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Monitoring of alcohol-based hand rubs in SARS-CoV-2 prevention by HS-GC/MS and electrochemical biosensor: A survey of commercial samples

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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, thus becoming the most widespread means for hand hygiene. Whereby, 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 work, a novel improvement of previous existent methods for the determination of alcohol content in such products was reported. In particular, two alternative sensitive and reproducible methods, such as an electrochemical screen-printed based enzymatic (*alcohol oxidase*) biosensor and a Headspace Gas Chromatography coupled with Mass Spectrometry (HS-GC/MS) were proposed. The electrochemical device represents a rapid, low-cost and accurate fraud screening method for alcohol-based hand rubs. The second technique confirms, identifies and simultaneously determines ethyl alcohol, isopropyl alcohol, n-propyl alcohol and methyl alcohol, improving their extraction through acidification in the sample pre-treatment step. The developed specific HS-GC/MS method was in-house validated according to ISO/IEC 17025 requirements. Analytical parameters such as limit of detection (LoD 0.13%v/v - 0.17%v/v), limit of quantification (LoQ 0.44% v/v - 0.57% v/v), inter-day repeatability (RSD_R 2.1–10.7%) and recovery (80–110%) were assessed. The relative expanded uncertainties range (between 0.1%v/v and 3.4%v/v) for all the analytes were evaluated. Results obtained using the different analytical approaches were compared and indicated that the two data sets were comparable (median; HS-GC/MS, 56%v/v; electrochemical biosensor, 62%v/v) and were not statistically different (one-way ANOVA test; $p = 0.062$). In addition, a good correlation (95%) was found. This study noticed that only 39% of the tested hand sanitiser products had the recommended average alcohol content, thus highlighting the need for analytical controls on this type of products.

1. Introduction

The spread of the Severe Acute Respiratory Syndrome Coronavirus 2, SARS-CoV-2, is responsible for the global diffusion of the virus-related disease officially named COVID-19 (CoronaVIrusDisease-2019) [1] and has emerged as a serious public health issue [2].

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 [3] and dedicated hand product use. Among these, hand sanitiser products/alcohol-based hand rubs (ABHRs) have found wide diffusion, becoming the most widespread means of obtaining rapid and effective hand hygiene [4–6].

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, which have been shown to be active against a wide variety of viruses and bacteria [7]. 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 a biocide in the European Economic Area (EEA), but the U.S. Food and Drug Administration (FDA, Silver Spring, MD, USA) has limited its content in ABHRs to 0.1 %v/v since it is not listed as an active agent for hand antisepsis and surgical hand preparation in the United States [7]. According to the principal

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health agencies, ABHRs must contain at least 60%v/v alcohol, to have an effect as disinfectants on pathogens, including SARS-CoV-2 [8]. 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 sanitiser applied on the hands [9,10].

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 sanitisers and galenic preparations are produced by Regulation (CE) N. 1223/2009 [11] and the European Pharmacopoeia protocols, respectively. Biocidal hand products must be authorized by the Italian Minister of Health [12], before being placed on the market and the active substances therein contained must be approved as disinfectants in compliance with Regulation (CE) N. 528/2012 for Biocidal Products [13]. Different formulations are also available as solutions, foams and gels, with the latter ones widely diffused among the population because of their manageability and ease of handling. Acrylate acrylates (Carbopol™, carbomer, acrylates/c10–30, and tea-carbomer) or cellulose derivatives (hydroxypropyl methylcellulose, hydroxyethyl cellulose and polyquaternium-7) are examples of frequently used gelling agents [14,15].

The sudden increase in the demand for ABHRs has favoured the spread of substandard products not aligned with the health agencies recommendations and in the literature, several analytical methods based on spectroscopy, spectrometry and flame ionization detection, have focused on the determination of alcohols in hand sanitisers [16–21].

In 2020, the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) has developed and evaluated different analytical methods (gas chromatography with flame ionization detection, liquid chromatography with ultraviolet absorbance detection, quantitative nuclear magnetic resonance spectroscopy, and attenuated total reflectance Fourier-transform infrared spectroscopy) for the quality control of ABHRs in terms of both alcohol concentration and impurities [20]. Indeed, depending on the purity of EtOH used for ABHRs production, the population may be exposed to harmful levels of substances not intended for use in ABHR that may be present as impurities [20–22].

