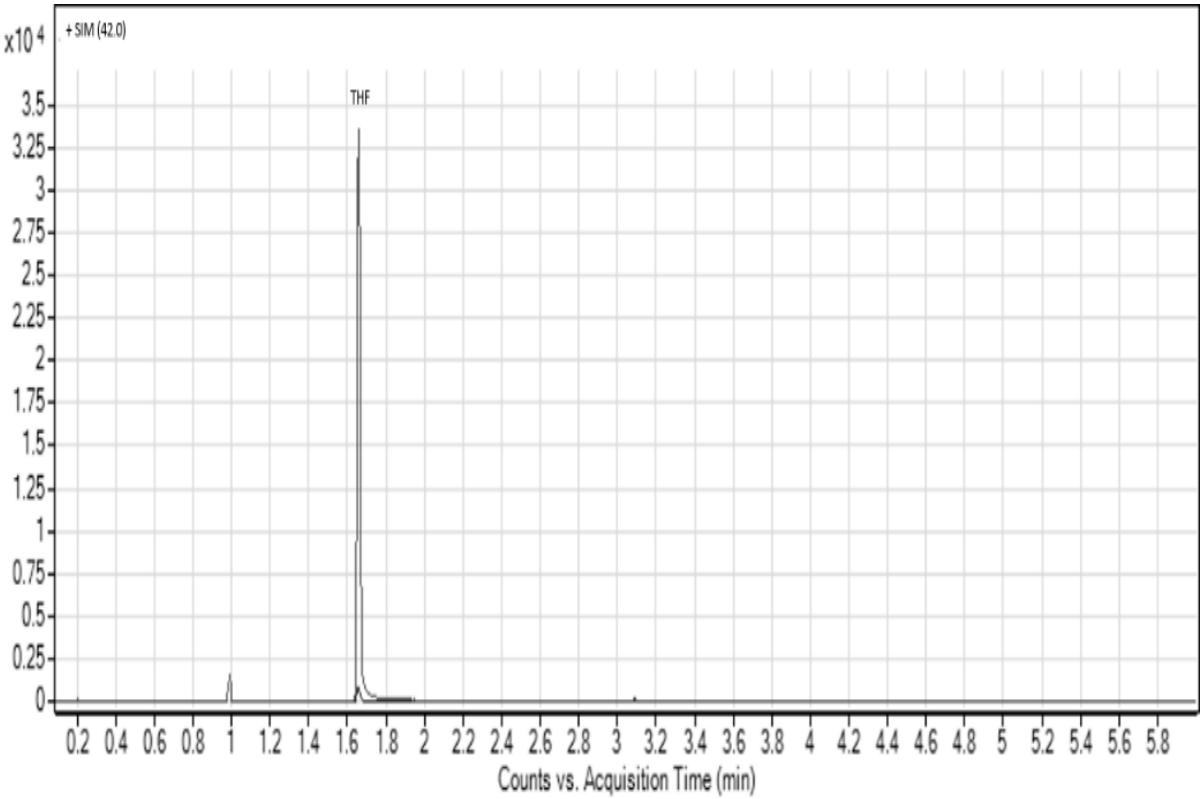
Carbomer

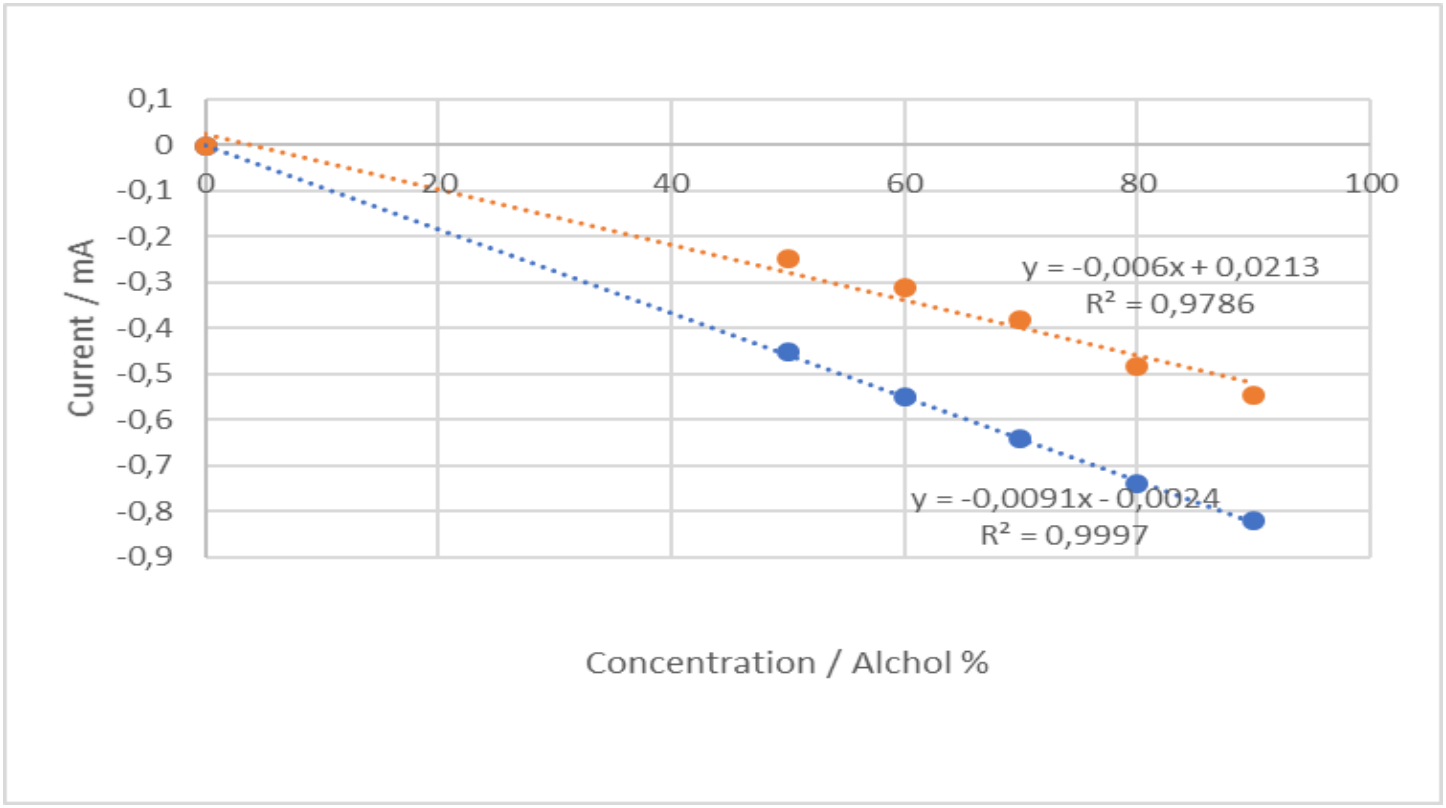

A


B


C


D


x10 4
+ SIM (42.0)
THF
3.5
3.25
3
2.75
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4 2.6 2.8 3 3.2 3.4 3.6 3.8 4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6 5.8
Counts vs. Acquisition Time (min)


0,1
0
-0,1
-0,2
-0,3
-0,4
-0,5
-0,6
-0,7
-0,8
-0,9
0
20
40
60
80
100
Current / mA
Concentration / Alchol %
y = -0,006x + 0,0213
R² = 0,9786
y = -0,0091x - 0,0024
R² = 0,9997

