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UV–vis spectrophotometric method for the quantitation of all the components of Italian general denaturant and its application to check the conformity of alcohol samples

Remo Bucci [a], Fabrizio Balestrieri [b], Andrea D. Magrì [a], Antonio L. Magrì [a], Federico Marini [a,*]

[a] *Dipartimento di Chimica, Università di Roma "La Sapienza", p.le Aldo Moro 5, I-00185 Roma, Italy*
[b] *Dipartimento di Chimica—Sez. di Merceologia, Università di Perugia, I-06123 Perugia, Italy*

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Abstract

A simple, fast and relatively inexpensive spectrophotometric method for the identification and the quantification of the individual components of the Italian general denaturant in alcohol samples is proposed.

In particular, it is shown that bitrex (a quaternary ammonium salt), whose UV spectrum is completely masked by those of the other denaturant components, can be identified using its reaction with disulphine blue VN-150 (an anionic dye indicator), which leads to the formation of an intensely colored ion-association complex (mole ratio 1:1), easily extractable in chloroform. As far as the quantitative detection is involved, it is however necessary to shake the chloroform phase in the presence of 1 mol L^{-1} $NaClO_4$ aqueous solution because of the fast adsorption of the ion pair on the walls of the glass cell. Perchlorate anion, due to mass action, substitutes the anionic dye indicator in the association complex: as a consequence, disulphine blue passes to the aqueous phase, where its absorbance at $\lambda = 640$ nm is measured. On the other hand, C.I. Reactive Red 24 dye is easily identifiable from the visible spectrum of the product without any further pretreatment: its concentration can be determined measuring the absorbance at $\lambda = 542$ nm. Thiophene, being significantly more concentrated than the other components, can be identified from the UV spectrum of a 1:100 diluted solution of the alcohol sample and quantitatively determined measuring the absorbance at $\lambda = 230$ nm. Lastly, methyl ethyl ketone (MEK) can be identified from the UV spectrum of a 1:5 diluted solution of the alcohol sample and quantitatively determined measuring the absorbance at $\lambda = 273$ nm. However, more accurate results can be obtained using a multiwavelength analysis in the range 220–250 and 250–310 nm for the determination of thiophene and MEK, respectively.

Validation on standard denatured alcohol samples has proven the method to be both accurate and sufficiently precise (within- and between-days repeatability <5%) to be applied to the analysis of real commercial samples.

Keywords: Denatured alcohol; Spectrophotometry; General denaturant components

1. Introduction

In all the EU countries, ethyl alcohol is liable to excise duty (i.e. to taxation for its production), unless it is destined to social and economically relevant uses (industrial, agricultural, pharmaceutical): in the latter case, facilitations up to the complete exemption are provided for. However, to prevent the tax-free alcohol from being used in a fraudulent way (particularly in the production of goods intended for the human consumption), when it is destined for industrial and commercial purposes, this product is required to be denatured, by adding a mixture of hardly eliminable substances, which make it easily recognizable (color and unpleasant smell) and not drinkable (bitter taste).

* Corresponding author. Tel.: +39 06 49913856; fax: +39 06 4457050.
E-mail address: fmmonet@hotmail.com (F. Marini).

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Each State adopts its own general denaturant (GD), controls the denaturing procedures and, in special cases, authorizes the use of denaturants different than the general one. The EU Regulation 2559/98 [1] provides that, in the case of Italy, the ethyl alcohol to be denatured must have an alcoholic grade not below 90% in volume and that the denaturation using the GD has to be carried out adding, per hectoliter of anhydrous alcohol: 125 g of thiophene, 0.8 g of denatonium benzoate (bitrex), 3 g of C.I. Reactive Red 24 aqueous solution at 25% (w/w), 2 l of methyl ethyl ketone (MEK).

It is then evident that the control of the denatured alcohol is an important issue for analytical chemistry applied to commodity science. This importance is two-fold: in fact, denatured alcohol can be an end-product (e.g. for skin disinfection) and as such its chemical composition has to meet the requirements specified by the Customs' Tariff. On the other hand, as the denaturation allows the alcohol to be tax-free, the analytical control on the product is extended to the cases where it is used as a solvent or a reactant.

As far as we know, no specific methods for the determination of all the components of Italian general denaturant in alcohol samples or alcoholic formulations is available. In fact, only studies and methodologies for the determination of its individual components are reported in the literature, almost always referring to different matrices and using separative techniques (HPLC, GC, CE), often coupled with a mass spectrometric detector [2–5]. In particular, the determination in alcohol samples has been investigated only in the case of bitrex, probably due to its being included in the denaturing formulations of many countries: after a first investigation by Glover and Blake using TLC [5], HPLC on a cyano-silica column [6] or, more recently, GC/MS have been successfully used for this analysis [7]. On the other hand, visible spectrophotometry is involved only when the dye is concerned: Yang et al. report a method for the quantitation of a mixture of dyes (including C.I. Reactive Red 24) using a PLS multivariate calibration of spectral data [8]. As a consequence, the analytical laboratories for the control of denatured alcohol have to set up their own methodologies, combining and adapting available methods which have been designed on other matrices.

Therefore, in order to realize an alternative analytical method which could be simpler, faster and cheaper, the possibility of using a single technique (UV–vis spectrophotometry, which requires an instrumentation present in almost all the analytical laboratories) to identify and quantify all the four components of the above mentioned general denaturant in alcohol samples has been studied. The method is essentially based on measuring the absorbance at fixed wavelengths; however, the advantages of using a multicomponent analytical approach, based on processing the spectrophotometric data in a selected range by a multiwavelength computational program are also exploited.

In addition, we have closely examined some aspects of the chemical, spectroscopic (UV, IR) and thermoanalytical (TG, DSC) behavior of each component.

2. Experimental

2.1. Instruments

UV–vis spectra were acquired on a Perkin-Elmer 320 spectrophotometer equipped with 1 cm quartz cells and connected to a model 3700 Data Station. The software "IF-320" and "CDS-13" were used for the instrument control and for the mathematical treatment of the spectra, respectively; multiwavelength analysis was performed by the package "QUEST", which minimizes the square root of the sum of the squared differences at each point between a linear combination of the "standards" and the "unknown" spectra (rms error), to find a least-squares best fit. The scan speed was adjusted at 60 nm min^{-1}, slit width = servo (gain = 5) and response time = 2 s. The spectrophotometer was connected with a "Colora" ultrathermostat.

An Orion EA 940 pH-meter with an Orion 81-02 Ross combination pH electrode, calibrated in concentration units, was used, so in this work the symbol pH corresponds to $-\log[H^+]$.

IR spectra were recorded using a Perkin-Elmer 1600 FTIR spectrometer.

