Gas-chromatographic Method for Volatile Congeners in Alcoholic Beverages Analysis

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Alcoholic fermentation of fruits and cereals is the primary source of alcoholic beverages production. The main component in these drinks is ethanol; but there are also many secondary products like glycerin or acetaldehyde, and mixtures of other higher-order alcohols called "fusel", also known as congeners. They are important for the characterization of alcoholic beverages and also for forensic medicine purposes, when drivers assertions concerning alcoholic beverages consumption have to be verified in order to prosecute them for driving under influence. We developed a gas-chromatographic method of qualitative and quantitative analysis of alcohol congeners in our toxicology laboratory. The method was validated in terms of linearity, precision, accuracy and detection limits. Our findings are consistent with other from the literature relative to this type of analytical determinations.

Key-words: alcohol congeners, gas-chromatography, validation

The ethanol from alcoholic beverages, in small doses is a stimulant of the digestive functions, a weak respiratory analeptic and partially substitutes some metabolites for interchange and thermogenesis. In high doses the toxic effect of alcohol is manifested primarily through the central nervous system depression. The alcohol intoxication is more serious as its concentration in blood is higher. [1-3]

Alcoholic beverages are obtained through chemical processes that occur during alcoholic fermentation. Beside the main product (ethanol), many secondary products result like acetic aldehyde, glycerin and a mixture of higher-order alcohols called "fusel".

Compounds of "fusel" are known as congeners. The term "congener" includes many substances: carbohydrates, tannins, phenols, metals, dyes, vitamins, minerals, histamine and other pharmacologically active agents. Congener content of commercial alcoholic beverages differs significantly depending on the type of beverage [1, 4]. Efforts have been made in order to investigate unstable phenolic compounds on derivatized (silylated) red wines samples using a GC/MS system [5].

Within the forensic toxicology field determination of congeners is a helpful tool in evaluation of drivers declarations prosecuted for driving under influence of alcohol concerning the type and amount of alcoholic beverage ingested. In Romania gas-chromatographic method for alcohol analysis is still not the routine, but an ongoing process of modernization is running based on research activities [6, 7].

The purpose of our researches is to develop and validate a gas chromatographic method for the identification and measurement of volatile congeners present in alcoholic beverages.

Experimental part

Material and method

Instrument: "Konica-HRGC 4000 B" GC

- gas chromatograph has been used for all gas chromatographic measurements.

Detector: flame ionization detector (FID)

Column: capillary column "Carbowax"-2.0μm – 30m - 0.53mm ID

Program: 70^0 C (5 min), 5^0 C/min to 110^0 C (5 min)

Carrier gas: nitrogen-flow 10 mL/min

Combustion gas: air –flow 220 mL/min
Hydrogen-flow 39 mL/min
Make up: nitrogen – flow 25 ml/min

Injection mode: split ratio: 5/1 ; 1μL

Injector: 250^0 C

Detector Temperature: 260^0 C

Results were registered and processed using a data acquisition system "Hewlet Pakard" - with a DDS Clarity Master software package.

Reagents:

Absolute ethanol – provided by Merck KGaA –Darmstadt, Germany;

Methanol– provided by Merck KGaA –Darmstadt, Germany;

1- butanol – provided by Merck KGaA –Darmstadt, Germany;

2- butanol – provided by Merck KGaA –Darmstadt, Germany;

Isobutanol – provided by Merck KGaA –Darmstadt, Germany;

2-methyl 1- butanol – provided by Merck KGaA –Darmstadt, Germany;

Acetone – provided by Merck KGaA –Darmstadt, Germany;

N-propanol-provided by Sigma-Aldrich Laborchemikalien GmbH,Germany;

Distilled water;

Samples of alcoholic beverages: spirits type: vodka, brandy and other spirits.

Samples were prepared in concentrations recommended in the Official Journal of the European Community [8]. The method was evaluated on the basis of precision, accuracy, linearity, specificity, limit of detection and quantification [9, 10].

