

TECHNIQUE IMPROVEMENT OF ETHANOL CHROMATOGRAPHING AND ITS DETECTION IN BIOLOGICAL FLUIDS AND AIR

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Purpose. To have elaborated the accurate and reproducible techniques of extraction, purification and qualitative assay for ethanol qualitative analysis in biological fluids (urine) and ambient air (out of working area) by means of gas-liquid chromatography (GLC) and liquid chromatography (LC) methods. **Methodology.** For choice of the optimum gas-carrier velocity, we have prepared a test reagent mixture. To this end, we have used alkyl nitrite technique of ethanol detection. We have applied a gas-liquid chromatography and liquid chromatography method for qualitative determination of ethanol in biological fluids and air. **Results.** We have taken urine from drunken person then have adjusted specimen to pH 9.0 by addition of 20% solution of Na₂CO₃ and have extracted ethanol by chloroform two times in 10 mL. Besides, we have filled an aspirator (or rubber bulb) with fifty liters of contaminated air with ethanol taken from working area. We have carried out the gas-liquid chromatography and the liquid reversed-phase chromatography for the extracts analyses out of urine and research of contaminated air. But formerly, we have calculated the graphic curves for selecting the optimal gas-carrier velocity using the dependencies based on the theory of gas-carrier velocity in GLC method. We have experimentally ascertained that the duration of LC analysis (1 min and 36 s) of urine (or of air) polluted with ethanol is at least two time less than it was for LGC analysis (4 min and 15 s). **Originality.** For the first time, we have elaborated the accurate and reproducible techniques of extraction, purification and qualitative assay for ethanol qualitative analysis in biological fluids (urine) and ambient air (out of working area). **Practical value.** The obtained results are well-reproducible, both introduced techniques are accurate, sensitive and demonstrative; the methods of LGC and LC analyses can be successfully used to detect ethanol in biological fluids and ambient air. *References 15, tables 1, figures 2.*

Keyword: detection technique, ethanol, biological fluids, ambient air, chromatography

УДОСКОНАЛЕННЯ МЕТОДИКИ ХРОМАТОГРАФУВАННЯ ЕТИЛОВОГО СПИРТУ ТА ЙОГО ВІЯВЛЕННЯ В БІОЛОГІЧНИХ РІДИНАХ І ПОВІТРІ

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Зважаючи на неабияку важливість екологічної хімії етанолу і факти, що найбільша кількість (90%) судово-хімічних експертиз стосується отруєнь етанолом, в статті представлені результати розробки надійних і відтворюваних методик виділення, очистки і якісного аналізу етилового спирту в біологічних рідинах і в повітрі за допомогою газо-рідинної (ГРХ) й рідинної хроматографії (РХ). Залежності, що ґрунтуються на теорії швидкостей газо-носіїв у методі ГРХ, дали змогу обчислити графічні криві для вибору оптимальної швидкості газо-носія. Експериментально встановлено, що тривалість РХ аналізу (1 хв. 36 с) стосовно визначення наявності домішки етилового спирту в сечі (чи повітрі) є, щонайменше вдвічі менша, ніж для ГРХ аналізу (4 хв. 15 с).

Ключові слова: методика виявлення, етиловий спирт, біологічні рідини, навколишнє повітря, хроматографія.

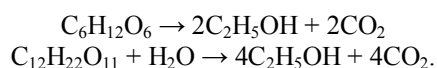
PROBLEM STATEMENT. Alcohol-containing beverages were known to human being more 4000 years ago. However, the first person who discovered pure alcohol (*spiritus*) by distilling wine was a well-known Persian (Iranian) alchemist by the name of

Muhammad ibn Zakariya al-Razi (854–925 CE). Afterwards, (1334 CE) alchemist Ramon Llull has obtained *aqua vino* – brandy. He was so amazed by its properties that he has named this discovery by doomsday harbinger, mainly, because of specific

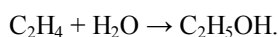
influence on a human body. This property [1–3] of drinking alcohol is first of all caused by physical and chemical features of ethanol. Ethanol, also commonly called ethyl alcohol, drinking alcohol, or simply alcohol is the principal type of alcohol found in alcoholic beverages, produced by the fermentation of sugars by yeasts. It is a neurotoxic [4] psychoactive drug and one of the oldest recreational drugs used by humans. It can cause alcohol intoxication when consumed in sufficient quantity. Besides that, ethanol is a volatile, flammable, colorless liquid with a slight chemical odor. Consequently, it is used as an antiseptic, a solvent, a fuel, and, due to its low freezing point, the active fluid in post-mercury thermometers. The molecule is a simple one, being an ethyl group linked to a hydroxyl group. Its structural formula, $\text{CH}_3\text{CH}_2\text{OH}$, is often abbreviated as $\text{C}_2\text{H}_5\text{OH}$ or EtOH .