The thermal measurements were carried out using a Perkin-Elmer TGS-2 Thermogravimetric Analyzer, connected to a model 3700 Data Station, and a Perkin-Elmer DSC-7 Differential Scanning Calorimeter (Perkin-Elmer, Shelton, CT, USA) equipped with a multitasking software for instrument control and data analysis. Unless otherwise specified, thermogravimetry (TG) and differential scanning calorimetry (DSC) runs were made on a sample of about 1–2 mg (TG) or 0.3–0.5 mg (DSC) in a stream of N_2 or O_2 (flow rate, 50 mL min^{-1}), heating rate 10 °C min^{-1}.

Pyrex glass tubes (15 mL), with screw-on caps, were used to extract the disulphine–bitrex ion-association complex.

2.2. Materials and standard solutions

The individual components of the general denaturant (denatonium benzoate, thiophene, methyl ethyl ketone and C.I. Reactive Red 24 dye) and a concentrated standard denaturing mixture (which requires a preliminary dilution with the proper volume of MEK prior to being used) have been kindly provided by the Customs Agency Chemical Laboratory in Rome. Stock standard solutions of each component were prepared directly by weighing (or by volumetric transfer in the case of MEK) and dilution with 90% ethyl alcohol to a known volume.

Two "standard" samples of denatured alcohol have been prepared in our laboratory to be used in the validation phase (see Section 3.6). The first one was prepared by adding accurately weighed amounts of each component to 90% ethyl alcohol, resulting in the following composition (the values are expressed as amount of denaturing agent in 1 L of anhydrous alcohol; see Section 3.6):

Thiophene	1.08 g L^{-1}
Denatonium benzoate (bitrex)	8.80 mg L^{-1}
C.I. Reactive Red 24 dye	8.63 mg L^{-1}
Methylethylketone (MEK)	22.2 mL L^{-1}.

The second standard denatured alcohol sample was prepared adding the standard concentrated denaturing mixture described in the EC regulation, after proper dilution with MEK, to an accurately measured volume of 90% (v/v) ethyl alcohol. The final composition of this sample was (the values are expressed as amount of denaturing agent in 1 L of anhydrous alcohol; see Section 3.6):

Thiophene	1.23 g L^{-1}
Denatonium benzoate (bitrex)	7.84 mg L^{-1}
C.I. Reactive Red 24 dye	7.35 mg L^{-1}
Methylethylketone (MEK)	19.6 mL L^{-1}.

Additionally, fourteen commercial samples of denatured alcohol for skin disinfection, produced by different brands and purchased from different stores in Rome were also analyzed.

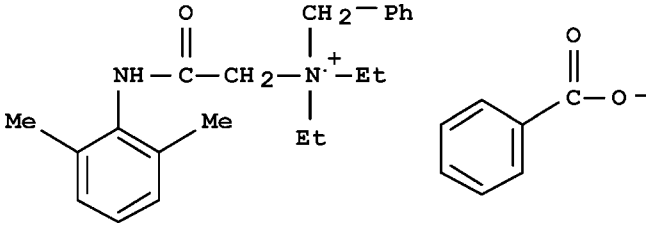
Stock standard aqueous solution of disulphine blue VN 150 was prepared dissolving the proper quantity of the dye ("Merck."; acid blue 1; C.I. 42045) preventively purified by double crystallization from absolute ethyl alcohol and dried in an oven at 105 °C.

All chemicals were analytical-reagent grade.

3. Results and discussion

Before describing in detail the procedure for the analytical control of the samples of denatured alcohol and the results obtained, a short presentation of any of the individual denaturing components (where some particular data acquired during the experimental setup have been added to complete and update the ones already published in the literature) is presented.

3.1. Denatonium benzoate (benzyldiethyl(2,6-xylylcarbamoylmethyl)ammonium benzoate, bitrex; $C_{21}H_{29}N_2O$ $C_7H_5O_2$; mw 446.59)



is a white very bitter crystalline powder soluble in water, alcohol, sparingly soluble in acetone and insoluble in ether.

The IR spectrum of bitrex (reproduced in Fig. 1) shows several bands, consistent with the chemical structure of the compound. The presence of the benzoate anion is witnessed by the two strong carboxylate stretching bands at 1566 cm^{-1} (antisymmetric) and 1374 cm^{-1} (symmetric). When turning

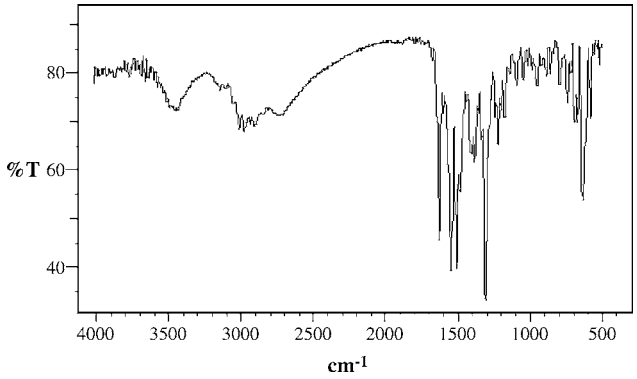


Fig. 1. IR spectrum of bitrex (KBr disc).

to the denatonium moiety, we can observe a strong amide I band at 1684 cm^{-1}; with respect to the equivalent aliphatic amides, the frequency of this band is shifted to higher frequencies, due to the ring competing for the lone pair on the nitrogen atom. The broad band with a maximum at 3433 cm^{-1} is also connected to the presence of an amidic bond (N–H stretching vibration). The presence of several phenylic residues results in the strong peak at 1606 cm^{-1} and in several peaks in the region 1500–1400 cm^{-1}.

The thermogravimetric analysis of the compound leads to identical results when using N_2 or O_2 (Fig. 2, curves a and b). In both cases, indeed, the anhydrous compound is stable up to about 175 °C, and then a steep mass loss, occurring in two overlapping steps (t_{max} = 185 and 215 °C), is detected. At 250 °C the residue is almost zero. A further attempt of improving the resolution of the two processes, reducing the rate of temperature variation (from 10 to 2 °C min^{-1}), has been unfruitful. On the other hand, DSC curves allow a better differentiation of the thermal decomposition process as

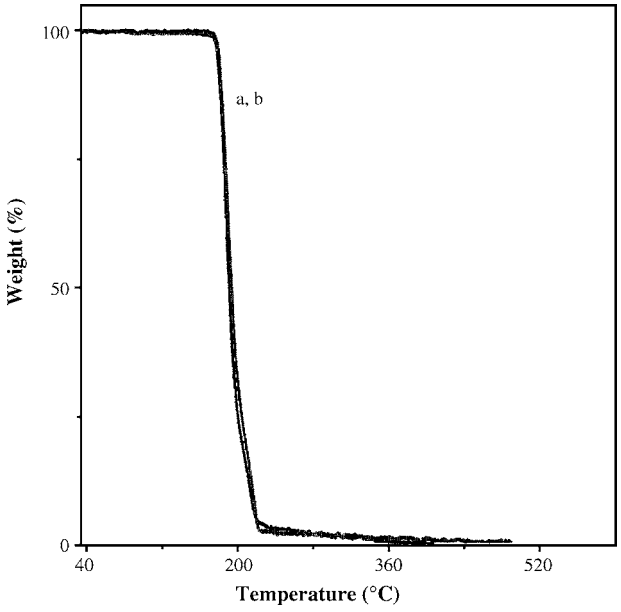


Fig. 2. TG of bitrex in nitrogen (a) and oxygen (b).