In early 2020 FDA investigated methyl alcohol (MeOH) contamination in ABHRs and stated that it cannot be safely used as an ingredient, or as a denaturant, in hand sanitiser [7]. In Europe Regulation 1223/2009/EC on Cosmetic Products, rules the presence of methanol in cosmetic products setting a volume fraction limit of 5% for MeOH content in cosmetics, calculated as EtOH or IPA denaturant on the basis of its harmonised classification in Part 3 of Annex VI to Regulation (EC) No.1272/2008 [23].

Considering all these critical issues, we conducted an Italian survey, for the safeguard of consumers, on ninety ABHRs of different formulations (foam, liquid and gel), commercially available to the public during the first period of the pandemic. Quality assessment of collected alcohol-based hand sanitisers was performed by HS-GC/MS combined with an innovative approach based on the electrochemical biosensor.

The simultaneous determination of EtOH, IPA, n-PA as active ingredients in hand sanitisers and of MeOH as impurity ruled by the Cosmetic Products Regulation [11], was investigated with a specific analytical method. Considering that the target compounds are volatile, HS-GC/MS was chosen for the analysis of hand sanitisers because it reduces the equipment contamination caused by other ingredients [24]. The headspace conditions (as equilibration temperature and equilibration time) and acidification sample treatment were studied and optimised. Method validation parameters were evaluated according to ISO/IEC 17025 requirements [25]. In addition, a rapid and inexpensive screening was conducted on collected samples by an electrochemical biosensor, based on the immobilisation of Alcohol oxidase on screen-printed electrodes (SPEs). This tool is normally used for ethyl alcohol determination in food matrices such as cheese and wine [26] and the application for primary alcohol determination in ABHRs is described for the first time in this paper. Results obtained on commercially available hand sanitisers products by biosensors and by HS-GC/MS were finally compared.

In this paper, the analyses of ninety commercial samples will evidence that quality assessment of these products is of primary importance for the safety of consumers. Furthermore, the study will show that the combination of HS-GC/MS and electrochemical biosensor is a fast and reliable tool, able to detect hand sanitisers with insufficient alcohol concentration.

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 (Bogomilovo, Bulgaria). Tetrahydrofuran (>99.9%) as internal standard (IS) and Chloridric acid (HCl≥37%) were purchased from Merck KGaA (Darmstadt, Germany) and Sigma Aldrich (St. Louis, MO, 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* (15 U/mg) were obtained from Sigma-Aldrich. All the solutions were of 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, were 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. 81% of the samples collected were produced in Italy, 8% were made in Europe, 4% in other countries and 7% of samples had no indications on the label. Formulations consisted of 87% gel, 12% liquid and 1% foam.

2.3. HS-GC/MS

2.3.1. Standard solutions and samples preparation

EtOH, IPA, *n*-PA and MeOH 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 0.1 M 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). The Sample was sonicated at room temperature for 10 min 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 EtOH, IPA, *n*-PA and MeOH. A non-alcohol based hand rub gel was used as a blank sample for the construction of the matrix-matched calibration curve for each analyte. The blank sample was subjected to all the 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 using an Agilent 7890B gas chromatograph

connected to an Agilent 5977A single-quadrupole mass spectrometer (Agilent Technologies, 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 ™ (30 m x 250 μm x 0.25 μm) (Phenomenex, Torrance, CA, USA). The carrier gas was helium (99.999%). Before HS-GC/MS analysis, vials were placed in a headspace oven thermostated at 60 °C with vial shaking set to off. Different conditioning times were evaluated in order to maximize the partitioning of the volatile portion of the sample into the vial headspace. The 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 min. 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^{-1}. The GC/MS oven temperature program was: 40 °C held for 1 min then ramped at 10 °C min^{-1} up to 90 °C (run time: 6 min); carrier gas (helium) was kept at a constant flow rate of 1.3mLmin^{-1}. 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 total scan (TIC) mode.