Ethanol is an important industrial ingredient. It has widespread use as a precursor for other organic compounds such as ethyl halides, ethyl esters, diethyl ether, acetic acid, ethyl amines ethylene and so forth. However, due to its thermal and environmental flammability index [5, 6] ethanol is utilized as an engine fuel and fuel additive. Ethanol as a fuel is not a source of greenhouse gases. It has a zero balance of carbon(IV) oxide. According to an industry advocacy group, ethanol as a fuel reduces harmful tailpipe emissions of carbon monoxide, particulate matter, oxides of nitrogen, and other ozone-forming pollutants. Argonne National Laboratory analyzed greenhouse gas emissions of many different engine and fuel combinations. According to their results, biodiesel/petrodiesel blend (B20) showed a reduction of 8%, conventional E85 ethanol blend a reduction of 17% and cellulosic ethanol – 64%, when compared with pure gasoline.

Currently, there are two basic ways to ethanol production [7]; it is a microbiological process and synthetic method [8]. On the first way, ethanol in alcoholic beverages and fuel is produced by fermentation. Certain species of yeast (*e.g.*, *Saccharomyces cerevisiae*) metabolizes sugar producing ethanol and carbon(IV) oxide. The chemical equations below summarize the conversion:



On the second way, ethanol for use as an industrial feedstock or solvent (sometimes referred to as synthetic ethanol) is made from petrochemical feed stocks, primarily by the acid-catalyzed hydration of ethylene:



The catalyst is most commonly phosphoric acid, adsorbed onto a porous support such as silica gel or diatomaceous earth. The reaction is carried out in the presence of high pressure steam at 300°C where a 10:6 ethylene to steam ratio is maintained. But, synthetic ethanol that has been produced indirectly by means of concentrated sulfuric acid forming intermediate ethyl sulfate, which was next hydrolyzed to yield ethanol and

regenerate the sulfuric acid turned out toxic to human. Therefore synthetic ethanol that is manufactured in many countries is denatured by adding various substances such as fuchsine, phthalic acid esters, formaldehyde, higher alcohols and others. According to the Pharmacological Committee demands, only that ethanol which is produced from food raw material may be employed in medicine.

Ethanol in the little amounts (4–5 g per day) permanently penetrates into the human body along with some food. In particular, ethanol content in yogurt, mare's milk, kvass and wheat bread amount to 0.003–0.005, 1.0–4.5, 0.6–2.2 and 0.40–0.45 % (V/V), respectively [9]. In the blood of any sober person there are always traces of ethanol formed as a result of biochemical processes. This is the so-called endogenous ethanol. The origin source of ethanol is acetaldehyde forming by pyruvic acid decarboxylation. The possibility of ethanol formation by intestinal microflora and respiratory tracts also must not be ruled out. Normative level of alcohol in the organism of a healthy person amounts to 0.018–0.030 % by weight of the human body [10].

Unlike eating, alcohol does not require digestion. It is easily absorbed by means of digestive tract, coming to blood and lymph. Brain cells whose weight amounts to only 2% of body weight absorb approximately 30–40 % of all consumed alcohol. The liver neutralizes a considerable part of alcohol. Remainders come out through the kidneys, lungs and sudoriferous glands. Alcohol is able to remain in the body for a very long time (20 days or more).

Since ethanol is widely used in everyday life and industry as well as it plays an important biological role in vital activity of organisms, therefore its detection is a mandatory procedure of the forensic-chemical examinations. That is why sensitive, rapid and reliable methods for the qualitative detection of ethanol are extremely required.

The largest number of the forensic-chemical examinations falls on ethanol poisoning (near 90%) [11]. There are described techniques of ethanol detection starting from reaction of iodoform formation; the sensitivity of this reaction is 0.04 mg in 1 mL of solution, but it is not specific. Ethanol can be detected by reaction of ethyl acetate formation. Here, detection limit amounts 15 µg of ethanol in 1 mL of solution (methanol interferes with ethanol detection) [12]. The interaction of ethanol with benzoyl chloride makes for ethyl benzoate formation that has the characteristic odor (the detection sensitivity is 2 mg in 1 mL of solution [12]. Ethanol is oxidized by potassium bichromate or permanganate till acetaldehyde which then is determined by reaction of sodium nitroprusside with morpholine (blue, sensitivity is 1 µg in sample) [13]. In this respect, the method of gas-liquid chromatography (GLC) prevails over different methods of ethanol detection [14, 15]. Nevertheless, final information about ethanol detection needs to be done over all reactions set taking into consideration their specificity and sensitivity.