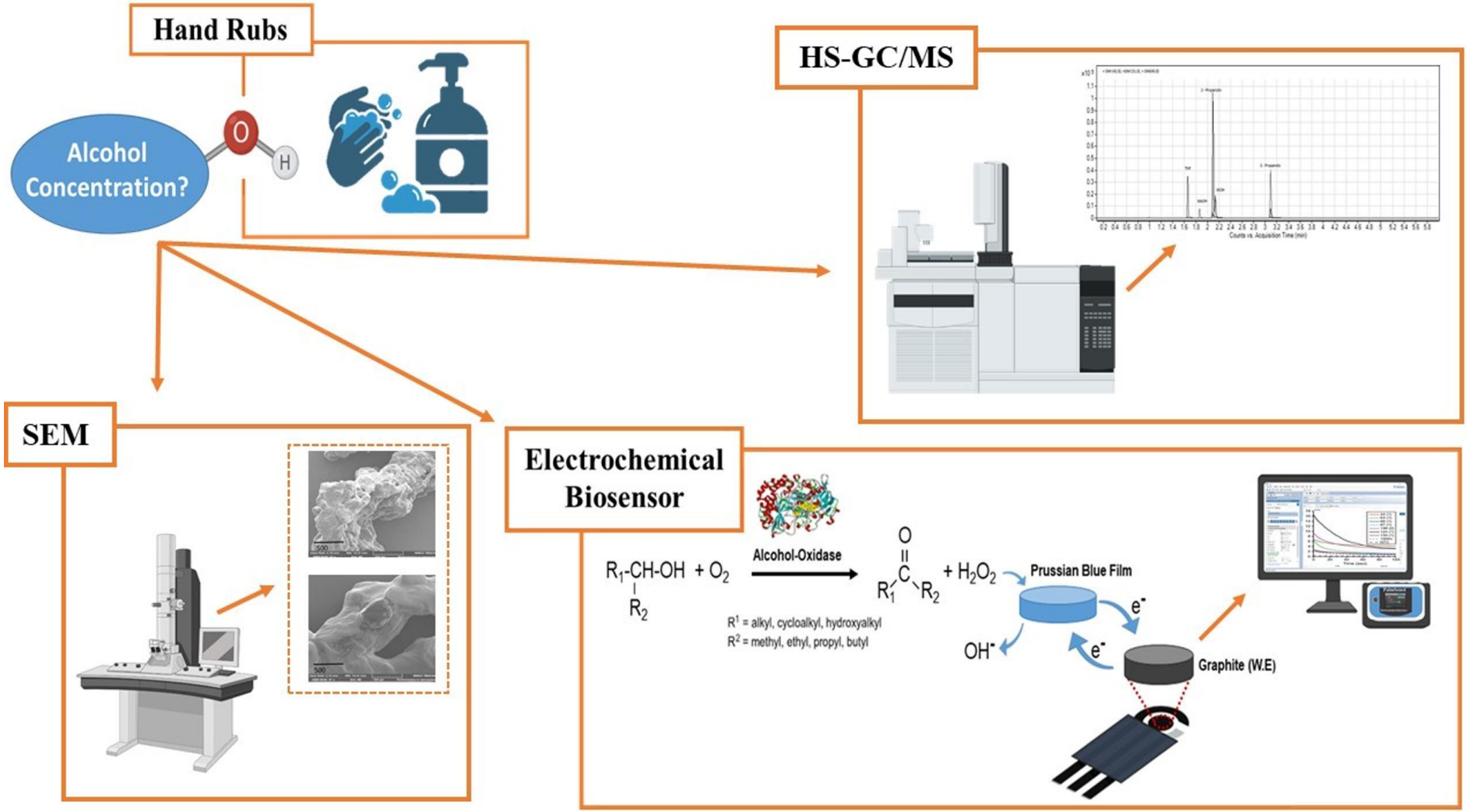

Hand Rubs
Alcohol Concentration?
O
H
HS-GC/MS
SEM
Electrochemical Biosensor
Alcohol-Oxidase
R_1-CH-OH + O_2
R_2
+ H_2O_2
R^1 = alkyl, cycloalkyl, hydroxyalkyl
R^2 = methyl, ethyl, propyl, butyl
Prussian Blue Film
e^-
OH^-
Graphite (W.E)

Table 1

	Characteristic ions (+m/z) (quantification ion underlined)	t_R (min)
MeOH	*31*, 29, 15	1.864
IPA	*45*, 27,43	2.098
EtOH	*31*, 45,46	2.132
n-PA	*31*, 27, 29	3.092
THF (IS)	*42*, 41, 72	1.665

Table 1. Analytes and IS characteristic m/z ions for SIM mode acquisition and retention times (tR)

Table 2

Biocidal sample ID	Formulation	EtOH detected in sample (%v/v)	U* (%v/v)	EtOH detected in acidified sample (%v/v)	U* (%v/v)	EtOH declared on label (%v/v)
045	Liquid	80.3	+/- 3.6	85.3	+/- 3.8	84
021	Gel	42.2	+/- 1.8	60.0	+/- 2.7	56
011	Gel	55.7	+/- 2.4	80.1	+/- 3.6	78
017	Gel	52.4	+/- 2.3	80.1	+/- 3.6	78
*U= expanded uncertainty						

Table 2. Results on biocidal products with different sample treatment.

Table 3

Validation parameters	Analyte				performance criteria
	MeOH	IPA	EtOH	n-PA	
Linearity – correlation coefficient	0.9998	0.9998	0.9998	0.9998	≥0.995
Regression equation	y = 7E-07x – 0.0016	y = 8E-06x + 0.0071	y = 2E-06x + 0.0004	y = 4E-06x + 0.0005	-
F[a]	5.95E+03	2.77E+04	6.31E+03	3.21E+04	≥7,71[b]
LOD (%v/v)	0.16	0.17	0.13	0.15	-
LOQ (%v/v)	0.53	0.57	0.44	0.50	-
Recovery (%)					
Level I (2 %v/v)	110	93	80	91	80-110
Level II (20 %v/v)	103	94	80	91	
Level III (60 %v/v)	103	96	81	93	
RSD_r					
Level I (1 %v/v)	2.9	9.1	8.0	8.9	HORRATr < 2
Level II (50 %v/v)	2.4	1.7	1.6	1.8	
Level III (70 %v/v)	3.9	3.4	3.5	3.3	
RSD_R					
Level I (1 %v/v)	4.9	10.7	9.3	8.4	HORRATr < 2
Level II (50 %v/v)	2.3	2.1	2.1	2.1	
Level III (70 %v/v)	3.8	2.9	3.6	3.2	

[a]*F* value obtained from ANOVA *F*-test. [b] Critical value of F (0.05,1,4)