2.3.4. 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) [27] 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 produced 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 a diffusional electrochemical mediator. This chemical deposition was carried out following an optimised procedure reported in previous works [28]. In particular, alcohol biosensors were obtained by immobilising alcohol oxidase (AOx) onto PB modified working electrodes. Specifically, a solution of AOx (1 mg mL^{-1}), Glutaraldehyde (1%) [29] and BSA (5%) in distilled water was prepared and cast.

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, Houten, Netherlands), which is a hand-held battery-powered potentiostat instrument for use with electrochemical sensors or electrochemical cells. In particular, amperometric measurements were carried out applying on AOx-PB/SPE a 50 mV potential for 40 s

2.4.3. Calibration curve and treatment of the sample

For the detection of the alcohols in hand sanitisers, 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 as HS-GC/MS) was used as a blank sample for the construction of the matrix-matched calibration curve of alcohol. Calibration curves were determined by plotting the current (sampled at t = 40 s) versus the analyte concentration. For each calibration point and for all samples, six measurements, using different biosensors, were carried out.

The hand rub samples were treated as follows: an equal amount of 50 mM phosphate buffer pH 7.4 was added to each sample (dilution 1:1 v/v); the mixed solutions were sonicated 60 min (50 Hz, 35 °C using a Hielscher UP100H) and then gently shaken overnight under controlled temperature in hermetic glass vials. For the analysis, the obtained solutions were directly analysed.

2.4.4. pH measurements

The pH value of each sample was measured after a dilution with distilled water 1:1 v/v and stirring for 1 h to avoid the matrix effect of the hydrogel. The instrument is pH8 + DHS (XS instruments, Carpi, 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) (SUPRATM 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., Laughton, UK) with a gold target. Microscope photos have been performed on a Celestron, Microcapture Pro apparatus (Celestron, Torrance, CA, USA) with 1600x magnification.

3. Results and discussions

3.1. HS-GC/MS instrumental and sample treatment optimization

In this study, an accurate and sensitive HS-GC/MS method for the simultaneous determination of EtOH, IPA, *n*-PA and MeOH in hand sanitiser products was developed. Headspace GC is a routinely used technique to investigate volatile analytes even though alcohols determination in several matrices may be carried out also using GC with direct sample injection [16,20]. However, headspace analysis is particularly appropriate for ABHRs considering their formulations, since GC parts fouling is reduced and cleaner extracts are obtained (Figure SI1) than with direct sample injection [24].

Since a Certified Reference Material (CRM) on this matrix was not commercially available, four biocidal ABHRs were spiked with a known amount of alcohol to assess the effectiveness of extraction because the alcohol content on the label of these products is mandatory [13].

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 thermostatic times (10, 20, 30 and 40 min). The temperature of both the gas-tight syringe and headspace oven was constant at 60 °C to avoid the volatilization of high boiling substances that would interfere with the analysis. Results revealed (data not shown) that *n*-PA and EtOH peak area ratios slightly increased in all samples as the equilibration time increased, though no substantial difference was observed between 30 and 40 min. MeOH and

IPA peak area ratios were constant in all samples for all tested times. Therefore, 30 min was chosen as vials conditioning time before analysis, even though in literature shorter equilibrium times for HS-GC/MS methods are reported [17,18].

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 based on their selectivity and abundance. The fragment 31 *m/z* was chosen as a quantifier for MeOH, EtOH and *n*-PA because of its highest intensity; while the 45 *m/z* ions were selected as a 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 the 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 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.

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. Results showed that EtOH content was lower than that reported on the label for all but one tested product, 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.

To break the polymer crosslinks, samples were acidified with HCl 0.1M since, as reported in the literature, polymer crosslinking patterns are affected by pH [20,30]. After this pre-treatment step, samples were analysed again keeping the other processing steps unchanged. The results showed that the alcohol contents, obtained by acidification of samples, were coherent with those reported on the 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.

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 the 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 SI1): no interferers were observed in the retention time window expected for each analyte. Fig. 1 shows the chromatogram obtained from the fortified blank sample at the analytes final concentration of 50 %v/v.

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

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

Table 2
Results on biocidal products with different sample treatment.