Results and discussions

The identification of volatile congeners based on their retention time is represented in figure 1. The quantitative

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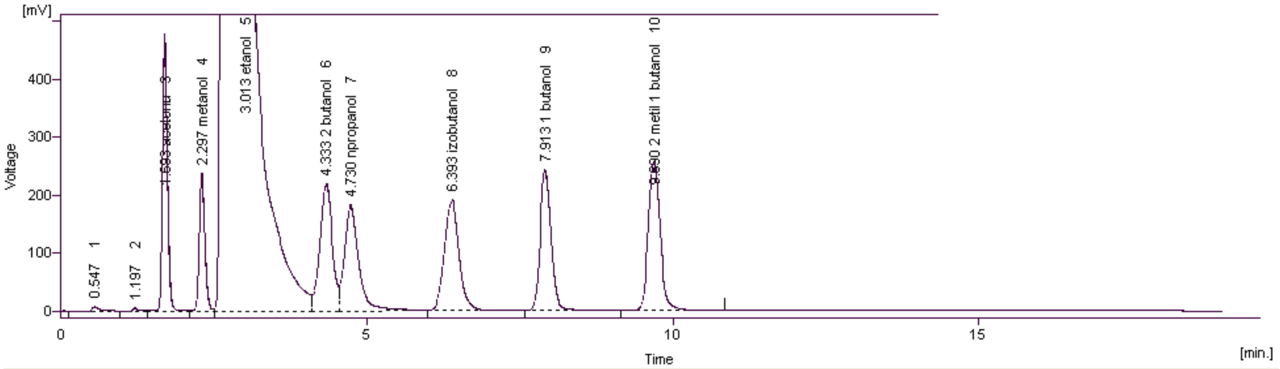

Fig. 1 Chromatogram of the volatile congeners identification

Table 1
VOLATILE CONGENERS CONCENTRATIONS (mg/dl) AND THE CORRESPONDING FID RESPONSES IN mV

Methanol		Acetone		Isobutanol		n-propanol	
Response (mV)	Conc. mg/dl	Response (mV)	Conc. mg/dl	Response (mV)	Conc. mg/dl	Response (mV)	Conc. mg/dl
86.3852	2.37	91.9059	2.37	147.4395	2.40	232.2241	2.40
149.7962	7.12	269.8583	7.11	406.8614	7.22	506.2007	7.22
335.2538	11.88	577.0282	11.85	717.1882	12.03	798.3434	12.03
748.0686	23.76	1371.9033	23.70	1673.0083	24.06	1649.4780	24.06
1001.2211	35.64	1726.1465	35.55	2179.3416	36.09	2084.7178	36.09
1236.2663	47.52	2148.0268	47.40	2645.2264	48.12	2548.8718	48.12
1-butanol		2-butanol		2-metil1-butanol			
Response mV	Conc. mg/dl	Response mV	Conc. mg/dl	Response mV	Conc. mg/dl		
91.1054	2.43	228.1340	2.43	132.8835	2.46		
356.3160	7.29	453.4533	7.29	391.2781	7.38		
664.3057	12.15	839.3206	12.15	741.2180	12.30		
1537.1497	24.30	1673.8691	24.30	1741.0882	24.60		
2063.5197	36.45	2049.5820	36.45	2320.4182	36.90		
2531.2211	48.60	2443.9184	48.60	2812.3356	49.20		

Table 2
LINEAR REGRESSION EQUATION, CORRELATION FACTOR (R), DETERMINATION FACTOR (R^2)

Volatile congener	Linear equation	**Correlation factor**	**Determination factor**
Acetone	Y = 1108.99* X+41.9551	0,9902	0,9805
Methanol	Y = 629.70* X+31.34143	0,9942	0,9885
2 butanol	Y = 1190.73* X+219.641	0,9860	0,9723
N propanol	Y = 1258.33* X+161.286	0,9921	0,9843
1 butanol	Y = 1333.82* X-1.10314	0,9937	0.9874
Isobutanol	Y =1381.37 * X+38.9774	0,9914	0,9830
2-metil 1- butanol	Y = 1446.51*X+71.2455	0,9924	0,9849

evaluation of these results is presented in table 1. The linearity regression data was determined, and the results are presented in table 2.

The congeners concentration in an unknown sample (uncertainty – response/concentration correlation) is presented in table 3. Statistical data for the analysis from unknown concentration samples are presented in table 4. Accuracy and precision are both performance criteria, the results being presented in table 5 and 6 respectively.