Table 3. Validation parameters of the method and performance criteria

Figure 1

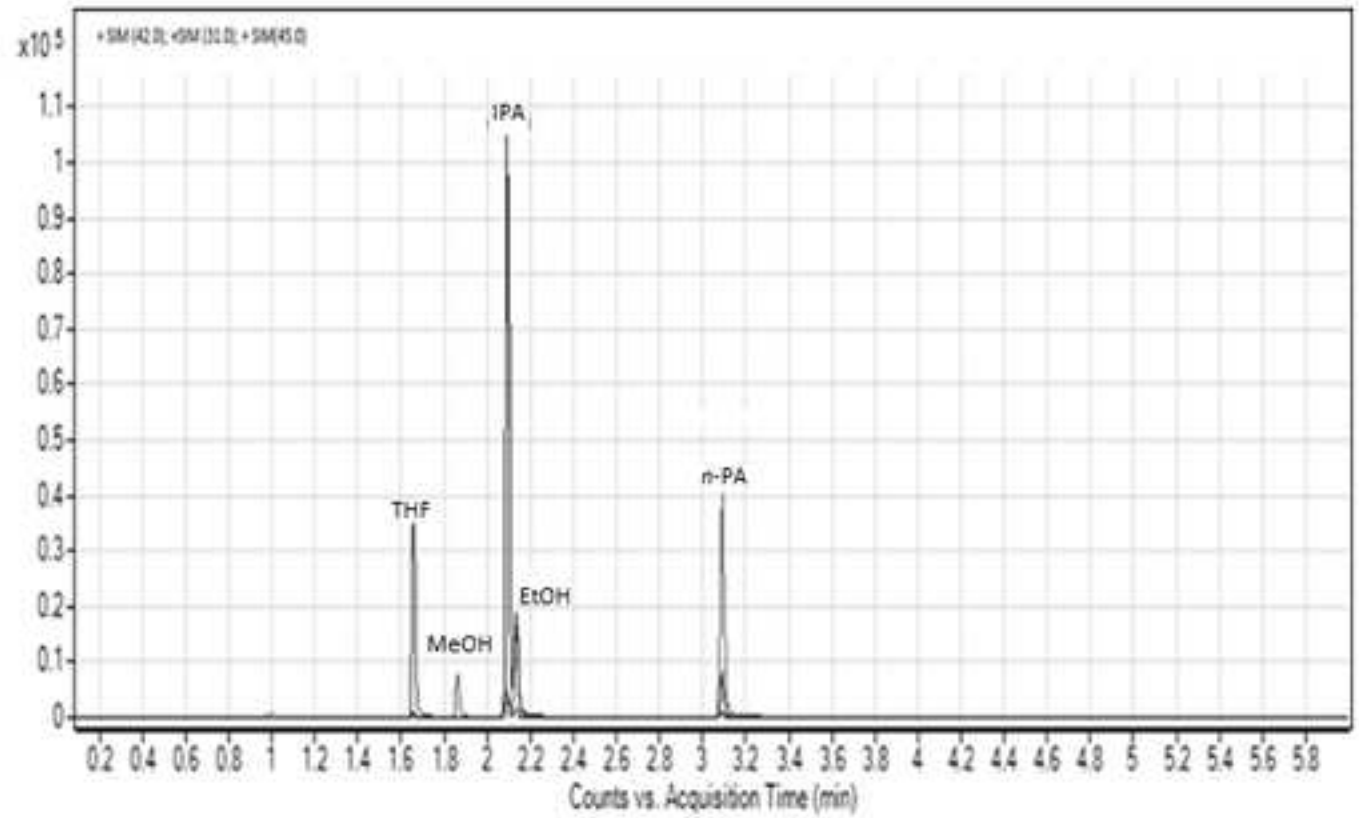
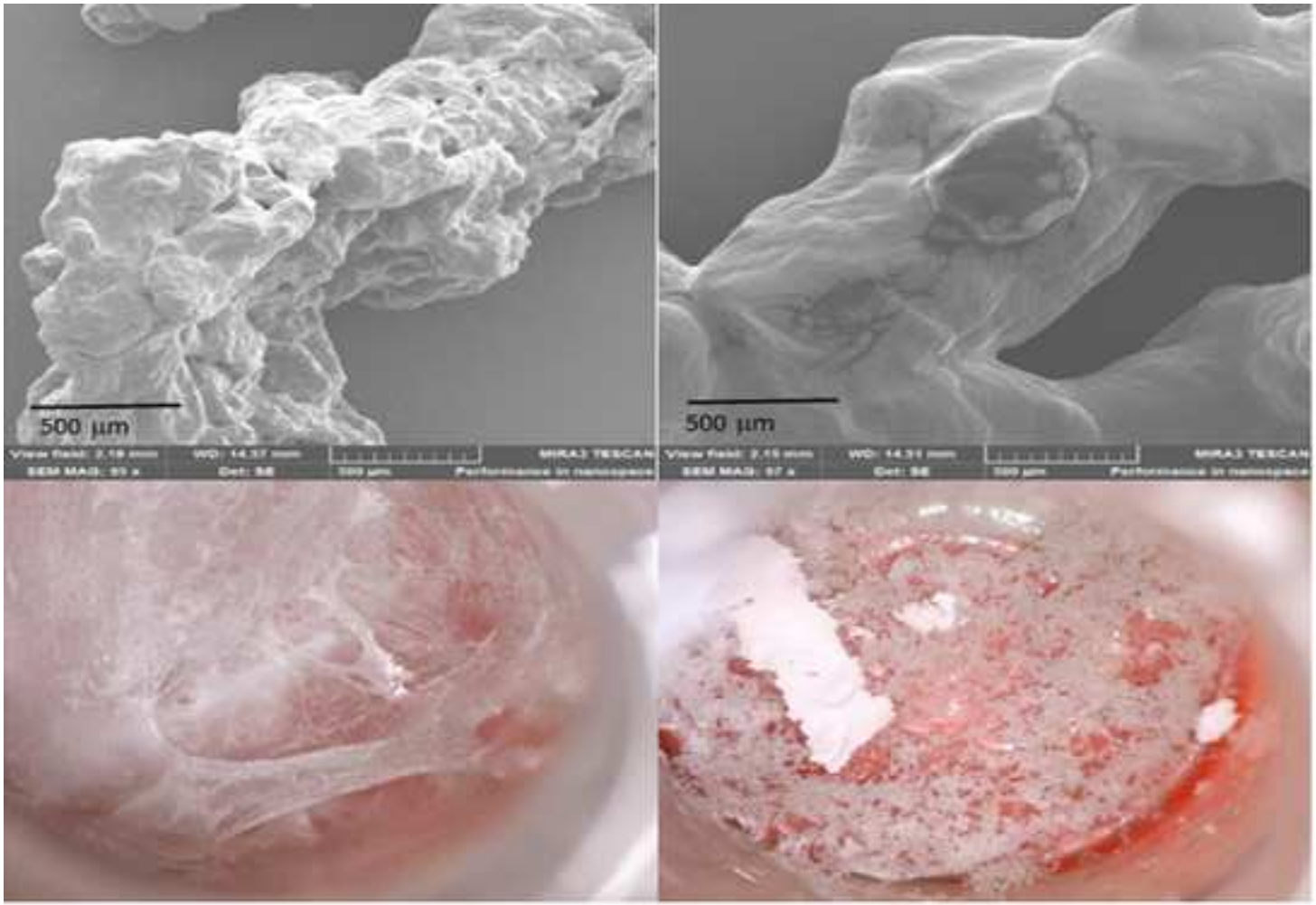

x10 5
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
IPA
n-PA
THF
EtOH
MeOH
0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4 2.6 2.8 3 3.2 3.4 3.6 3.8 4 4.2 4.4 4.6 4.8 5 5.2 5.4 5.6 5.8
Counts vs. Acquisition Time (min)