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

Detection (LoDs) and quantification (LoQs) limits were determined considering the approach described in Eurachem Guide Fitness for Purpose [31] according to which they were calculated by considering the standard deviation obtained from the analysis of 10 independent blank samples, spiked at a 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 through 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 on 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 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 than the critical value of *F*, deduced from the table at the significance level $\alpha = 0.05$ and $\nu = 4$ degrees of freedom (Table 3). The equations of EtOH, IPA, *n*-PA and MeOH, obtained from the least-squares elaborations, were

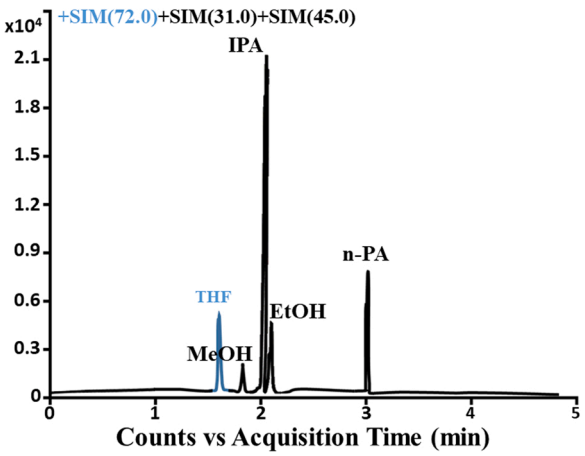

Fig. 1. Chromatogram of the blank sample and the IS with the analytes at the concentration of 50% v/v.

Table 3
Validation parameters of the method and performance criteria.

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)

used to quantify these analytes in real samples (Figure SI2).

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% and 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.

The relative expanded uncertainties ranged for all the analytes between 0.1%v/v and 3.4%v/v by considering all the relevant sources of uncertainty of the overall analytical procedure. The measurement uncertainty evaluation was determined by using the GUM (bottom-up) approach.

3.3. Collected samples: morphology and pH measurement

To evaluate the applicability of the validated method to real samples, 90 ABHRs were selected and analysed. About 72% of samples reported the presence of polymers on the label including acrylates/C10–30 alkylacrylatecrosspolymer, tea carbomer, polyacrylatecrosspolymer-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 (Fig. 2a) showed that the hand rub samples based on carbomer and hydroxyethylcellulose had