Nowadays, GLC methods are widely used at the qualitative and quantitative analysis of ethanol that is

isolated out of biological materials, biological fluids, working air, and foodstuffs. For example, 0.3 % of ethanol that is detected by GLC method in a person blood testifies to absence of intoxication; such its quantity appears in an organism owing to fermentation process. It is known, that in GLC method a gas is mobile phase whereas a liquid is stationary phase. At the heart of chromatographic fractionation lies the process of the compounds distribution in a mixture between a gas mobile phase and a liquid stationary phase which in the form of thin film is coated on the solid carrier possessing the big specific surface.

However, a liquid chromatography (LC) is more up-to-date method. Here, a liquid is mobile phase while a solid substance or a liquid applied on the solid carrier modified by means of organic groups is stationary phase.

Purpose of work. Considering all above-stated, we have undertaken an attempt to elaborate a reliable technique for ethanol qualitative analysis in biological fluids (urine) and ambient air (out of working area) by means of GLC and LC methods.

EXPERIMENTAL PART AND RESULTS OBTAINED. *Preparing of the test mixture.* For choice of the optimum gas-carrier velocity, it was necessary to prepare a test reagent mixture. For this purpose, alkyl nitrite technique of ethanol detection was used. 0.5 mL of a 50% of solution of trichloroacetic acid and 1 mL of a 2% solution of ethanol have been put into a flask and closed up by rubber cork. Then the flask has been fastened on the holder; its contents have been stirred and the 0.5 mL of a 30% solution of sodium nitrite has been injected by means of syringe. It once again was thoroughly stirred and left for a 1 min to obtain the test mixture of alkyl nitrites. Ready test mixture has been utilized for selection of GLC analysis conditions.

Extracting of ethanol out of urine. At first the standard solution is prepared by means of adding 2 mL of ethanol toward 50 ml of person urine. Next the 50 mL of urine was sampled from drunken person; specimen was left for the one day. Later, all specimens were adjusted to pH 9.0 by addition of 20% solution of Na_2CO_3 and were extracted by chloroform two times in 10 mL. Each of the two types (standard solution and urine) of chloroform extracts were combined and chloroform was evaporated up to 1 mL [14]. These solutions were utilized for chromatographic analysis.

Sampling of contaminated air. Fifty liters of contaminated air with ethanol is taken from working area using aspirator (or rubber bulb). Gathered certain quantity of air was examined by means of GLC and LC methods.

Chromatographic analysis. The extracts analyses out of urine and air research have been carried out by methods of both gas-liquid chromatography and of the reversed-phase chromatography for liquid.

Gas-liquid chromatography: chromatograph of the Chrom-5 model equipped by thermal conductivity detector referred to as katharometer (amperage of Wheatstone's bridge amounts to 0.09 A), metal chromatographic column (200×0.3 cm) with the solid support Chromaton N-AW (0.16–0.20 mm) was exploited at the following conditions of measurement: a

liquid stationary phase (Squalan, 15%), a gas-carrier (N_2), volumetric gas-carrier velocity ($F_{\text{vol.}} = 30 \text{ mL}\cdot\text{min}^{-1}$), vaporizer temperature (75°C), column temperature (70°C), and detector temperature (75°C).

Reversed-phase chromatography: the liquid chromatograph of the Cvet-304 model with ultra-violet detector ($\lambda = 254 \text{ nm}$) and chromatographic column made of stainless steel (10×0.4 cm) was used. The sorbent is a silica gel C-3 ($S = 260 \text{ m}^2\cdot\text{g}^{-1}$) with modified *n*-alkyl-chains C_{16} (particle size of a silica gel are 10 μm). The eluent is a mix of the isopropyl alcohol and water (at a ratio of 35 to 65) with added ammonia aqueous solution (0.25 %). The elution rate, pressure, and thermostat temperature are $1 \text{ cm}^3\cdot\text{min}^{-1}$, 40 atm, and 50°C , respectively.