Figure 2a



Hydroxycellulose

Carbomer

Figure 2b

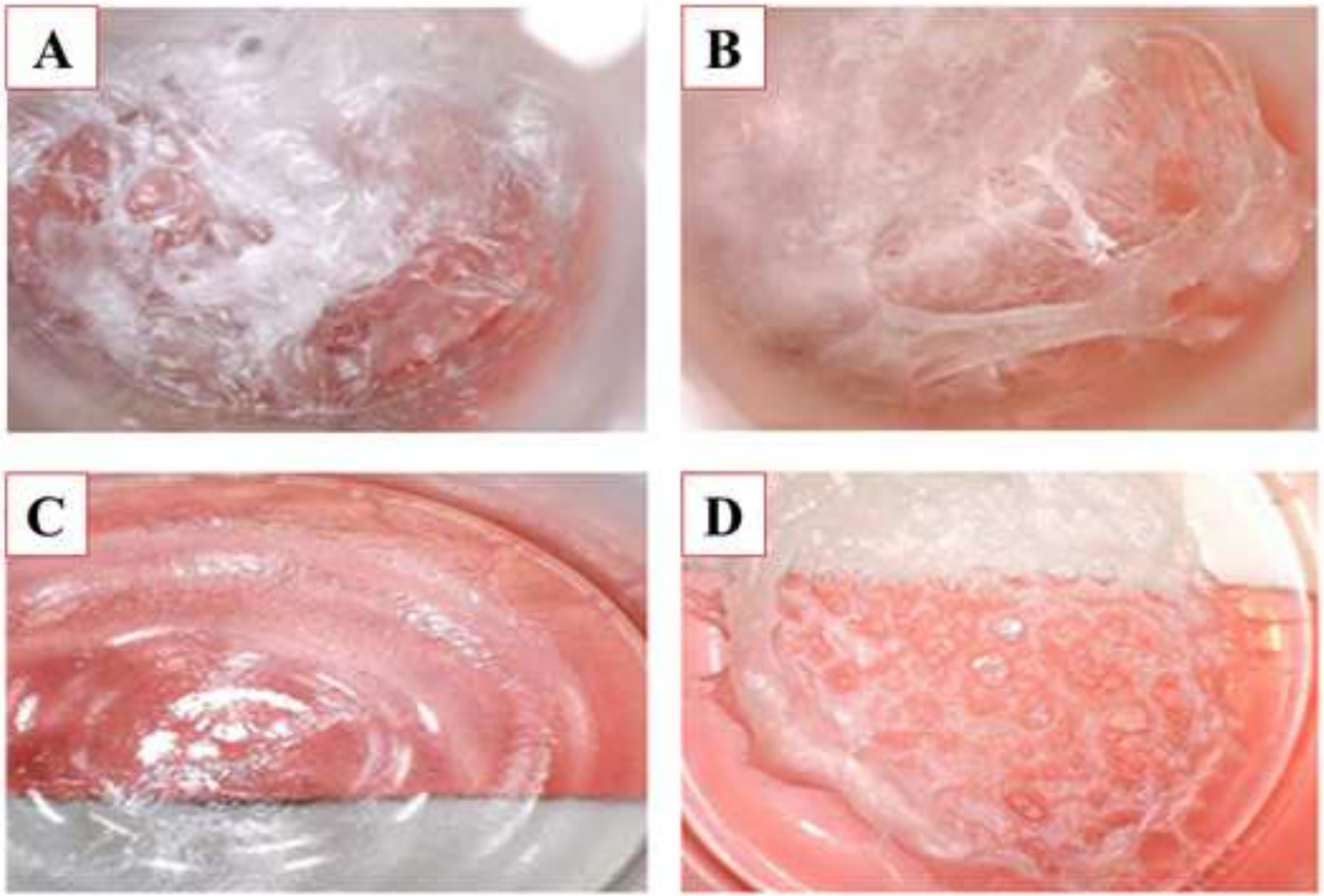


Figure 3

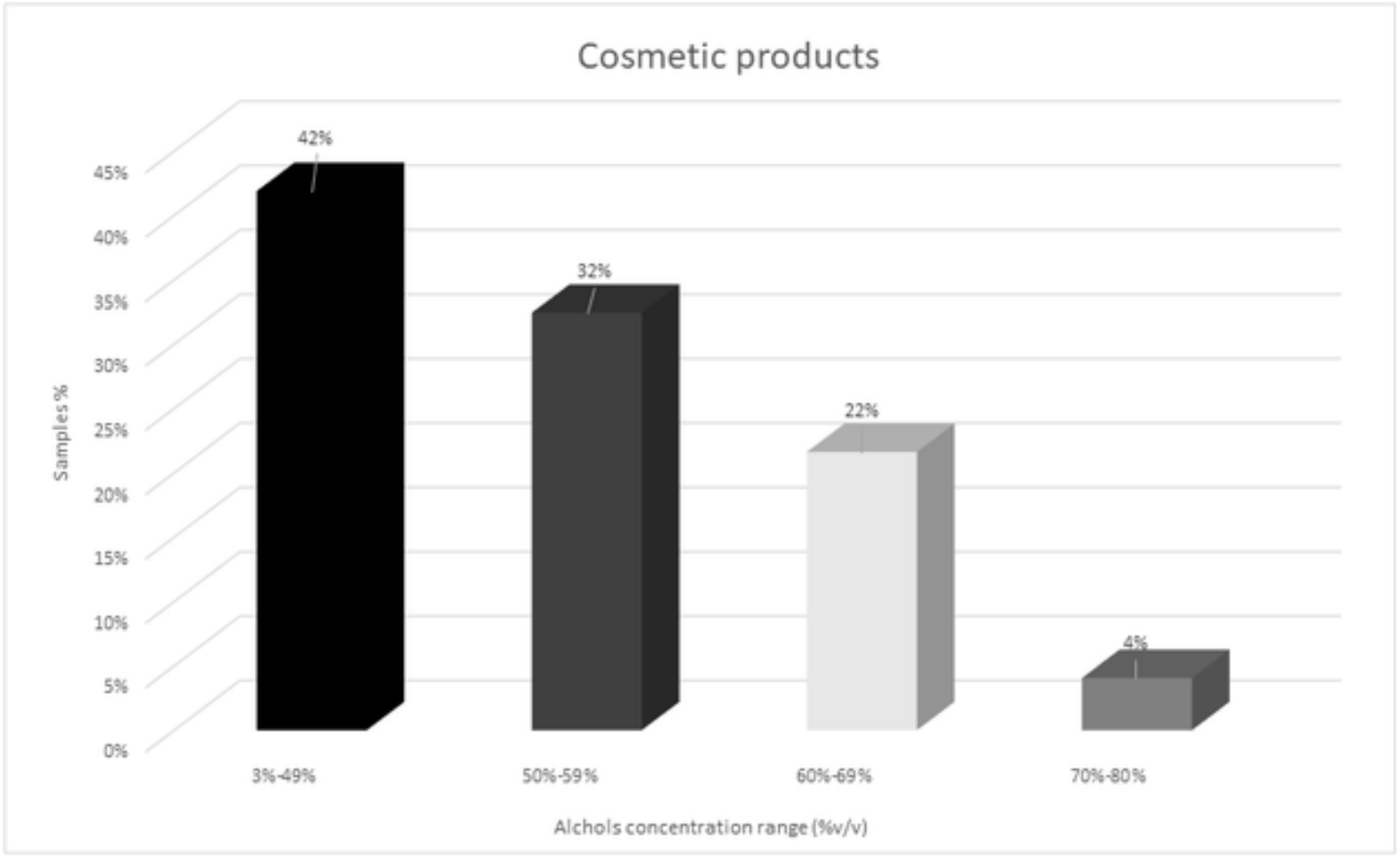

Cosmetic products
42%
32%
22%
4%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Samples %
3%-49%
50%-59%
60%-69%
70%-80%
Alchols concentration range (%v/v)

Figure 4

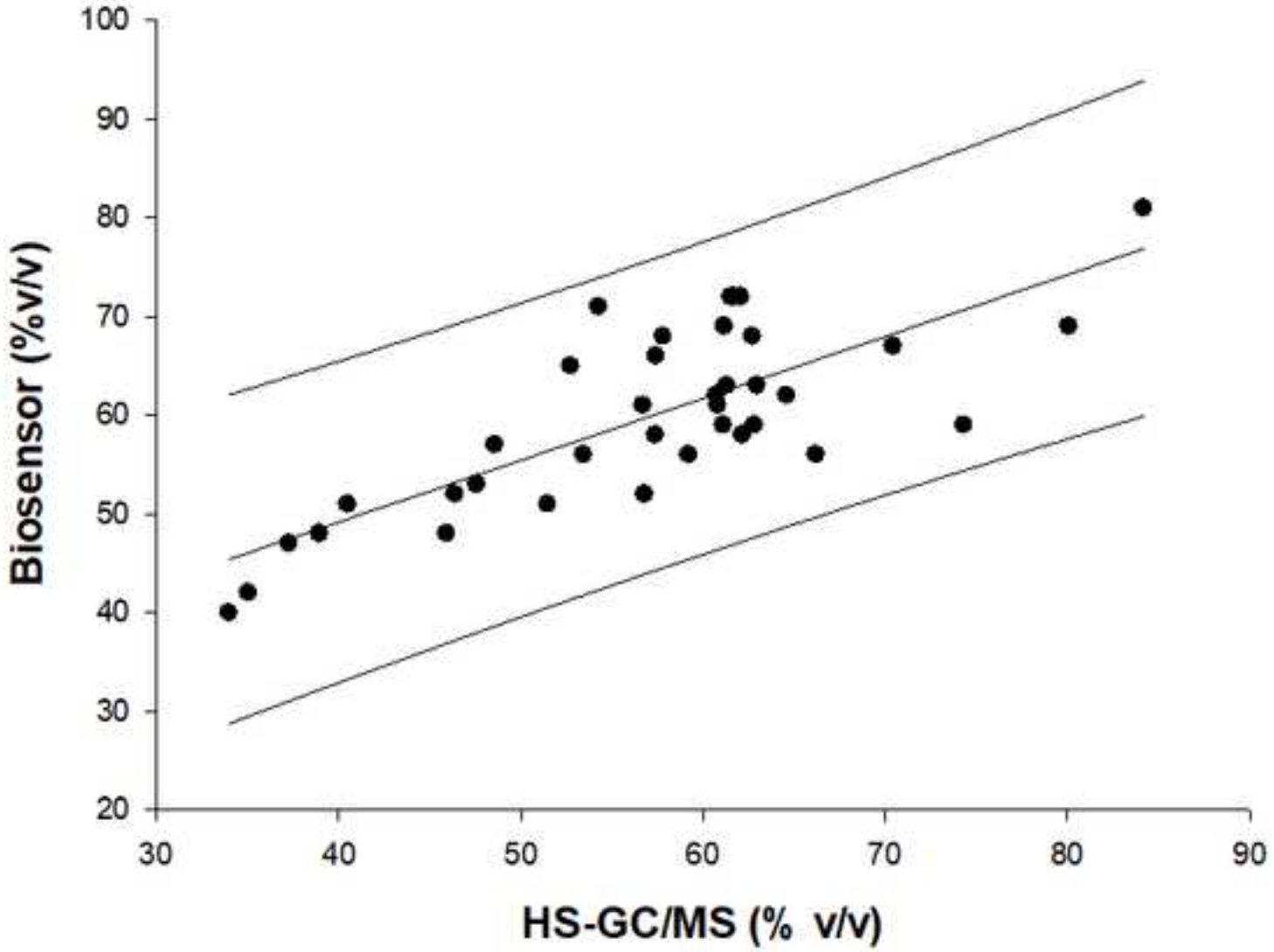

Biosensor (%v/v)
HS-GC/MS (% v/v)
100
90
80
70
60
50
40
30
20
30
40
50
60
70
80
90

Figure SI 1

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Supplementary Material
Figure SI 1.tif

Figure SI 2

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Supplementary Material
Figure SI 2.tif

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Severe Acute Respiratory Syndrome Coronavirus 2, SARS-CoV-2
- alcohol-based hand rubs (ABHRs) have found wide diffusion
- polymer crosslinks can affect the alcohol release on hands
- hand rub samples based on carbomer and hydroxyethylcellulose
- different behavior was observed in function of the used crosslinker