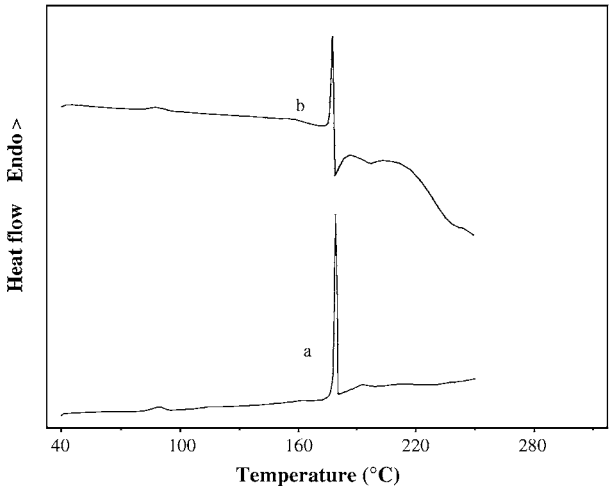


Fig. 3. DSC of bitrex in nitrogen (a) and oxygen (b).

a function of the atmosphere of the furnace (inert or oxidizing, Fig. 3 curves a and b). In fact, in N_2 a first narrow endothermal peak ($t_p = 178\,^{\circ}C$; $\Delta H = 89\,J\,g^{-1}$), ascribable to the fusion of the substance, is evidenced: as a confirmation, when the experiment is repeated in a sealed capsule, which does not allow the elimination of the decomposition products, the peak is anticipated ($t_p = 173\,^{\circ}C$), but the associated enthalpy is only a little lower ($\Delta H = 81\,J\,g^{-1}$). At least two other endothermal peaks between 180 and 250 °C concur to the complete thermal decomposition of the product. In O_2, instead, the endothermal peak due to the fusion is rapidly transformed in an exothermal one, because of the fast initial oxidative decomposition of the substance, which is then followed by other exothermic processes up to about 250 °C.

A solution of bitrex in 90% (v/v) ethanol shows a characteristic UV spectrum with several partially overlapped absorption bands but only two evident maxima at $\lambda = 264$ and 270 nm; at $\lambda < 250$ nm the absorbance increases rapidly but no maximum appears up to 210 nm (Fig. 4, curves b and b′).

However, this spectrum cannot be directly used to its qualitative and quantitative analysis in the case of denatured

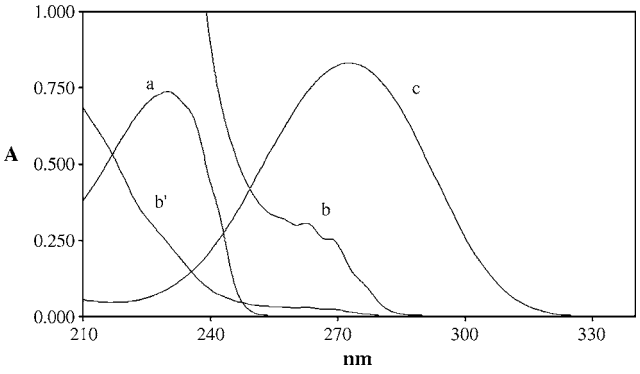


Fig. 4. UV absorption spectra of thiophene ($c = 10\,mg\,L^{-1}$) (a), bitrex ($c = 100$ and $10\,mg\,L^{-1}$) (b and b′) and MEK ($c = 4\,mL\,L^{-1}$) (c) in ethanol 90% (v/v).

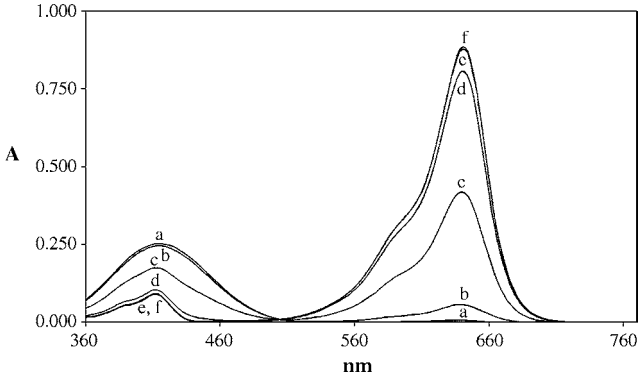
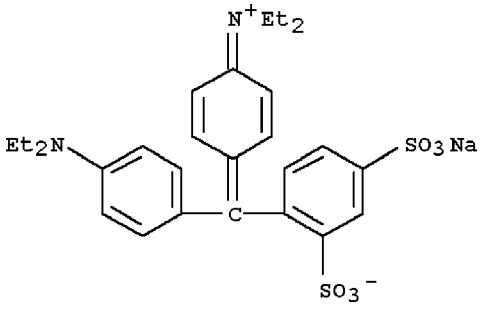


Fig. 5. Visible absorption spectra of disulphine blue VN 150 ($c = 8.93\,\mu mol\,L^{-1}$ in $1\,mol\,L^{-1}$ $NaClO_4$ aqueous solution) at pH = 1.0 (a); pH = 1.7 (b); pH = 2.6 (c); pH = 3.7 (d); pH = 4.6 (e); pH = 5.5 (f); blank = $1\,mol\,L^{-1}$ $NaClO_4$ aqueous solution; $t = 25\,^{\circ}C$; $b = 1$ cm.

alcohol samples because it is completely masked by those of the other denaturant components. So we tried an alternative procedure based on the knowledge that cationic surfactants form ion-association compounds with some intensely colored anionic counter ions, which extracted into an organic solvent, can be determined by visible spectrophotometry [9–26]. To verify if bitrex could be determined applying the above method, we studied the reaction of this quaternary ammonium salt with disulphine blue VN150, an anionic dye indicator, which seems to show the higher sensitivity among the reagents employed for cationic surfactants determination [13,14,19] and that, some years ago, we ourselves successfully used to determine benzalkonium chloride, *N*-cethylpyridinium and *N*-alkyl-*N*,*N*,*N*-trimethylammonium sulphate (empigen CM) in aqueous solutions (unpublished work).

3.1.1. Study of the reagent (anionic dye indicator)

Disulphine blue VN 150 (*N*-(4-{[4-(diethylamino) phenyl][2,4-disulfophenyl]methylene }-2,5-cyclohexadien-l-ylidene)-*N*-ethylethanaminium hydroxide, inner salt, sodium salt; C.I. acid blue 1; $C_{27}H_{31}N_2O_6S_2$ Na; mw 566.66).



is a violet powder soluble in water, partly in ethanol and insoluble in chloroform.