Selection of conditions for GLC analysis. The time interval of GLC analysis amounts a few minutes owing to rapid reaching of equilibrium between phases. Characteristic qualitative parameters in GLC are retention time and retention distance. Quantitative GLC analysis is carried out by means of measuring peak's parameters (height and area) (Figure 1). Fast response of GLC method as well as elementary construction of thermal conductivity detector makes possible the determination of substance at trace amount (0.01%).

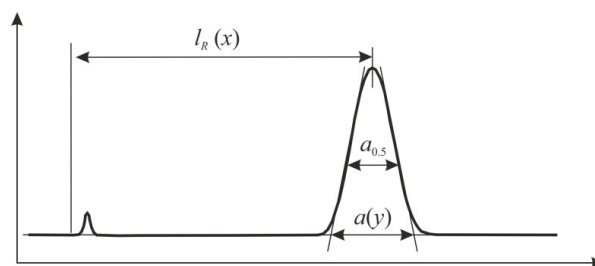


Figure 1 – Valuation of peak's parameters at a chromatogram

To elaborate the gas chromatography techniques of ethanol detection we have carried out investigations at the selection of chromatographic analysis conditions (gas-carrier rate selection *etc.*). Discrimination or effectiveness of chromatographic column is characterized by number of theoretical plates (N) that can be evaluated according to the formula:

$$N = 16 \left(\frac{l_R}{a} \right)^2 = 5.54 \left(\frac{l_R}{a_{0.5}} \right)^2 \text{ or } N = 16 \left(\frac{x}{y} \right)^2,$$

where l_R (or x) [mm] is interval from line of bringing in sample to point of maximal peak; a (or y) [mm] is width of peak in base; $a_{0.5}$ [mm] is half-width of peak.

Number of theoretical plates is calculated using value of t_R :

$$N = 16 \left(\frac{t_R}{rv} \right)^2 = 5.54 \left(\frac{t_R}{r_{0.5}} \right)^2,$$

$$r_{0.5} = \frac{a_{0.5}}{B}; \quad rv = \frac{a}{B},$$

where t_R [s] is retention time for substance; B [$\text{mm}\cdot\text{s}^{-1}$] is rate of chart-ribbon motion.

Column efficiency can be expressed through height that is equivalent to the number of theoretical plates (h). It displays which portion of column length accounts for one plate; h [arb. u.] can be described by a formula:

$$h = \frac{L}{N},$$

where L [cm] is column length; N is the number of theoretical plates in column.

Based on the obtained results, the dependence of h from linear velocity of carrier gas has been plotted (Figure 2). The linear velocity of gas-carrier (μ [cm·s⁻¹]) is determined by the ratio:

$$\mu = \frac{L}{t_R'}$$

where t_R' [s] is retention time of gas-carrier.

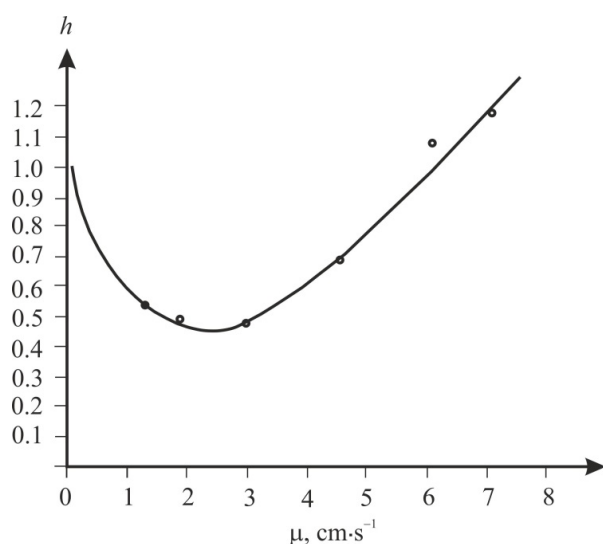


Figure 2 – Relationship between h and μ

According to this plot, we determine the gas-carrier rate whose value specifies the efficiency of separation. For that we use the previously prepared test mixture: 2 mL of alkyl nitrites vapors we draw out of flask by means of syringe and inject to chromatograph. Next, we determine retention parameters for ethyl nitrite of the test mixture, gradually altering the volumetric velocity of gas-carrier. These retention parameters we used for computation of h and the $h - \mu$ diagram constructing (Table).

Data of Table and Figure 2 testifies that optimal rate (μ) correspond to minimum on the $h - \mu$ curve, where chromatographic column operates a most effectively. However, owing to ability of gas to compressing, a linear velocity of gas-carrier is non-constant along the full length of chromatographic column. Therefore, the maximal operating efficiency takes place only at a little part of this column. As a matter of fact, when we perform analysis using the velocities that exceed the optimal value we in such a way save GLC examination time and at the same time practically do not influence onto h value.