Manuscript Click here to view linked References

Alcohol-based hand rubs against SARS-CoV-2: Analysis of ninety commercial samples by HS-GC/MS and electrochemical biosensor

Costanza Majorani[1], Claudia Leoni[1], Laura Micheli[2], Rocco Cancelliere[2], Marco Famele[1], Roberta Lavalle[1], Carolina Ferranti[1], Luca Palleschi[1], Luca Fava[1], Rosa Draisci[1], Sonia D'Ilio[1]

[1] *National Centre for Chemicals, Cosmetic Products and Consumer Health Protection, Istituto Superiore di Sanità, Rome, Italy*

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Keywords:

CoViD-19; Alcohol-based-hand-rubs; polymers; gas chromatography; electrochemical biosensor

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Abstract

Alcohol-based hand rubs (ABHRs) have found large diffusion during the Severe Acute Respiratory Syndrome Coronavirus 2, SARS-CoV-2, becoming the most widespread means for hand hygiene. For this reason, it is fundamental to assess the alignment of commercial ABHRs to the indications provided by the principal health agencies regarding alcohol content and possible impurities. In this study, a survey was conducted on ninety ABHRs, purchased on the Italian market, with the aim of obtaining an overview of products available to the public during the first period of the pandemic. Samples were firstly analysed in terms of pH and by Scanning Electron Microscopy and optical microscopy, in order to investigate their morphology and the effect of polymer crosslinks on alcohol release. Alcohols in samples were determined by static Headspace Gas Chromatography Coupled with Mass Spectrometry (HS-GC/MS) and by an electrochemical biosensor, based on the immobilisation of Alcohol oxidase on screen printed electrodes (SPEs). The analytical approaches were compared through a correlation study, showing a screening method by biosensors and a quantification one by HS-GC/MS. The survey has evidenced that 26% of the tested cosmetic products had the recommended average alcohol content, confirming the importance of analytical controls on this type of products.

1. Introduction

The spread of the Severe Acute Respiratory Syndrome Coronavirus 2, SARS-CoV-2, is responsible of the global diffusion of the virus related disease officially named COVID-19 (CoronaVIrusDisease-2019) (Ludwig and Zarbock, 2020) and has emerged as a serious public health issue (Kooraki et al., 2020). Studies and investigations conducted since the outbreak of the pandemic, have stated that individual-to-individual transmission, mainly occurs through the droplets and the small particles, called *aerosol*, expelled from an

individual infected with SARS-CoV-2 while coughing, sneezing, or speaking (Saadat et al., 2020). Once in human body, the virus exhibits symptoms like fever, fatigue, muscle soreness, altered taste and smell (Li et al., 2020) and, in some cases, the infection can cause pneumonia, severe acute respiratory syndrome and renal failure up to the death of the individual (Kooraki et al., 2020; Li et al., 2020).

To counter the spread of the virus, the WHO and the major public health agencies have recommended the use of adequate personal protective equipment (PPE) together with careful personal hygiene to be sought especially through frequent hand washing (WHO, 2020) and dedicated hand products use. Among these, alcohol-based hand rubs (ABHRs) have found wide diffusion although they were already routinely employed in healthcare settings. The use of these products in the last months has significantly increased, making them the most widespread means of obtaining rapid and effective hand hygiene (CDC, 2020; Hadaway, 2020; Berardi et al., 2020a).

The sanitising/disinfecting action of ABHR is due to the presence of alcohols, whose primary targets are the proteins in cell plasma membranes of pathogens that have demonstrated to be active against a wide variety of viruses and bacteria (WHO, 2009). Ethyl alcohol (EtOH), isopropyl alcohol (IPA) and n-propyl alcohol (*n*-PA) are, alone or in a combination, the most used alcohols in ABHR formulations. It is remarkable to highlight that *n*-PA is approved for the use as biocide in the European Economic Area (EEA), but FDA has limited its content in ABHRs to 0.1 % v/v since it is not listed as active agent for hand antisepsis and surgical hand preparation in the United States (Price, 1939; WHO, 2009; FDA, U.S., 2020).

According to the principal health agencies, ABHRs must contain at least 60 %v/v alcohol, to have effect as disinfectants on pathogens, including SARS-CoV-2 (WHO, 2006; Leslie et al., 2021). Scientific literature has evidenced that, in addition to the alcohols concentration, other factors that contribute to sanitation must be taken into account, such as the minimum friction time and the amount of sanitizer applied on the hands (CEN, 2013; Macinga et al.2014; Wilkinson 2017; Voniatis et al. 2021; Kenters 2020).

To cope with the new health emergency and limit infections, a wide variety of ABHRs has been placed on the market as cosmetics, biocidal products and galenic productions. Cosmetic hand sanitizers and galenic preparations are produced in accordance to Regulation (CE) N. 1223/2009 (Regulation (EC) N. 1223/2009, 2009) and the European Pharmacopoeia protocols, respectively. Biocidal hand products must be authorised by the Italian Minister of Health (Decreto del Presidente della Repubblica n. 392/1998, 1998), before being placed on the market and the active substances therein contained must be approved as disinfectants in compliance to Regulation (CE) N. 528/2012 for Biocidal Products (Regulation (EU) N. 528/2012, 2012). The declaration of the alcohol content, as active substance in ABHRs, on the label is mandatory for biocidal products and galenic preparations, while is not required for cosmetic products.

Different formulations are also available as solutions, foams and gels, with the latter ones widely diffused among population because of their manageability and ease of handling (Abuga, 2021). Acrylate acrylates (Carbopol ™, carbomer, acrylates/c10-30, and tea-carbomer) or cellulose derivatives (hydroxypropylmethylcellulose, hydroxyethylcelluloseand polyquaternium-7) are examples of frequently used gelling agents (Islam et al., 2004; Shalaby and Shukr, 2011).

However, the sudden increase in the demand for ABHRs has favoured the spread of substandard products that may be not aligned with health agencies indications for the alcoholic fraction in order to protect population from infections (Berardi et al., 2020b).

Some studies have therefore focused on the development of analytical methods for the quality control of ABHRs, with particular attention to cosmetic hand sanitizers, to ensure their conformity to the indications of the sanitary authorities and aim at raising the issue of population safeguard from non-effective products. In Fonseca *et al.* (2020) two methods employing mid and near infrared spectroscopy were developed and applied to 34 ABHRs samples for EtOH determination, while Da Silva *et al.* (2020) developed a portable near infrared spectrometer, combined with classification chemometric tools, to be used in the construction of models to identify conforming and non-conforming commercial and laboratory synthesised hand sanitizer samples. Berardi *et al.* (2020b) and De Lacerda *et al.* (2020) developed GC/FID based methods to determine the EtOH content in seven cosmetic and biocidal hand sanitisers. Additionally, depending on the purity of EtOH used for ABHRs production, population may be exposed to harmful levels of substances not intended for use in ABHR that may be present as impurities (Emami et al., 2020; Dear et al., 2020; Tse et al., 2021; Guntner et al., 2021). In particular, in early 2020 FDA investigated about methyl alcohol (MeOH) contamination in AB-HRs and stated that it cannot be safely used as an ingredient, or as a denaturant, in hand sanitizer (FDA, U.S., 2020). In Europe, Regulation (EU) 2020/1683 amended Annex III of Regulation 1223/2009/EC on Cosmetic Products (Commission Regulation (EU) 2020/1683, 2020), setting a volume fraction limit of 5% for MeOH content in cosmetics, calculated as EtOH or IPA denaturant.

Considering all these critical issues, the assessment of compliance of ABHRs available on the market to the indications provided by the health agencies and the assessment of population' exposure to hazardous substances that may be contained in these products are fundamental during the coronavirus pandemic

A survey was conducted for the very first time concerning ninety ABHRs, purchased on the Italian market between April and November 2020, among cosmetic products, biocidal products and galenic preparations. The aim was to obtain an overview of products available to the public during the first period of the pandemic, with particular attention to cosmetic ABHRs for which there is no obligation to report the alcohol concentration on label.

Samples were firstly characterised in terms of pH and three-dimensional and topographical images by Scanning Electron Microscopy (SEM) and optical microscopy, in order to investigate their morphology and whether polymer crosslinks can affect the alcohol release on hands.

All collected samples were analysed with a sensitive analytical technique based on static Headspace Gas Chromatography Coupled with Mass Spectrometry (HS-GC/MS) and a specific method that was in-house developed for the simultaneous determination of EtOH, IPA, *n*-PA and MeOH, respectively. The headspace conditions (as equilibration temperature and equilibration time), acidification sample treatment and method validation parameters were studied and optimised according to ISO/IEC 17025 requirements (ISO/IEC 17025).