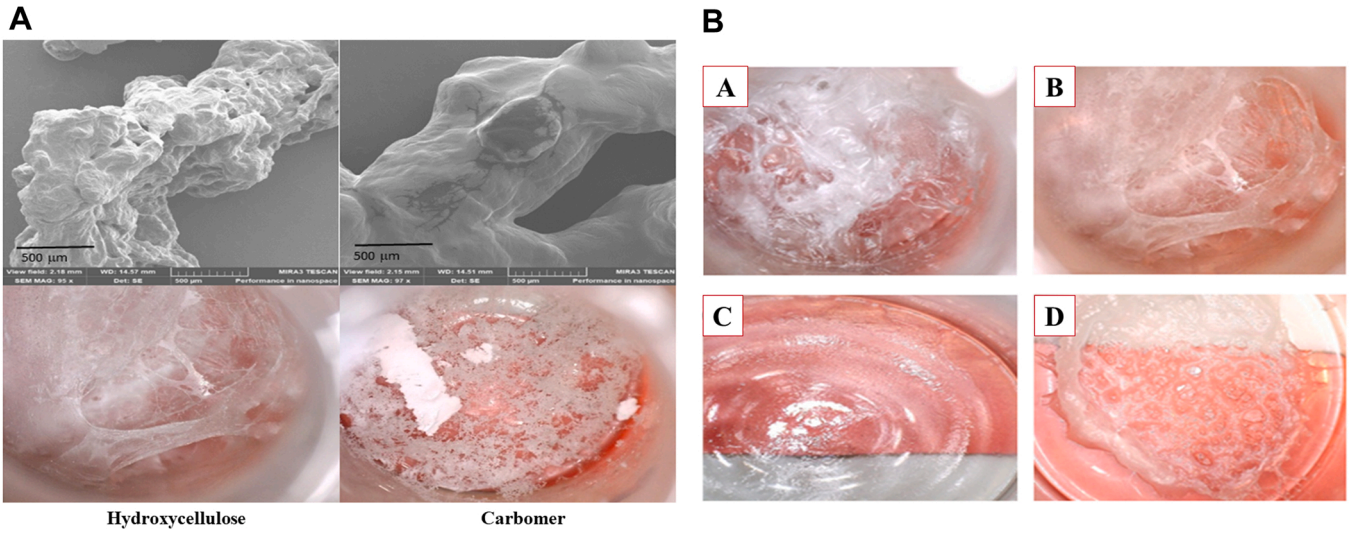

Fig. 2. a. SEM and optical images (100x) of hydroxyethylcellulose and carbomer hand rubs. b. Optical images of different hand rubs gel (100x magnitude) containing polyacrylate crosspolymer-6 (A), Polyquaternium-7 (B), acrylates/C10–30 alkyl acrylate crosspolymer (C), poly(methyl methacrylate) (D).

a filamentous structure as confirmed by optical microscope analysis (Fig. 2b), where the lyophilised samples generated small white flakes [32] or uneven transparent film, typical of the derivate of cellulose [33], respectively. For the other lyophilized polymers (acrylates/C10–30 alkylacrylatecrosspolymer, polyacrylatecrosspolymer-6, poly(methylmethacrylate) and polyquaternium-7) used for hand rubs, different behavior was observed in the function of the used cross linker as reported in the literature [34,35].

Before measuring the alcohol content for all the samples at our disposal, a pH control was carried out, obtaining values ranging from 4.9 to 7.3.

3.4. Determination of alcohols

Ninety ABHRs, purchased from the Italian market, were analysed by HS-GC/MS and by biosensor.

The results of seventy-four cosmetic products by HS-GC/MS analysis are shown in Fig. 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 ± 3%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 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.

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 only 47% of them was coherent with the 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 ± 2%v/v the lowest determined alcohol concentration and 85.3 ± 3.8%v/v the highest. Alcohol concentrations declared on the 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 ± 1%v/v was determined. A possible reason for 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 ± 2%v/v and 63 ± 3%v/v, confirming the value on labels. EtOH was used in all the preparations tested, except for only one sample in which IPA was determined. Neither MeOH nor *n*-PA were determined.

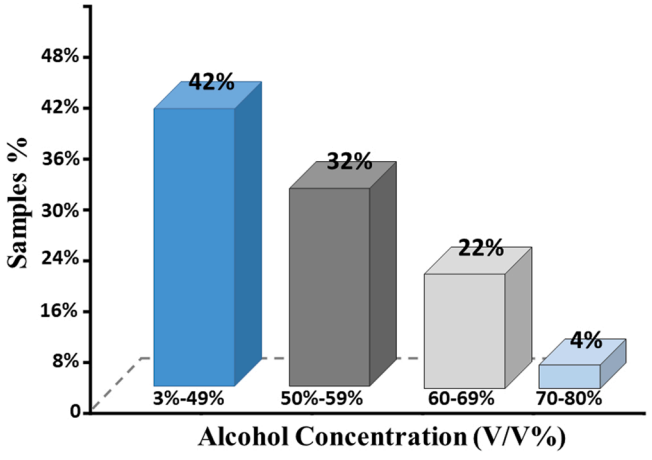

Fig. 3. Cosmetic products: average alcohol concentration expressed as %v/v.

The survey pointed out that 61% of the analysed samples (74 cosmetic, 10 biocidal and 6 galenic products) collected in Italy contained alcohols below 60% v/v. This finding is in contrast with those of other studies conducted in the same period on hand sanitisers [17,19,20] and two explanations may be evaluated.

The first consideration is attributable to the wide diffusion of substandard products not aligned with the health agencies recommendations in the first period of the pandemic, as also evidenced by Da Costa et al. [17]. Otherwise, the manufacturing processes must be considered, in which losses of alcohols may occur leading to final concentrations lower than the expected values [16]. Even though the quality of analysed samples was questionable for active ingredients content, MeOH content was below LoQ value in all samples.