Fig. 5 shows the visible spectra of diluted aqueous solutions of the reagent (acid–base indicator) at different pH

Table 1
Spectrophotometric characteristics and acid–base equilibrium constant of disulphine blue VN 150 ($t = 25\,^{\circ}C$; $I = 1\,mol\,L^{-1}$ $NaClO_4$)

Species	ε ($L\,mol^{-1}\,cm^{-1}$)	pK_a
HL	0 ($\lambda = 640$ nm)	2.44 ± 0.05
L^-	$(2.8 \pm 0.1)\,10^4$ ($\lambda = 414$ nm)	
	$(9.9 \pm 0.2)\,10^4$ ($\lambda = 640$ nm)	
	$(1.0 \pm 0.1)\,10^4$ ($\lambda = 414$ nm)	

The precision is reported as the standard deviation of the mean ($n = 5$; $\alpha = 0.05$).

values. At high acidity (pH ≤ 1) the yellow solution shows a single absorption band with maximum at 414 nm. Increasing the pH up to 6.5 the solution turns to blue and a new more intense absorption band appears with maximum at 640 nm while the first band is shifted hypsochromically (maximum at 410 nm) with hypochromic effect. The acid–base equilibrium is characterized by an isosbestic point at 502 nm. The equilibrium constant and the molar absorptivities of the two species (HL, L^-) were calculated by the analysis of the curves A versus pH (at 640 and 414 nm) [27,28] and the results are reported in the Table 1.

In alkaline medium, a slow irreversible alteration of the color of the solution is indicative of the degradation of the dye indicator.

3.1.2. Study of bitrex–disulphine reaction

Disulphine blue VN 150 does not extract in chloroform from a neutral aqueous solution but in presence of bitrex the organic phase assumes a blue color proving the formation of an ion-association compound between the two reagents. The spectrum of the organic phase is slightly different with respect to that of the neutral aqueous solution of the reagent mixture ($\lambda_{max} = 628$ nm) but its color is not stable in time, because of the absorption of the ion-association compound on the glassware which remains colored also after short periods of contact. Only in the employment of pyrex glassware it was possible to eliminate this interference during the extraction process. However, the phenomenon repeats itself on quartz or glass optical cells (after 5 min the absorbance is almost halved) making impossible a quantitative analysis. To avoid this, taking into account the studies of Biswas and Mandel [29] on the interference of inorganic ions in the formation of cationic surfactant-anionic dye compounds, we tried to extract again the chloroform phase with an equal volume of $1\,mol\,L^{-1}$ $NaClO_4$ aqueous solution. Disulphine blue VN 150 quantitatively passes in the aqueous solution while the organic one, containing the denatonium in perchlorate form, remains perfectly colorless. Absorbance of the aqueous solution is now stable.

Using this procedure on aqueous solutions containing bitrex and disulphine blue at different molar ratios the stoichiometry (1:1) of the ion-association compound and the excess of dye indicator necessary to quantitative extraction of bitrex (five time at least) were determined (Fig. 6).

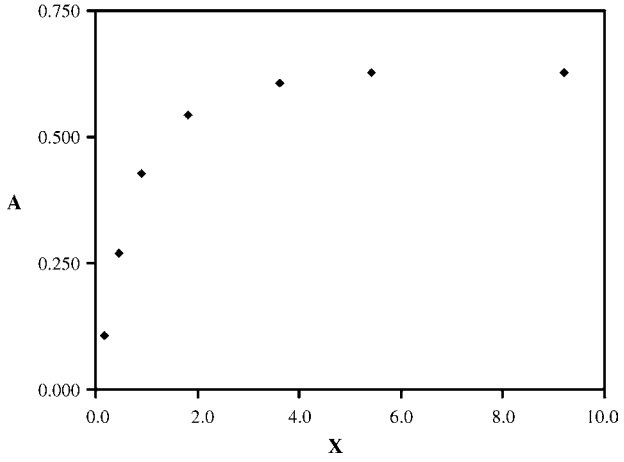


Fig. 6. Absorbance vs. disulphine blue VN150/bitrex molar ratio; $C_{bitrex} = 5.32\,\mu mol\,L^{-1}$; $\lambda = 640$ nm; blank = $1\,mol\,L^{-1}$ $NaClO_4$ aqueous solution; $t = 25\,^{\circ}C$; $b = 1$ cm.

3.1.3. Calibration curve

Aliquots (0.1–0.5 mL) of a standard solution of bitrex in 90% ethanol ($C = 80\,mg\,L^{-1}$) are placed in pyrex glass tubes (15 mL), with screw-on caps and the solvent is evaporated in a bain-marie. The residue is dissolved into 1 mL of a disulphine blue VN 150 solution (C about $10^{-3}\,mol\,L^{-1}$) and extracted in 10.0 mL of $CHCl_3$ (the two phases are carefully shaken for 1 min). After removing the aqueous phase, 5.0 mL of the organic phase are transferred to a second test tube, containing 5.0 mL of a $NaClO_4$ aqueous solution ($1\,mol\,L^{-1}$). The tube is then carefully shaken until the complete disappearance of the blue coloration from the organic phase. The absorbance of the aqueous solution at 640 nm is then measured, using glass cells with 1 cm optical path (blank = $1\,mol\,L^{-1}$ $NaClO_4$ aqueous solution).

The resulting calibration curve has been determined to be:

$$A_{640} = (-0.020 \pm 0.011) + (0.2557 \pm 0.0049\,L\,mg^{-1})C;$$
$$R^2 = 0.9967$$

Visual inspection of the residuals and analysis of variance ($F = 2712$, corresponding to $p = 1.78 \times 10^{-12}$) have proven the linear model to be correct. Anyway, a t test on the intercept value has demonstrated that this value is not statistically different from zero ($p < 0.05$); therefore, the calibration curve to be used for the successive analysis has been computed using a linear model without intercept:

$$A_{640} = (0.2484 \pm 0.0030\,L\,mg^{-1})C; \quad R^2 = 0.9955$$

Also in this case, the analysis of residuals and ANOVA ($F = 2227$, corresponding to $p = 4.40 \times 10^{-13}$) have been used for regression diagnostics.

When this calibration curve had to be used to analyze the validation and commercial samples of denatured alcohol, its stability was controlled by measuring each time the absorbance of at least one standard concentration ($1.50\,mg\,L^{-1}$) prior to the successive analyses.