Furthermore, as a comparison, the ninety ABHRs samples were analysed by an electrochemical biosensor, based on the immobilisation of Alcohol oxidase on screen printed electrodes (SPEs) (Boujtita et al., 2000) that isknown to be a rapid and inexpensive monitoring tool. The biosensor is normally used for ethyl

alcohol determination in food matrices as cheese and wine (Azevedo et al., 2005), and in this paper the application for primary alcohol determination in ABHRs is described for the first time.

Results obtained on the samples from the different analytical approaches were finally compared through a correlation study, showing a screening method by biosensors and a quantification one by HS-GC/MS able to selectively recognize the single alcohols and quantitatively determine them all.

2. Materials and Methods

2.1 Reagents and samples

Methyl alcohol (98.7%), isopropyl alcohol (99.8%), ethyl alcohol (99.7%) and n-propyl alcohol (99.5%) were purchased from C.P.A. chem (Bulgaria). Tetrahydrofuran (>99.9%) as internal standard (IS) and Chloridric acid (HCl≥37%) were purchased from Merck KGaA (Germany) and Sigma Aldrich (USA) respectively.Distilled water was used for reference solutions and sample dilution.

Ferric chloride, potassium ferricyanide, glutaraldehyde, hydrogen peroxide and Alcohol Oxidase (AOx, EC 1.1.3.13, definition Alcohol: oxygen oxidoreductase) from *Candida boidinii* (15U/mg) were obtained from Sigma-Aldrich (USA). All the solutions were analytical grade.

2.2 Sample collection

Ninety ABHR samples, which were randomly collected from several shops (supermarkets and pharmacies) in the city of Rome from April to November 2020, stored at 25°C and subsequently analysed for this study. These ABHRs of different types and brands were selected and analysed to determine their alcohol content. About 82% of samples purchased were cosmetic products, 11% were biocidal products and 7% were galenic preparations. Most of samples collected were produced in Italy in a percentage of 81%, for the remaining, 8% were made in Europe, 4% in other countries and 7% of samples had no indications on label. Formulation consisted of 87% gel, 12% liquid and 1% foam.

2.3 HS-GC/MS

2.3.1 Standard solutions and samples preparation

MeOH, IPA, EtOH and *n*-PA working solution of 0.4 %v/v was obtained by dissolving 200 µL of each alcohol in 50 ml of distilled water. IS working solution of 0.02 %v/v was prepared by dissolving 10 µL of THF in 50 ml of distilled water. These working standard solutions were used to make the spike addition to the blank matrix for the construction of matrix-matched calibration curves. Working solutions were daily prepared. A 50 µL aliquot of hand rub gel and 100 µL HCl 0.1M were dissolved in 25 ml of distilled water by vortex-mixing. 1 ml was drawn from this latter solution, transferred into a 20 mL HS vial and finally added to 0.5 ml of IS solution at 0.02 %v/v and 0.5 mL of distilled water (final volume: 2 ml). Sample was sonicated at room temperature for 10 minutes and analysed by HS-GC/MS.

2.3.2 Calibration curve and quality control sample

A five point calibration curve was obtained in a concentration range from 1% v/v to 80%v/v for MeOH, IPA, EtOH and *n*-PA. A non-alcohol based hand rub gel was used as blank sample for the construction of the matrix-matched calibration curve for each analyte. Blank sample was subjected to all sample processing steps. Calibration curves were determined by plotting the peak area ratio of the analytes to IS versus the analyte concentration. Quality control samples were prepared at concentrations of 50 %v/v.

2.3.3 Instrumentation and conditions

Analyses were performed by using an Agilent 7890B gas chromatograph connected to an Agilent 5977A single-quadrupole mass spectrometer (Agilent Tecnologies, Santa Clara CA, USA) equipped with an automated HS sampler (Pal System, CTC120 Analytics AG, Zwingen, Switzerland). Separation was performed on capillary column Zebron™ ZB-WAXPLUS ™ (30m x 250µm x 0.25 µm) (Phenomenex, Torrance CA, USA). The carrier gas was helium (99.999 %). Before HS-GC/MS analysis, vials were placed in headspace oven thermostated at 60°C with vial shaking set to off. Different conditioning times were evaluated in order to maximize partitioning of the volatile portion of the sample into the vial headspace. Time conditioning effect for each analyte was studied in four selected biocidal samples. Since EtOH was the only active substance in the selected biocides, they were fortified with the working solution in order to investigate the response of each analyte. The analyses were conducted in duplicate at the following conditioning times: 10, 20, 30 and 40 minutes. The gas tight syringe, heated at 60°C, sampled and injected the steam (250 µL) in split mode (split ratio 40:1). Septum purge flow was 3 ml/min. The GC/MS oven temperature program was: 40 °C held for 1 min then ramped at 10° C/min up to 90°C (run time: 6 minutes); carrier gas (helium) was kept at a constant flow rate of 1.3 ml/min. The electron impact energy was 70 eV and the quadrupole, ionization source and injector temperatures were set at 150°C, 230°C and 90°C respectively. The mass analyser was set in the selected ion monitoring (SIM) mode and in total scan (TIC) mode.

2.3.3 Method Validation

Performance characteristics such as sensitivity, specificity, limit of detection (LoD), limit of quantification (LoQ), linearity, precision (repeatability and intermediate precision), accuracy and measurement uncertainty, were assessed according to well-established requirements of ISO/IEC 17025, Guide to the expression of Uncertainty in Measurement (GUM) (ISO, 2008) and internal performance criteria.

2.4 Biosensor for alcohol detection

2.4.1 Screen-printed electrodes

Screen-printed electrodes (SPEs) were home-made with a 245 DEK (High performance multi-purpose precision screen printer, Weymouth-UK) screen-printing machine. The electrodes, printed on a folding polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy), were produces in foils of 48. Graphite-based ink (Elettrodag 421) from Acheson (Milan, Italy) was used to print the working and the counter electrode, while silver ink (Acheson Elettrodag 4038 SS) was used for the reference electrodes. The diameter

of the SPE's working electrode was 0.3 cm resulting in an apparent geometric area of 0.07 cm^2. The application of an insulating print (Argon Carbonflex 25.101S) defines the actual surface area.

2.4.2 AOx (Alcohol Oxidase) screen-printed based biosensor

Screen-printed platforms were modified using Prussian Blue (PB, $Fe_4(Fe(CN)_6)^3$) as diffusional electrochemical mediator. This chemical deposition was carried out following an optimised procedure reported in a previous work (Cancelliere et al., 2020). In particular, alcohol biosensors were obtained by immobilising alcohol oxidase (AOx) onto PB modified working electrodes. Specifically, a solution of AOx (1mg/ml), Glutaraldehyde (1%) and BSA (5%) in distilled water was prepared and casted In the recent years, several studies were devoted to the development and use of AOx based biosensors, which indicates how important is this branch of bioanalytical chemistry (Alferov et al., 2011; Prasanna Kumar et al., 2020).

The sensor presented in this work exploits the reaction reported below. The electrochemical measurement and therefore the current signal obtained from the hydrogen peroxide discharge is proportional to the concentration of alcohol present in the sample.

$$RCH_2OH + O_2 \leftrightarrows RCHO + H_2O_2$$

Electrochemical experiments (Amperometry) were performed using a PalmSens (Palm Instruments BV, Electrochemical Sensor Interfaces, Netherlands), which is a hand-held battery powered potentiostat instrument for use with electrochemical sensors or electrochemical cells. In particular, amperometric measurement were carried out applying on AOx-PB/SPE a 50mV potential for 40 s.

2.4.3 Calibration curve and treatment of sample

For the detection of the alcohol present in gel, the calibration curve was constructed using IPA in 50 mM phosphate buffer + 0.1 M KCl, pH 7.4 as standards (50, 60, 70, 80, 90 and 99.8 %; $y = -0.0077x + 0.3841$ $R^2 = 0.0997$). A non-alcohol based hand rub gel (the same of HS-GC/MS) was used as blank sample for the construction of the matrix-matched calibration curve of alcohol. Calibration curves were determined by plotting the current at 40s versus the analyte concentration. For each calibration points and for all samples, six measurements, using different biosensor, were carried out.

The hand rub gel samples were treated as following: an equal amount of 50 mM phosphate buffer pH 7.4, was added to each samples; the mixed solutions were sonicated 60 minutes (50Hz, 35°C using an Hielscher UP100H) and after gently shacking overnight under controlled temperature in hermetic glass vials. For the analysis, the obtained solutions were directly analysed.