Samples were analysed also by the electrochemical biosensor, selective for the class of primary alcohols and 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 an increasing chain length of the alkyl (R) group. To avoid the influence of the different pH of the hand rubs on the enzymatic reaction of the alcohol biosensor, all samples were diluted in buffer (50 mM phosphate buffer, pH 7.4) and before the analysis was treated as reported in paragraph *2.4.3*. The amount of alcohol present in the hand sanitisers was extrapolated from the calibration line (Figure SI3, constructed used IPA as standard) by adding known concentrations of IPA to an alcohol-free hand rub (the same used for HS-GC/MS measurements), in order to minimize the matrix effect on the electrochemical measurement. Moreover, the analytical performances of AOx-based biosensors in the above reported study were investigated. In particular, the screen-printed-based devices showed a good reproducibility (RSD <10%), sensitivity (LoD=27%v/v) and reusability (the signal loss is <11%). This latter was evaluated up to 10 successive measurements with relative standard deviation (RSD) ranging from 10%.

The alcohols concentrations (%v/v), in terms of mean and standard deviation, median, geometric mean, 5th and 95th percentiles, obtained with the two different techniques in 90 commercial hand sanitisers are reported in Table 4. The two analytical methods gave results very similar to each other, with a slight trend of the electrochemical biosensor to overestimate with respect to HS-GC/MS.

Using SigmaPlot ver 11, the 99% prediction interval for the percentage of alcohol content obtained with both methods is calculated using the following equation:

$$y = y_0 \pm \left(\frac{t(n-p-1)s}{1 + X_0'(X'X)^{-1}X} \right) \quad (1)$$

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 results of biosensor were in accordance with those of HS-GC/MS for about 90% of all analysed ABHRs (compared to HS-GC/MS, Fig. 4 where only the most significant results are reported), in particular when carbomer and acrylates were used as gelling agents. 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%. This result provided a predicting range for the future analysis of the ABHRs.

In addition, the distribution of the differences between the two analytical approaches for each sample showed a Gaussian pattern, as

Table 4
Alcohols content of 90 commercial hand sanitisers. Concentrations in % v/v.

	Mean	Standard Deviation	Median	Geometric mean	5th percentile	95th percentile
HS-GC/MS	56	11	56	55	39	73
Electrochemical Biosensor	61	9	62	60	48	77

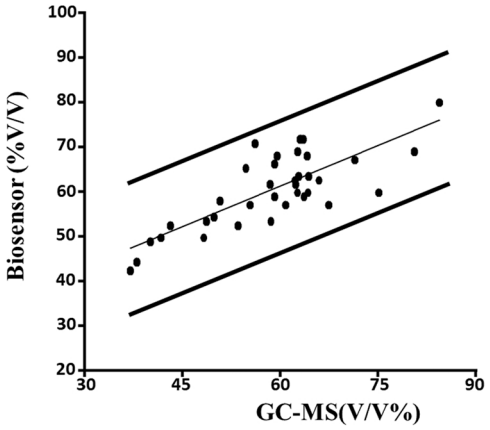

Fig. 4. Prediction interval of the comparison of the most significant data point selected among 90 hand rubs analysed by electrochemical biosensors and HS-GC/MS.

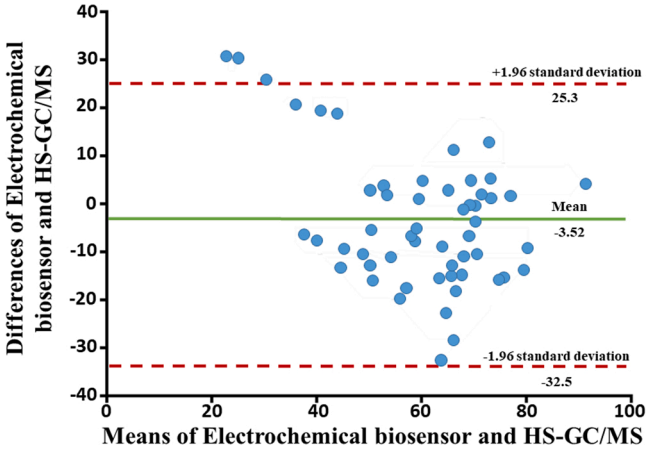

Fig. 6. Bland-Altman plot.

well proved in Fig. 5. Thus, the distribution of the values was evaluated by the Bland-Altman plot (Fig. 6) in which the differences are plotted against the corresponding averages of the two techniques. The x-axis reported the average of the two measurements for each sample.