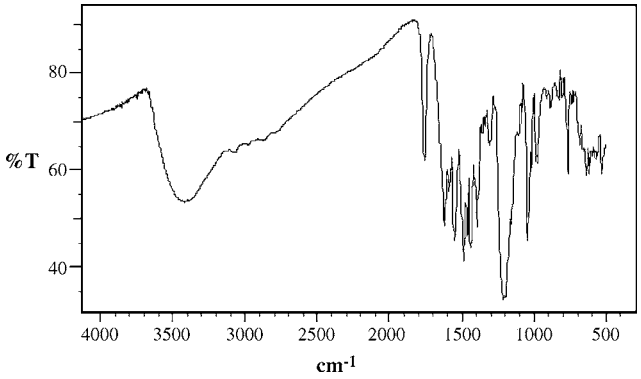
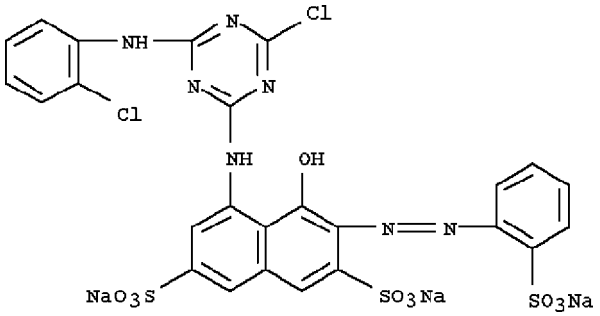


Fig. 7. IR spectrum of C.I. Reactive Red 24 dye (KBr disc).

3.2. C.I. Reactive Red 24 (2,7-naphthalenedisulfonic acid, 5-[[4-chloro-6-[(2-chlorophenyl)amino]-1,3,5-triazin-2-yl]amino]-4-hydroxy-3-[(2sulfophenyl)azo]-, trisodium salt; $C_{25}H_{14}Cl_2N_7O_{10}S_3Na_3$; mw 808.48)



is a red crystalline powder soluble in water and alcohol.

The IR spectrum of Red 24, reported in Fig. 7, shows several bands, consistent with the chemical structure reported above. In particular, the broad band at 3421 cm^{-1} results from the overlap of the two aminic N–H stretching and the naphtolic O–H stretching. The presence of an aromatic alcohol moiety results also from the medium/strong band at 1354 cm^{-1}. The strong peak at 1548 cm^{-1} is instead due to the substituted triazinic residue (stretching of the C=N bond). Lastly, the three sulphonate anions give rise to a strong stretching peak at 1196 cm^{-1}.

The thermal stability of Red 24 was studied by TG and DSC in oxygen and nitrogen atmospheres. TG curves (Fig. 8) show that in O_2 the anhydrous compound is stable up to about 280 °C and then it decomposes, rapidly, by several overlapped steps (t_{max} = 350, 455 and 536 °C); at 760 °C the residue consists mainly of sodium sulphate as confirmed by its IR spectrum. In N_2, the first decomposition process occurs at the same temperature but the successive decomposition step is delayed (t_{max} = 470 °C) and no constant weight is obtained up to 840 °C. According to DSC curves (Fig. 9), the decomposition of Red 24 occurs in O_2 with evident exothermic reactions which are almost absent in N_2.

A solution of Red 24 in 90% (v/v) ethanol shows a characteristic UV–vis spectrum with six main maxima at λ = 243, 279, 315, 370, 510 and 542 nm (Fig. 10 curve a), which can be used for the qualitative and quantitative analysis of the dye if interfering species are absent or negligible (as in the case of denatured alcohol samples: see Section 3.5.2). In fact, in the concentration range from about 5 to 30 mg L^{-1}, the spectra

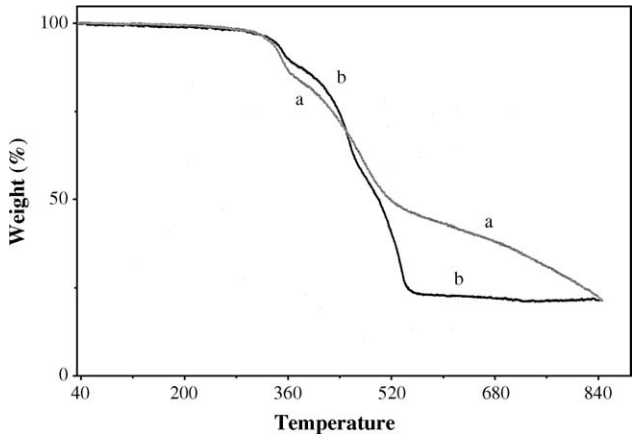


Fig. 8. TG of C.I. Reactive Red 24 dye in nitrogen (a) and oxygen (b).

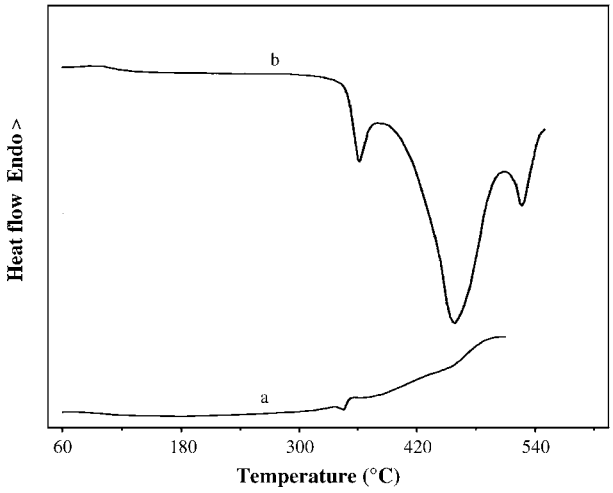


Fig. 9. DSC of C.I. Reactive Red 24 dye in nitrogen (a) and oxygen (b).

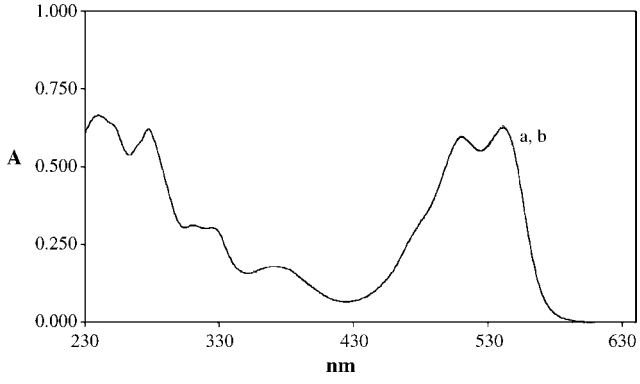


Fig. 10. UV–vis absorption spectra of C.I. Reactive Red 24 dye (c = 15 mg L^{-1} in ethanol 90% (v/v)): experimental (a) and calculated (b).