We established the limit of detection for each congener: 0.67ug/L (acetone), 1.02ug/L (methanol), 0.37ug/L (2-butanol), 0.76ug/L(1-propanol), 1.30ug/L (isobutanol), 0.64ug/L (1-butanol) and 1.43 ug/L (2-methyil 1 -butanol).

We obtained specific retention times for each congener, with no interference from other excipients. The lack of intereferneces is important for an analytical method to be considered specific [11].

The correlation coefficient values (R) presented in table 2 indicate a very strong association between variables. The determination factor (R^2) shows a good linear association between variables X and Y [12, 13].

In gas chromatography the peak area is expressd in mV while the peak height expresses the analyte concentration. Such signals are obtained at different concentrations using standard calibration solutions [14].

Table 2 contains the equations of linear regression for each congener, in an equation Y = aX. + b, where a is the intercept while b is the slope [15].

If we take the example of acetone which has a determination coefficient of R^2=0. 9885, meaning that 98.85% of the total variation of Y can be explained by a linear relationship between X and Y, while the remaining 1.15% of the variation continues to be unexplained. We can find similarities for other congeners as well: methanol, 2-butanol, n-propanol, 1-butanol, isobutanol, 2-methyl 1-butanol.

Table 7 contains the following statistical data: the intercept **a**, slope **b**, with standard deviations $\mathbf{s_b}$, standard error of regression $\mathbf{s_{y/x}}$, **F**-Fischer function and were calculated using the LINEST function in Microsoft Office Excel.

The number of degrees of freedom (n – 2) was determined by calculations of two parameters, namely slope b and intercept a as seen in table 7.

F-statistic as we see in table 7 is significantly bigger than 1; if we take the example of acetone which is the

Table 3
VOLATILE CONGENERS CONCENTRATIONS IN A UNKNOWN CONCENTRATION SAMPLE (mg/dL)

Methanol		Acetone		Isobutanol		n-propanol	
Response (mV)	Conc. mg/dl	Response (mV)	Conc. mg/dl	Response (mV)	Conc. mg/dl	Response (mV)	Conc. mg/dl
280.976	9.73	475.864	9.92	535.996	8.26	535.967	6.82
284.572	9.86	470.562	9.81	578.791	9.01	530.585	6.71
268.449	9.26	455.084	9.49	539.760	8.32	504.473	6.21

1-butanol		2-butanol		2-metil 1-butanol	
Response (mV)	Conc. (mg/dl)	Response (mV)	Conc. (mg/dl)	Response (mV)	Conc. (mg/dl)
510.275	9.06	461.499	5.30	582.565	9.04
565.798	10.08	487.100	5.82	625.164	9.69
516.408	9.17	455.173	5.17	579.603	8.99

Table 4
STATISTICAL DATA OF UNKNOWN CONCENTRATION SAMPLE

Statistical functions	Acetone	Methanol	2-butanol	n-propanol	isobutanol	1-butanol	2-metil 1 butanol
Unknown concentration	9.51	9.80	6.46	7.45	8.81	9.51	9.52
Standard deviation	1.80	1.46	2.33	1.80	1.68	1.46	1.62
I.C.95%	7.72	6.30	10.02	7.72	7.23	6.30	6.70

Table 5
ACCURACY – PERFORMANCE CRITERION

Acetone (47.40mg/dl)	**Metanol (47.52 mg/dl)**	**2-butanol (48.60 mg/dl)**	**Npropanol (48.12 mg/dl)**	**Izobutanol (48.12 mg/dl)**	**1-butanol (48.60 mg/dl)**	**2-metil 1-butanol (49.20 mg/dl)**
41.873	46.772	49.256	49.146	47.109	47.863	47.898
44.716	46.374	49.860	48.126	48.691	48.598	49.425
45.741	46.022	49.975	48.732	50.121	49.370	50.899
37.524	40.898	42.449	41.885	42.623	42.932	43.857
49.804	49.692	54.830	53.014	54.365	53.574	55.533
39.168	44.050	45.713	45.538	44.190	44.480	44.827
43.482	50.209	52.808	52.377	51.058	52.212	52.408
44.739	47.290	46.756	49.780	47.410	44.727	43.179
44.324	46.585	49.867	47.853	47.584	47.616	48.971
44.425	49.943	52.419	52.373	51.968	51.808	52.766
Mean						
43.5796	46.7835	49.3933	48.8824	48.5119	48.318	48.9763
Accuracy						
3.82	**0.74**	**0.79**	**4.25**	**0.39**	**0.29**	**0.23**
CP precision 10%						
4.74	**4.752**	**4.86**	**4.812**	**4.812**	**4.86**	**4.92**
Acceptance						
yes	**Yes**	**yes**	**yes**	**yes**	**Yes**	**yes**