Ethanol detection in urine and contaminated air using GLC technique. A flask has been filled 0.5 mL of either chloroform extracts (either from standard or examinee solution) or 2 mL of contaminated air out of aspirator. Next, 0.5 mL of solution of trichloroacetic acid has been added into the flask. The contents of a flask closed up by rubber cork have carefully been stirred and 0.5 mL of sodium nitrite solution has been injected by syringe. It has again been stirred and left for a 1 min. The ethyl nitrite vapors (2 mL) produced from ethanol have been sampled and injected in chromatograph. After chromatogram recording, the retention parameters of ethyl nitrite have been specified in the chloroform extracts out of urine and in the samples from contaminated air. These results compare with corresponding parameters of the standard solution. In case of the parameters coincidence we can draw a conclusion about ethanol presence in the urine or air. On the contrary, absence of peaks on the chromatogram of investigated material gives grounds for asserting that samples have not ethanol.

Table – Choice of optimal rate of the gas-carrier (column length – 100 cm)

μ [cm·s ⁻¹]	t_R (air) [s]	t_R (ethyl nitrite) [s]	x [mm]	y [mm]	N	h
1.30	75.0	105	28.0	8.2	186.08	0.54
1.92	52.0	107	21.5	6.0	205.12	0.49
3.00	33.0	87	13.0	3.6	207.40	0.48
4.55	22.0	43	9.0	3.0	144.00	0.69
6.10	16.5	32	6.0	2.5	92.20	1.08
7.14	14.0	26	7.0	3.0	85.00	1.18

Thus, in desired conditions of GLC analysis, ethanol retention time in standard solution comes to 4 min 05 s while one in the chloroform extracts out of urine or in the samples of contaminated air amounts to 4 min 15 s. It is necessary to notice that peaks differ among themselves only by the area values.

LC detection of ethanol. We have also the adapted technique of ethanol detection by reversed-phase chromatography method. Either 0.5 ml of a chloroform extract out of urine or 2 ml of air from an aspirator are necessary to the carrying out analysis. The same chemical agents that used at the accomplishment of GLC analysis have been added to the examined sample. We records chromatogram under indicated condition of LC analysis. The ethanol retention time for standard solution comes to 1 min 32 s, and for the chloroform extracts out of urine as well as for the samples of contaminated air this parameter amounts to 1 min 46 s.

CONCLUSIONS. The results presented in the article may be summarized as follows:

1. Data analysis relating to ecological chemistry of ethanol, its influence on human organism and detection modes has been scrutinized.

2. The ethanol extraction and its detection techniques in biological fluids (urine) and contaminated air (working range) with application of LGC and LC methods have been elaborated and adapted for qualitative analysis realization.

3. It has been ascertained that duration of LGC analysis is almost twice as long as it is for LC analysis (4 min 15 s by comparison with 1 min 36 s).

4. The obtained results are well-reproducible, both introduced techniques are accurate, sensitive and demonstrative; the methods of LGC and LC analyses can be successfully used to detect ethanol in biological fluids and ambient air.

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УСОВЕРШЕНСТВОВАНИЕ МЕТОДИКИ ХРОМАТОГРАФИРОВАНИЯ ЭТИЛОВОГО СПИРТА И ЕГО ВЫЯВЛЕНИЕ В БИОЛОГИЧЕСКИХ ЖИДКОСТЯХ И ВОЗДУХЕ

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Учитывая огромную важность экологической химии этилового спирта и факта, что наибольшее количество (90%) судебно-химических экспертиз касается отравления этиловым спиртом, в статье представлены результаты разработки надежных и воспроизводимых методик выделения, очистки и качественного анализа этилового спирта в биологических жидкостях и в воздухе с помощью газожидкостной (ГЖХ) и жидкостной хроматографии (ЖХ). Зависимости, основанные на теории скоростей газ носителя в методе ГЖХ, позволили рассчитать графические кривые для выбора оптимальной скорости газ носителя. Экспериментально установлено, что продолжительность ЖХ анализа (1 мин. 36 сек.) по определению наличия примеси этилового спирта в моче (или воздухе) является, по крайней мере, в два раза меньше, чем для ГЖХ анализа (4 мин. 15 сек.).

Ключевые слова: методика выявления, этиловый спирт, биологические жидкости, окружающий воздух, хроматография.