Table 2
Values of the parameters used to generate the UV–vis spectrum of C.I. Reactive Red 24 dye according to Equation $A_i = \sum_k a_{ik}C$ (where $a_{ik} = a_{\max,k} \exp[-0.5(\lambda_i - \lambda_{\max,k})^2/\sigma_k^2]$); λ = 230–640 nm; C = 15.0 mg L^{-1} in ethyl alcohol 90% (v/v)

Gaussian curve	$a_{\max,k}C$	$\lambda_{\max,k}$	σ_k	Gaussian curve	$a_{\max,k}C$	$\lambda_{\max,k}$	σ_k
1	0.664102	239.6481	22.68025	7	0.064975	329.9850	6.264588
2	0.0716115	255.8357	5.130162	8	0.178097	372.3295	26.98587
3	0.102927	266.6914	4.467734	9	0.064277	476.4178	9.742807
4	0.353828	277.4802	6.742465	10	0.241113	495.1009	36.79784
5	0.175358	289.3680	6.446741	11	0.360745	510.8427	14.87648
6	0.291339	310.5087	18.83211	12	0.492683	544.6054	12.68538

obey Beer's law and the calibration curve at λ = 542 nm is:

$$A_{542} = (-0.0053 \pm 0.0057) + (0.04191 \pm 0.00030\ \mathrm{L\,mg^{-1}})C; \quad R^2 = 0.9995.$$

The goodness of fit was proven by visual inspection of the residuals and analysis of variance (F = 19001, corresponding to $p = 9.89 \times 10^{-18}$). Furthermore, the intercept value resulted not to be statistically different from zero according to a t-test ($p < 0.05$); therefore, the calibration curve to be used for the successive analysis has been computed using a linear model without intercept:

$$A_{542} = (0.04164 \pm 0.00012\ \mathrm{L\,mg^{-1}})C; \quad R^2 = 0.9994$$

Also in this case, the analysis of residuals and ANOVA (F = 19210, corresponding to $p = 3.45 \times 10^{-19}$) have been used for regression diagnostics.

When this calibration curve had to be used to analyze the validation and commercial samples of denatured alcohol, its stability was controlled by measuring each time the absorbance of at least one standard concentration (15.0 mg L^{-1}) prior to the successive analyses.

As in a previous work by ourselves on the analysis of diclofenac [30], the spectrum of the dye has been deconvolved into a set of gaussian curves, so that anyone lacking of the pure substance can compute a theoretical spectrum, almost identical to the experimental one (Fig. 10 curve b). In the Table 2, the optimal parameters defining one of the possible sets of gaussians (12 in the proposed case), which allow to calculate the spectrum of Red 24 dye in 90% (v/v) ethanol in the interval λ = 230–640 nm (C = 15.0 mg L^{-1}) are reported. As also apparent from the figure, the two spectra (experimental and computed) are perfectly overlapped (std. error < 0.002 and $R^2 > 0.9999$).

3.3. Thiophene (divinylene sulfide; C_4H_4S; mw 84.14)

is a colorless liquid ($d_4^{25} = 1.0573$; bp_{760} = 84.4 °C) with slight aromatic odor, insoluble in water; miscible with most organic solvents.

A solution of thiophene in 90% (v/v) ethanol has a characteristic UV spectrum (Fig. 4, curve a) which can be used for its qualitative and quantitative analysis, if interfering species are absent or negligible (as in the case of general denatured alcohol samples: see Section 3.5.2). In fact, in the concentration range from about 2 to 15 mg L^{-1}, the spectra obey Beer's law and the calibration curve at λ = 230 nm is:

$$A_{230} = (0.004 \pm 0.011) + (0.0726 \pm 0.0012\ \mathrm{L\,mg^{-1}})C; \quad R^2 = 0.9994.$$

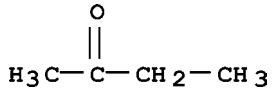
The validity of the model was checked by visual inspection of the residuals and analysis of variance (F = 3523, corresponding to $p = 2.57\ 10^{-8}$). Additionally, the intercept value was proven not to be statistically significant by performing a t-test ($p < 0.05$); therefore, the calibration curve to be used for the successive analysis has been computed using a linear model without intercept:

$$A_{230} = (0.07095 \pm 0.0052\ \mathrm{L\,mg^{-1}})C; \quad R^2 = 0.9994$$

Also in this case, the analysis of residuals and ANOVA (F = 5284, corresponding to $p = 4.56\ 10^{-10}$) have been used for regression diagnostics.

When this calibration curve had to be used to analyze the validation and commercial samples of denatured alcohol, its stability was controlled by measuring each time the absorbance of at least one standard concentration (6.00 mg L^{-1}) prior to the successive analyses.

3.4. Methylethylketone (Butanone; MEK; $C_4\ H_8\ O$; mw 72.11)



is a colorless liquid ($d_4^{20} = 0.805$; bp_{760} = 75.6 °C) with acetone-like odor, soluble in about four parts of water; miscible with alcohol, ether and benzene.

A solution of MEK in 90% (v/v) ethanol has a characteristic UV spectrum (Fig. 4, curve c) which, if interfering species are absent or negligible (as it occurs in the case of general denatured alcohol samples: see Section 3.5.2), can be used for its qualitative and quantitative analysis. In fact, in the concentration range from about 0.2 to 6 mL L^{-1}, the spectra obey Beer's law and the calibration curve at λ = 273 nm is a

straight line:

$$A_{273} = (-0.0013 \pm 0.0032) + (0.20867 \pm 0.00095\,\mathrm{L\,mL^{-1}})C; \quad R^2 > 0.9999$$

Visual inspection of the residuals and analysis of variance ($F = 47945$, corresponding to $p = 2.12 \times 10^{-16}$) have proven the linear model to be correct. Anyway, a *t*-test on the intercept value has demonstrated that this value is not statistically different from zero ($p < 0.05$); therefore, the calibration curve to be used for the successive analysis has been computed using a linear model without intercept:

$$A_{273} = (0.20837 \pm 0.00053\,\mathrm{L\,mL^{-1}})C; \quad R^2 > 0.9999$$

Also in this case, the analysis of residuals and ANOVA ($F = 60479$, corresponding to $p = 4.40\ 10^{-18}$) have been used for regression diagnostics.

When this calibration curve had to be used to analyze the validation and commercial samples of denatured alcohol, its stability was controlled by measuring each time the absorbance of at least one standard concentration (3.00 mL L^{-1}) prior to the successive analyses.

3.5. Proposed method

Based on the chemical, spectroscopic and thermal properties reported above, the following method for the analytical control of denatured alcohol samples has been set up. In some cases, together with the simpler and faster procedure for the determination of each analyte, an alternative one based on the mathematical processing of the spectra (multiwavelength analysis) is also reported with the aim of improving the accuracy of the results.

3.5.1. Identification and quantitation of bitrex

The analytical procedure is almost identical to what described for the calculation of the calibration curve (see Section 3.1.3), the only difference being the amount of sample to be withdrawn for the successive analysis (3.0 mL). Consequently, the identification and quantification of bitrex in samples of general denatured alcohol is carried out, after reaction with disulphine blue and extraction, by recording the visible spectrum of the aqueous phase and measuring the absorbance at $\lambda = 640$ nm.

3.5.2. Identification and quantitation of the dye, thiophene and MEK

As anticipated in the previous sections, all the three components can be directly determined in denatured alcohol samples without any pre-treatment.