Table 6
PRECISION – PERFORMANCE CRITERION

Acetone (47.40mg/dl)	**Methanol (47.52 mg/dl)**	**2-butanol (48.60 mg/dl)**	**N-propanol (48.12 mg/dl)**	**Isobutanol (48.12 mg/dl)**	**1-butanol (48.60 mg/dl)**	**2-metil-1-butanol (49.20 mg/dl9**
41.873	46.772	49.256	49.146	47.109	47.863	47.898
44.716	46.374	49.860	48.126	48.691	48.598	49.425
45.741	46.022	49.975	48.732	50.121	49.370	50.899
37.524	40.898	42.449	41.885	42.623	42.932	43.857
49.804	49.692	54.830	53.014	54.365	53.574	55.533
39.168	44.050	45.713	45.538	44.190	44.480	44.827
43.482	50.209	52.808	52.377	51.058	52.212	52.408
44.739	47.290	46.756	49.780	47.410	44.727	43.179
44.324	46.585	49.867	47.853	47.584	47.616	48.971
44.425	49.943	52.419	52.373	51.968	51.808	52.766
Mean						
43.5796	46.7835	49.3933	48.8824	48.5119	48.318	48.9763
Standard deviation σ_r						
3.437	**2.854**	**3.649**	**3.401**	**3.538**	**3.551**	**4.099**
2.8x σ_r						
9.626	7.992	10.217	9.522	9.906	9.940	11.477
RSD%						
7.888	6.101	7.389	6.958	7.294	7.349	8.369
CP precision 10%						
4.74	**4.752**	**4.86**	**4.812**	**4.812**	**4.86**	**4.92**
Aceptance						
yes	**yes**	**yes**	**yes**	**yes**	**yes**	**yes**

Table 7
RESULTS OF STATISTICS PROCESSING WITH CI 95%

Statistic fuctions	Acetone	Methanol	2-butanol	n-propanol	isobutanol	1 butanol	2metil-1 butanol
Slope b	47.48	26.95	51.32	53.44	57.30	54.58	59.99
Intercept a	15.45	14.04	136.11	125.15	46.35	11.61	24.15
Standard deviation s_b	2.81	1.30	3.84	3.12	3.13	2.57	3.10
Standard deviation s_a	69.47	32.15	97.45	78.22	78.51	65.17	79.48
F-statistic (Fisher)	284.84	430.91	177.83	293.37	334.75	449.66	374.39
$SS_{regresi}$	4348954	1408898	5343033	5678621	6527224	6041693	7481361
Standard deviation of regresion $s_{y/x}$	123.563	57.18	173.33	139.12	139.63	115.91	141.35
Degree of freedom n-2	5	5	5	5	5	5	5
$SS_{rezidual}$	76339.1	16347.67	150222.8	96781.04	97493.7	67179.6	99912.63