($X_i = \frac{(X_{1i} + X_{2i})}{2}$), while the y-axis indicated the difference ($d_i = X_{1i} - X_{2i}$). Horizontal lines represent the mean difference and the limits of the agreement are defined as the mean difference plus and minus 1.96 times the standard deviation of the differences. Information on the comparability of the two techniques is given by the position of the points in the graph. The plot in Fig. 6 showed that the two techniques could be considered interchangeable as almost all points were between the limits of agreement. Furthermore, the differences between the two measurements had a slightly better agreement at lower alcohols mean concentration values. This allows considering the electrochemical biosensor as a complementary tool with screening purposes for the identification of hand sanitisers suspected to have low total alcohols content. Thus, rapid monitoring of commercial samples could be achieved as a more accurate and sensitive analytical technique, as the HS-GC/MS, might be used for confirmation analyses.

The differences between the measurements obtained with the two

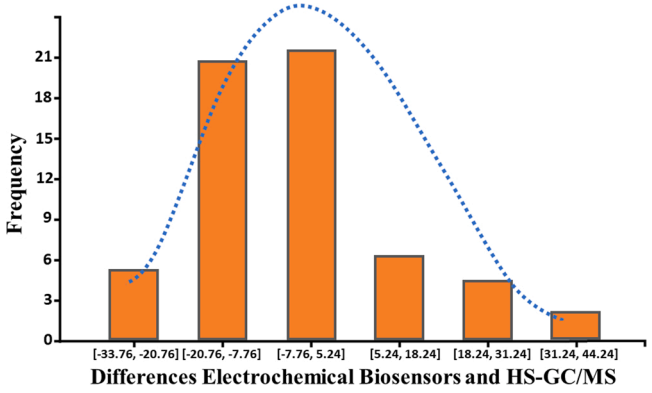

Fig. 5. Distribution pattern of the differences between the electrochemical biosensor and HS-GC/MS.

analytical approaches were related to the treatment of the sample (dilution for biosensor and acidification in HS-GC/MS) and different conditions of analyses (liquid for biosensor and steam in HS-GC/MS). This result, carried out on the most significant samples was confirmed by t-test and ANOVA one way (Turkey test) where, in both case, the differences in the median values among the treatment groups were not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($p = 0.062$).

4. Conclusion

In this work, a novel electrochemical screen-printed based biosensor and an HS-GC/MS in-house validated (according to ISO/IEC 17,025) method for the determination of alcohol content in ABHRs were reported. The analyses were conducted on ninety ABHRs (differing in formulation and brands) purchased on the Italian market from April to November 2020. All the 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 a diverse variety of commercial hand sanitisers. Moreover, encouraging results in terms of sensitivity (LoD 27%), reproducibility (RSD <10%) and reusability were obtained using an AOx-based biosensor. By comparing the results collected using the above-reported methods a good correlation was observed (95%). In addition, it was observed that only 39% of the tested products had an average concentration of at least 60%v/v of alcohol. Among the cosmetic sanitisers, the percentage of products containing the recommended alcohol levels was only 26%, while all biocidal products and galenic preparations analysed were aligned with the health agencies indications.

This study highlighted that the combination of biosensor and HS-GC/MS would give a powerful tool for the fast analysis of hand sanitisers, in which the first method is directly usable on the market, lowering the analysis costs and avoiding consumer fraud. Furthermore, the survey confirmed the need to increase analytical controls as executive actions for this type of products, in order to protect the consumer from

formulations in which the concentration of alcohol is not clearly stated on the label.

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CRediT authorship contribution statement

Costanza Majorani: Writing – original draft, Conceptualization, Methodology, Investigation. **Claudia Leoni:** Writing – original draft, Methodology, Investigation, Validation. **Laura Micheli:** Writing – original draft, Methodology, Investigation, Formal analysis. **Rocco Cancelliere:** Writing – original draft, Investigation, Resources. **Marco Famele:** Writing – review & editing, Resources. **Roberta Lavalle:** Writing – review & editing, Resources. **Carolina Ferranti:** Writing – review & editing. **Luca Palleschi:** Writing – review & editing. **Luca Fava:** Writing – review & editing. **Rosa Draisci:** Supervision. **Sonia D'Ilio:** Writing – review & editing, Supervision.

Declaration of Competing Interest

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.114694.

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