In fact, the dye is the only species which absorbs in the visible part of the spectrum, so it can be easily identified from a spectrum of the product without any dilution. Its quantification relies on measuring the absorbance of this solution at $\lambda = 542$ nm and computing the concentration by means of the calibration curve reported in Section 3.2.

The identification and quantitation of thiophene and MEK from their UV spectra can be operated in a similar way. Although all the four components of the general denaturant absorb in this spectral region, their relative concentrations and their spectrophotometric properties are such that, working on appropriately diluted solutions, the mutual interferences are practically negligible (within the limits commonly accepted for this kind of controls). Being thiophene significantly more concentrated than the other denaturant components, it can be identified from the UV spectrum (220–250 nm) of the sample diluted 1:100 and quantitatively determined measuring the absorbance at $\lambda = 230$ nm (see the calibration curve in Section 3.3). The quali-quantitative determination of MEK is carried out by recording the UV spectrum (250–300 nm) of a diluted (1:5) solution of the sample and measuring the absorbance at $\lambda = 273$ nm (see the calibration curve in Section 3.4).

3.6. Validation and conclusions

In order to evaluate the accuracy and precision (expressed both as within-day and intermediate repeatability) of the proposed method, we have analyzed two standard samples of denatured alcohol prepared in our laboratory—one by adding known amounts of each component and the other by proper dilution of the official denaturing mixture (see Section 2.2), according to what prescribed by the EU regulation.

In particular, within-day precision was checked by performing five replicate measurements on each sample during the same day, while between-days (intermediate) precision was estimated by repeating the same analysis on the same samples for five successive days, having care to check the stability of the calibration curves measuring the spectra of fresh reference material each day.

Furthermore, to achieve consistency with the EU regulation on alcohols [1], the alcoholic grade of each sample was also measured and both the expected and measured concentration values have been corrected accordingly, to be expressed as amount of denaturing agent in 1 L of anhydrous alcohol.

The obtained results, gathered in Table 3, show that the recovery of all the four components can be considered as fully satisfactory, even if in the case of MEK (and to a lesser extent of thiophene) the measured values are slightly greater than expected (about 8 and 2%, respectively). This is due to the absorbances of the other components at the selected wavelengths being not completely negligible. So, if necessary, more accurate results can be obtained using a multiwavelength analysis in the range 220–250 and 250–310 nm for the quantitation of thiophene and MEK, respectively.

This kind of multivariate analysis relies on using an appropriate software to compute the best fit of the unknown spectrum (the program QUEST, developed by Perkin-Elmer was used in our study) and requires a number of standard spectra at least equal to that of the analytes to be simultaneously determined. As our goal was to determine a single analyte

Table 3
Validation of the proposed method on standard denatured alcohol samples

Sample	Red 24		Bitrex		Thiophene			MEK		
	Claimed mg L^{-1}	Found λ = 542 nm	Claimed mg L^{-1}	Found λ = 640 nm	Claimed g L^{-1}	Found λ = 230 nm	Found λ = 220–250 nm	Claimed mL L^{-1}	Found λ = 273 nm	Found λ = 250–310 nm
(a) Results of five replicate measurements performed on the same day[a]										
Laboratory standard 1	8.6	8.7 ± 0.1	8.8	8.7 ± 0.3	1.08	1.09 ± 0.01	1.09 ± 0.01 [<0.002]	22.2	23.8 ± 0.3	21.8 ± 0.3 [<0.005]
Laboratory standard 2	7.4	7.3 ± 0.1	7.8	7.9 ± 0.3	1.23	1.24 ± 0.01	1.23 ± 0.01 [<0.002]	19.6	21.5 ± 0.3	19.7 ± 0.3 [<0.005]
(b) Result of five replicate measurements performed on five successive days[b]										
Laboratory standard 1	8.6	8.6 ± 0.1	8.8	8.9 ± 0.3	1.08	1.10 ± 0.01	1.09 ± 0.01 [<0.002]	22.2	23.1 ± 0.3	21.9 ± 0.3 [<0.005]
Laboratory standard 2	7.4	7.3 ± 0.1	7.8	7.7 ± 0.3	1.23	1.25 ± 0.01	1.23 ± 0.01 [<0.002]	19.6	21.7 ± 0.3	19.6 ± 0.3 [<0.005]

[a] Precision is reported as repeatability standard deviation of the mean ($n = 5$; $\alpha = 0.05$); rms error (multiwavelength method) is reported in the brackets.
[b] Precision is reported as intermediate repeatability (between days) standard deviation of the mean ($N = 5$ days; $\alpha = 0.05$); rms error (multiwavelength method) is reported in the brackets.

Table 4
Determination of general denaturant components in commercial samples of alcohol for skin disinfection

Samples	Red 24		Bitrex		Thiophene			MEK		
	Expected mg L^{-1}	Found λ = 542 nm	Expected mg L^{-1}	Found λ = 640 nm	Expected g L^{-1}	Found λ = 230 nm	Found λ = 220–250 nm	Expected mL L^{-1}	Found λ = 273 nm	Found λ = 250–310 nm
C1		7.3 ± 0.1		8.1 ± 0.3		1.23 ± 0.01	1.22 ± 0.01 [<0.001]		21.0 ± 0.3	19.7 ± 0.3 [<0.004]
C2		7.4 ± 0.1		8.1 ± 0.3		1.24 ± 0.01	1.23 ± 0.01 [<0.002]		21.4 ± 0.3	19.6 ± 0.3 [<0.003]
C3		7.2 ± 0.1		7.8 ± 0.3		1.22 ± 0.01	1.22 ± 0.01 [<0.002]		20.6 ± 0.3	19.1 ± 0.3 [<0.005]
C4		7.3 ± 0.1		8.0 ± 0.2		1.26 ± 0.01	1.25 ± 0.01 [<0.003]		22.1 ± 0.3	20.4 ± 0.3 [<0.002]
D1		7.4 ± 0.1		7.7 ± 0.3		1.23 ± 0.01	1.22 ± 0.01 [<0.002]		21.4 ± 0.3	19.6 ± 0.3 [<0.004]
D2		7.2 ± 0.1		7.6 ± 0.3		1.24 ± 0.01	1.23 ± 0.01 [<0.002]		21.6 ± 0.3	19.9 ± 0.3 [<0.005]
D3		7.5 ± 0.1		8.0 ± 0.2		1.25 ± 0.01	1.24 ± 0.01 [<0.002]		22.1 ± 0.3	20.2 ± 0.3 [<0.002]
B1	7.4	7.3 ± 0.1	7.8	7.7 ± 0.3	1.23	1.22 ± 0.01	1.21 ± 0.01 [<0.003]	19.6	22.4 ± 0.3	20.1 ± 0.3 [<0.003]
B2		7.5 ± 0.1		8.2 ± 0.3		1.23 ± 0.01	1.23 ± 0.01 [<0.001]		21.8 ± 0.3	19.8 ± 0.3 [<0.004]
B3		7.4 ± 0.1		7.6 ± 0.2		1.25 ± 0.01	1.24 ± 0.01 [<0.002]		22.0 ± 0.3	20.7 ± 0.3 [<0.005]
A1		6.0 ± 0.1		8.3 ± 0.3		1.03 ± 0.01	1.01 ± 0.01 [<0.002]		19.1 ± 0.3	17.7 ± 0.3 [<0.002]
M1		4.0 ± 0.1		6.6 ± 0.3		0.76 ± 0.01	0.73 ± 0.01 [<0.002]		43.6 ± 0.3	41.9 ± 0.3 [>0.1]
M2		4.4 ± 0.1		4.3 ± 0.2		0.78 ± 0.01	0.76 ± 0.01 [<0.002]		20.1 ± 0.3	17.4 ± 0.3 [<0.002]
A2		4.5 ± 0.1		8.8 ± 0.3		0.93 ± 0.01	0.93 ± 0.01 [<0.002]		24.4 ± 0.3	22.6 ± 0.3 [<0.01]