2.4.4 pH measurements

The pH values of each sample were measured after a dilution with distilled water 1:1 v/v and stirring for 1h in order to avoid matrix effect of hydrogel. The instrument is pH8 + DHS (XS instruments, Carpi, MO – Italy).

2.4.5 Scanning electron microscopy (SEM) and optical microscope

Scanning Electron Microscopy (SEM) was used to investigate the morphology of hydrogel samples. The experiment was performed by using a field emission scanning electron microscope (FE-SEM) (SU-PRATM 35, Carl Zeiss SMT, Oberkochen, Germany), using as operating parameters of the instrument 10 keV as gun voltage and a working distance of about 8 mm, while the detector used was the second electron one. Samples were previously metalised to allow electronic conduction on the sample surface. The metallisation, (1 min at 25 mA), was performed using a sputter coater (EMITECH K550X, Quorum Technologies Ltd., West Sussex, UK) with a gold target. Microscope photos have been performed on a Celestron, Microcapture Pro apparatus (Celestron, Torrance, USA) with 1600x magnification.

3. Results and Discussions

3.1 HS-GC/MS Instrumental and sample treatment optimization

The aim of this study was to develop an accurate and sensitive HS-GC/MS method for the simultaneous determination of MeOH, IPA, EtOH and *n*-PA in ABHRs. To the best of our knowledge, no other similar investigations concerning these analytes have been reported in literature in such matrices so far.

Since a Certified Reference Material (CRM) on this matrix was not commercially available, four biocidal ABHRs were spiked with known amount of alcohol to assess the effectiveness of extraction.

Before the sample extraction, the effect of time was evaluated by plotting the ratio of the corresponding peak area obtained for each analyte to the IS peak area, versus different thermostating times (10, 20, 30 and 40 minutes), maintaining the temperature of both gas tight syringe and headspace oven constant at 60°C. Results revealed (data not shown) that *n*-PAand EtOH peak area ratios slightly increased in all samples as the equilibration time increased, no substantial difference was observed between 30 and 40 minutes. MeOH and IPA peak area ratios were constant in all samples for all tested times. In conclusion, in method validation, 30 minutes was selected as vials conditioning time before HS-GC/MS analysis.

As concerns MS conditions, the most prominent and characteristic fragment masses were selected from the Total Ion Current (TIC) mode spectrum of the pure analytical standard of each analyte. In particular, one quantifier ion and two qualifier ions were selected for each compound on the basis of their selectivity and abundance. The fragment 31 m/z was chosen as quantifier for MeOH, EtOH and *n*-PA because of its highest intensity; while the 45 m/z ion was selected as quantifier for IPA. The same approach was adopted for IS quantifier ion selection. Table 1 shows the retention times and characteristic m/z ions selected for the acquisition in SIM mode of analytes and IS. Analytes qualitative identification was assessed by the combination of chromatographic separation and mass spectrometry criteria. According to the first, the relative retention time (i.e. the ratio between the chromatographic retention times (t_R) of the analyte and of the IS) of the analytes was compared with that obtained from the calibration curve of each analyte with a tolerance of 0.5 %. As for the mass spectrometry criteria, the ratios between the quantifier ion and the two qualifiers, detected in SIM mode during sample analysis, were compared with those obtained from the standards in the calibration curve.

[Please insert table]

Table 1. Analytes and IS characteristic m/z ions for SIM mode acquisition and retention times (t_R).

Once the instrumental conditions had been optimised, selected biocidal samples were analysed by HS-GC/MS, after being diluted in distilled water, added of IS, and sonicated. However, the results showed that EtOH content was lower than that reported on the label for all tested products with the exception of one different in formulation, being this liquid while the other three samples were gels. Thus, the presence of gelling agents, which act as blockers to avoid alcohols evaporation that would compromise the sanitising properties of these products, required a different sample treatment to make the alcohol extraction effective.

As reported in literature (Shimoda and Smela, 1998), since polymer crosslinking patterns are affected by pH, samples were then acidified with HCl 0.1M and analysed again, keeping the other sample processing steps unchanged. The results showed that the alcohol contents, obtained by acidification of samples, were coherent with those reported on products labels. It is noticeable that the liquid biocidal product was not affected by the acidification since, given its formulation, it did not contain polymers. Optimised sample treatment and instrumental conditions were then applied to the blank sample for the conduction of the validation studies, as well as to the samples collected from the market. Table 2 shows results obtained for the selected biocidal products at each different sample treatment.

[Please insert table]

Table 2. Results on biocidal products with different sample treatment.

3.2 Method validation

The performances of the analytical method were evaluated in terms of specificity, selectivity, detection limit, quantitation limit, linearity, precision, accuracy and measurement uncertainty. Validation studies were carried out by providing the optimised instrumental conditions and using a non-alcohol based hand rub as blank sample which was subjected to all sample processing steps.

The specificity of the method was assessed by monitoring in SIM mode the characteristic ions of each investigated compound in the blank sample chromatogram (Figure SI 1): no interferers were observed in the retention time window expected for each analyte. Figure 1 shows the chromatogram obtained from the fortified blank sample at analytes final concentration 50 % v/v.

[Please insert figure]

Figure 1. Chromatogram of the blank sample and the IS with the analytes at the concentration of 50% v/v

Detection (LoDs) and quantification (LoQs) limits were determined considering the approach described in Eurachem Guide Fitness for Purpose (Magnusson and Örnemark, 2014) according to which they were calculated by considering the standard deviation obtained from the analysis of 10 independent blank samples, spiked at concentration of 1 %v/v for all investigated alcohols. The achieved LoDs and LoQs were 0.16 %v/v and 0.4 %v/v for MeOH, 0.17 %v/v and 0.57 %v/v for IPA, 0.13 % v/v and 0.44 %v/v for EtOH, 0.15 %v/v and 0.50 %v/v for *n*-PA, respectively.

Linearity was assessed trough five-point matrix matched calibration curves, prepared by spiking blank samples at analytes concentrations of 1, 10, 50, 70 and 80 %v/v and run in three different days. For each compound, the calibration curve was determined by plotting the ratio of the corresponding peak area to the IS peak area, versus the analyte concentration. The correlation between concentration and detector response for each analyte was determined by a linear regression model using the method of ordinary least squares. As shown in table 3, linear regressions were adequate as the correlation coefficients were not less than 0,999 for each compound. An ANOVA *F*-test was also applied in order to ensure the linearity of the method. The test confirmed that the method was linear for each compound in the concentration range selected as the observed values of *F* were greater then the critical value of *F*, deduced from the table at the significance level $\alpha = 0.05$ and $\nu = 4$ degree of freedom (table 3). The equations of MeOH, IPA, EtOH and *n*-PA, obtained from the least squares elaborations, were used to quantify these analytes in real samples.

Since a Certified Reference Material (CRM) was not commercially available, ten replicates of fortified blank samples were run in order to conduct recovery studies. Blank samples were spiked at three different concentration levels (2, 20 and 60 %v/v) selected within the concentration range of the calibration curves and the accuracy of the method was assessed considering the percentage of recovery of each analyte at each fortification level. For each analyte recovery values were between 80% - 110% and complied with the internal performance criteria.

Three validation levels (1, 50 and 70 %v/v) were chosen for precision studies. Intraday repeatability was evaluated by analysing six replicates of blank samples fortified at each validation level; intermediate precision was established by extending the same approach to three different days for an overall number of 18 replicates analysed for each validation level. Method precision was expressed as the relative standard deviation (RSD_R) of the obtained results and it ranged between 4.9 % and 10.7 % at the first validation level (1 %v/v), between 2.0 % and 2.3 % at the second validation level (50 %v/v), between 3.0% and 3.8% at the third validation level (70 %v/v). The relative standard deviation values were less than 10% for each analyte at the tested validation levels for intra-day repeatability (RSD_r). Precision studies were acceptable since HORRATr values were less than 2 for each analyte. All the investigated analytes passed the criteria selected for precision studies. Results of validation studies are summarised in table 3.

[Please insert table]

Table 3. Validation parameters of the method and performance criteria

The measurement uncertainty evaluation was determined by using the GUM (bottom up) approach. All relevant sources of uncertainty of the overall analytical procedure were evaluated and expressed as relative standard uncertainties. The combined uncertainties obtained at each validation level by the relative standard uncertainties, were multiplied by a coverage factor (k) of 2, considering a 95% level of confidence, in order to obtain the relative expanded uncertainties that ranged for all the analytes between 0.1% v/v and 3.4% v/v.

3.3 Collected samples: morphology and pH measurement

With the aim of evaluating the applicability of the validated method to real samples, 90 ABHRs were selected and analysed to determine their alcohol content. About 72% of samples reported the presence of polymers on the label including acrylates/C10-30 alkylacrylatecrosspolymer, tea carbomer, polyacrylatecrossplymer-6, hydroxyethylcellulose, poly(methylmethacrylate) and polyquaternium-7. The morphological analysis of these samples showed similar behavior in function of the main component of the polymeric structure. The Scanning Electrone Microscope (SEM) images (figure 2a) show that the hand rub samples based on carbomer and hydroxyethylcellulose have a filamentous structure as confirmed by optical microscope analysis (figure 2b), where the lyophilised samples generated a small white flakes (Kumara et al., 2015) or uneven transparent film, typical of the derivate of cellulose (Orhan et al., 2018; Chávez-Guerrero et al., 2019), respectively. For the other lyophilised polymers (acrylates/C10-30 alkyl acrylate crosspolymer, polyacrylate crosspolymer-6, poly (methyl methacrylate) and polyquaternium-7) used for hand rubs, different behavior was observed in function of the used crosslinker as reported in literature (León et al., 2006; Tavares Gregolin et al., 2010).

Before measuring the alcohol content, the pH values of all samples were determined resulting in the interval 4.9 -7.3 in function of the polymer concentration, the percentage of thickening and crosslinker agent, the amount of thickening agent, etc. used for their preparation.

[Please insert figure]

Figure 2a. SEM and optical images of hydroxyethylcellulose and carbomer hand rubs

[Please insert figure]

Figure 2b. Optical images of different hand rubs gel (100x magnitude) containing polyacrylate crosspolymer-6 (A), Polyquaternium-7 (B), acrylates/C10-30 alkyl acrylate crosspolymer (C), poly(methyl methacrylate) (D)

3.4 Determination of alcohols

Seventy-four ABHRs, sold as cosmetic products, were analysed by HS-GC/MS and the results are shown in figure 3. Samples were plotted based on the average alcohol concentration, due to the contribution of all tested alcohols, expressed as %v/v. Concentrations ranged between 3.0 ± 0.1 %v/v and 80.0 ± 3.0 %v/v. The majority of samples (42%) had an alcohol concentration less than 49% v/v while 32% of samples were in the range 50 %v/v - 59 %v/v. Only 26% of samples had an average alcohol content greater or equal than 60 %v/v and, among these, only in 4% of samples an alcohol concentration in the interval 70 %v/v – 80 %v/v was measured.

[Please insert figure]

Figure 3. Cosmetic products: average alcohol concentration expressed as %v/v.

The most widely used alcohol for the production of selected cosmetic ABHRs was EtOH, which was found in 92% of the analysed samples while IPA was determined in 26% of samples alone or in combination with EtOH. MeOH and *n*-PA were below LoQ values (0.53 %v/v and 0.50 %v/v respectively) in all tested

samples. Alcohol concentration was declared on 49% of cosmetic ABHRs labels but in only 47% of them was coherent with declared values.

Analysis of 10 biocidal samples instead revealed that the average alcohol content was almost within the recommended range for these products, being 60.0 ± 2.0 %v/v the lowest determined alcohol concentration and 85.0 ± 3.0 %v/v the highest. Alcohol concentrations declared on products labels were also confirmed. Among the biocidal products purchased for the study, one sample was collected from a public distributor, available to people, and analysed. The result obtained did not match with the 70% v/v alcohol concentration declared on the label, as only 40.0 ± 1.0 % v/v was determined. A possible reason of this disagreement could be that the product was more exposed to spoilage, in terms of alcohol dispersion.

EtOH was determined in all biocidal products and for three of them the bactericidal activity was due to a combination of EtOH and IPA. As for cosmetic products, MeOH and *n*-PA were below LoQ values in all tested samples. The smallest portion of the analysed samples consisted of six galenic preparations whose alcohol concentrations ranged between 55.0 ± 2.0 % v/v and 63.0 ± 3.0 % v/v confirming the value on labels. EtOH was used in all preparations tested, with the exception of only one sample in which IPA was determined. Neither MeOH nor *n*-PA were detected.

Samples were analysed also by electrochemical biosensor, selective for the class of primary alcoholsand able to quantify the total alcohol content present in a sample. The biosensor was developed with the immobilization of alcohol oxidase (AOx) on the working electrode of SPEs. This enzyme has the highest affinity for methyl alcohol with the affinity decreasing with increasing chain length of the alkyl (R) group. In order to avoid the influence of the different pH of the hand rubs on enzymatic reaction of the alcohol biosensor, all samples were diluted in buffer (50mM phosphate buffer pH 7.4) and before the analysis were treated as reported in paragraph *2.4.3*. The amount of alcohol present in the gels was extrapolated from the calibration line (figure SI 2) by adding known concentrations of IPA to an alcohol-free gel (the same used for HS-GC/MS measurements), in order to minimize the matrix effect on electrochemical measurement. The results of biosensor are in accordance with those of HS-GC/MS for about 90% of all analysed hand rubs (compared to HS-GC/MS, fig. 4 where only the most significant results are reported), in particular when carbomer and acrylates were used as gel. Using Sigma Plot ver 11, the 99% prediction interval for the percentage of alcohol content obtained with both methods is calculated using the following equation (Sahai and Thompson, 1974):

$$y = y_0 \pm t\,(n\text{-}p\text{-}1)\; s\backslash 1 + X'_0\,(X'\,X)^{-1}\,X$$

where y_0 is the *y* value predicted for any x_0, *t* value for (n-p-1) degrees of freedom, *n* is the number of the data point, *p* is the order polynomial regression, *s* is correlate to the variance about the regression and X' and X'_0 is the (p +1)*1 vector, X is the n*(p +1) design matrix.

The elaboration of the results, obtained with both analytical methods, showed that all experimental data fall inside the calculated prediction interval of 35% v/v and 85% v/v (Fig.4) according to 99% (Sahai and Thompson, 1974). This result provided a predicting range for the future analysis of the ABHRs. The differences between the results obtained with the two analytical approaches were related to the treatment of

the sample (dilution for biosensor and acidification in HS-GC/MS) and different condition of analyses (liquid for biosensor and steam in HS-GC/MS). The results highlighted that the electrochemical biosensors can be a very useful tool for screening analyses of commercial hand rubs and may be used in combination with a more accurate and sensitive analytical technique, as the HS-GC/MS, achieving a rapid monitoring of all samples.

[Please insert figure]

Figure 4. Prediction interval of the comparison of the most significant data point selected among 90 hand rubs analysed by electrochemical biosensors and HS-GC/MS

4. Conclusion

The results of the survey conducted on ninety ABHRs differing in formulation and brands, purchased on the Italian market from April to November 2020, were obtained by applying a specifically developed and in-house validated method according to ISO/IEC 17025 requirements. All analytical parameters and sample preparation steps were explored and optimised obtaining a sensitive and specific HS-GC/MS based method, for the simultaneous determination of EtOH, IPA, *n*-PA and MeOH. From the validation study, excellent trueness and good precision were assessed and the method can be considered as a valuable and reliable tool for quantifying the alcohols content in ABHRs. It was observed that in only 26% of the tested cosmetic products the average alcohol concentration was, as recommended by the health agencies, at least 60% v/v. Analyses confirmed the alcohol content reported on the label for 47% of samples. Biocidal products and galenic preparations tested were aligned with the requirements of the EU legislation and the content of alcohols declared on their labels was confirmed. MeOH was not detected in all analysed samples. The same results were obtained by means of alcohol biosensor, a well-known analytical tool for its application in monitoring the alcohol level in food or wine production. The electrochemical tool shows the advantage, respect to the chromatographic system, of being at lower cost and performing a quick analysis, with the only limitation to be able to determine the total alcohol content, without discriminating the different substances. However, it can be used as a rapid *in situ* investigation system, able to evaluate the sanitising power of the gels, which must be subsequently analysed by HS-GC / MS as confirmatory method for the identification of the different alcohols therein contained. This study highlighted that the combination of biosensor and HS-GC/MS would allow to have a powerful tool for the analysis of alcohol in gels, being the first directly usable on the market, lowering the analysis costs and avoiding consumer fraud.

This survey revealed the importance of performing analytical controls on this type of products, especially for those in which the concentration of alcohols is not clearly stated on label and, on the other hand, suggests to have an effective sanitising action. A correct information on the label of ABHRs together with a more fitting alcohol content are essential to achieve a correct hand hygiene practice that is the basis to protect population from circulation of viruses.

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