Precision is reported as standard deviation of the mean ($n = 5$; $\alpha = 0.05$); rms error (multiwavelength method) is reported in the brackets.

(thiophene or MEK) in the presence of the other three interferents, it was sufficient to provide the program with a standard spectrum of thiophene (or MEK), while those of the other three components (pure or as a mixture) have been processed as samples in which the analyte concentration was zero. This procedure leads to more accurate concentration values and, additionally, can reveal unexpected interferences by means of an high rms error.

Additionally, both the within- and the between-day repeatability of the method were very good even for the quantitative analysis of bitrex which, being the more complex, results in the largest relative standard deviation ($\leq$5%). It can be easily noticed from the comparison of these values that no significant difference between the within- and the between-days repeatability was observed.

Successively, the method was applied to quantitate the amount of each of the four components of Italian general denaturant in 14 commercial samples of denatured alcohol for skin disinfection (the results are reported in Table 4). Also in this case, the final results have been corrected by the alcoholic grade to be expressed as concentration in the corresponding volume of anhydrous alcohol.

When inspecting the results in Table 4, it can be observed that in most of the cases the recovered concentrations appear to be consistent with the values expected for well denatured samples. However, in the case of the last four samples in the table, even if the qualitative identification of all the denaturing components has been correctly performed, their concentration is significantly different than expected (the anomalous MEK concentration found in the sample M1 is almost certainly due to the presence of an interferent, as suggested by the large value of the rms error in the parallel multicomponent analysis). The previous validation studies have shown the method to be accurate and sufficiently precise on standard samples, so the measured concentrations reflect the actual composition of these four denatured alcohol samples under analysis. Therefore, the discrepancy between the prescribed and the recovered values for these latter four samples could be due either to imperfect denaturation or to degradation of the components due to bad conservation. At present, investigating the causes of this finding goes beyond the scopes of the present paper; however, we think that disposing of a simple, fast and accurate analytical method, in addition to the ascertaining of the conformity of the product to its specification, could be useful also for this purpose.

With this perspective, we think that this work could represent a contribution to the solution of both these problems.

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References

[1] EU Commission, Official J. Eur. Commun. L320 (1998) 27.
[2] M.E. Alvarez-Pineiro, M.J. Lopez de Alda-Villaizan, P. Paseiro-Losada, M.A. Lage-Yusty, J. High Res. Chromatogr. 20 (1997) 321–324.
[3] A. Stumpf, K. Tolvaj, M. Juhasz, J. Chromatogr. A 819 (1998) 67–74.
[4] A. Brega, P. Prandini, P. Villa, A. Quadri, G. Ital. Chim. Clin. 16 (1991) 175–179.
[5] M.J. Glover, A.J. Blake, Analyst 97 (1972) 891–896.
[6] J. Kovar, M. Loyer, J. Liq. Chromatogr. 7 (1984) 2163–2177.
[7] L-K. Ng, M. Hupe, J. Harnois, A.H. Lawrence, Anal. Chem. 70 (1998) 4389–4393.
[8] Y. Yang, D. Cheng, G. Zhang, J. Hou, Fenxi Ceshi Xuebao 15 (1996) 48–51.
[9] M. Aoki, Y. Iwayama, Yakugaku Zasshi 79 (1959) 522–526.
[10] E. Ruf, Z. Anal. Chem. 204 (1964) 344–355.
[11] G.V. Scott, Anal. Chem. 40 (1968) 763–768.
[12] I. Sheiham, T.A. Pinfold, Analyst 94 (1969) 387–388.
[13] A. Arpino, C. Ruffo, Riv. Ital. Sost. Grasse 53 (1976) 395–398.
[14] J. Waters, W. Kupfer, Anal. Chim. Acta 85 (1976) 241–251.
[15] L.K. Wang, D.F. Langley, Arch. Environ. Contam. Toxicol. 5 (1977) 447–456.
[16] V.P. Denisenko, V.S. Brovarets, Khim. Farm. Zh. 13 (1979) 109–111.
[17] J. Kawase, M. Yamanaka, Analyst 104 (1979) 750–755.
[18] H.P. Sagaster, G. Roebisch, Z. Chem. 22 (1982) 225–226.
[19] H. Hellmann, Fresenius' Z. Anal. Chem. 310 (1982) 224–229.
[20] T. Sakai, N. Ohno, Bunseki Kagaku 32 (1983) 302–307.
[21] H. Hellmann, Fresenius' Z. Anal. Chem. 323 (1986) 29–32.
[22] I. Kasahara, M. Kanai, M. Taniguchi, A. Kakeba, N. Hata, S. Taguchi, K. Goto, Anal. Chim. Acta 219 (1989) 239–245.
[23] M.M. Bonilla Simon, A.A. De Elvira Cozar, L.M. Polo Diez, Analyst 115 (1990) 337–339.
[24] E. Nakamura, K. Ishiwata, H. Namiki, Bunseki Kagaku 39 (1990) 845–847.
[25] M. Wu, Y. Liu, M. Zeng, Jingxi Huagong Zhongjianti 32 (2002) 55–57.
[26] S. Li, S. Zhao, Anal. Chim. Acta 501 (2004) 99–102.
[27] L. Sommer, J. Kucerova, H. Prochazkova, M. Hnilichova, Publ. Fac. Sci. Univ. Purkyne, Brno 464 (1965) 249–277.
[28] E. Chiacchierini, V. Petrone, A.L. Magrì, Gazz. Chim. Ital. 105 (1975) 205–220.
[29] H.K. Biswas, B.M. Mandel, Anal. Chem. 44 (1972) 1636–1640.
[30] R. Bucci, A.D. Magrì, A.L. Magrì, Fresenius' J. Anal. Chem. 362 (1998) 577–582.