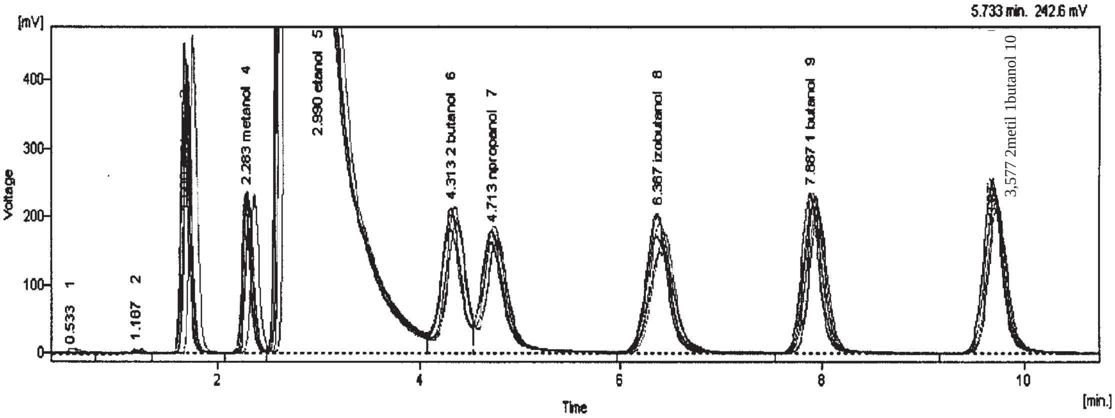

Fig. 2 Chromatograms overlay

F = 284.84, this means that the regression is significant and there is a linear relationship between dependent and independent variable [15].

The limit of detection was determined by performing measurements on increasingly diluted solutions until we obtained results that satisfy the criteria; as the concentration of the analyte gives a signal, which is significant enough to be detectable in comparison with the signal of a blank [16].

Determinations based on the statistical method is not suitable for gas chromatography because in this case to measure by the signal-noise method "and this only defines the ability to measure nothing" [16, 17] instead of measuring the lowest concentration. In general in gas chromatography the limit of detection is calculated with a response signal / noise ratio of 2 or 3.

The limit of quantification shows the smallest amount of congeners analyzed and measured with reasonable accuracy; statistically is 10 times the standard deviation of the detection limit [17].

Accuracy was demonstrated by performing a large number of congeners determinations of solutions of a certain level and is characterized by the systematic error, expressed as a mean difference between values obtained by repeated measurements and the real value. Figure 2 shows a good overlay of the chromatograms obtained from a large number of tests performed on the same solution. The chromatogram also reveals the specificity of the method, bit-sites appear to be well defined, no interference is observed, and symmetrical shape of the peaks is present.

To determine the measurement uncertainty we analyzed a sample of unknown concentration of congeners, and after three measurements we obtained the results presented in table 3.

The relationship between accuracy and precision is featured in figure 3: a high level of accuracy and precision does not necessarily imply high accuracy because if overcrowding results are present in one direction even if the chart indicates that high level results are away from the target point [12, 15]. Thus to prove the accuracy of the method a greater number of determinations on the same solution at concentrations of congeners was performed and standard deviation was calculated.

Repeatability was demonstrated by performing a set of measurements by the same analyst, on the same

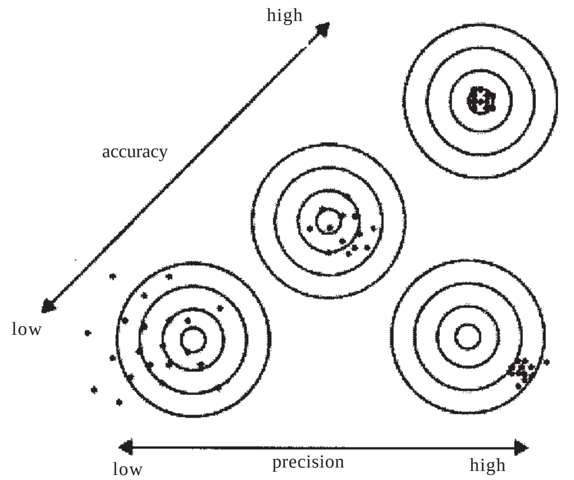

Fig. 3 Relationship between accuracy and precision [8]

equipment under the same conditions on samples of known concentration. After setting a reasonable standard deviation two routine measurements were repeated and standard deviation obtained from this two measurements were compared with the repeatability standard deviation mentioned above. So if specifically noted by s_r standard deviation of repeatability standard deviation resulting from the difference between the two measurements is expressed $\surd\,2\sigma_n$ and considering 95% confidence interval $\pm 1.96 \times \surd$ get $2\sigma r = 2.8\ \sigma_r$ [15].

The congeners constellation may indicate the nature of the ingested beverage (e.g. extremely high methanol and 1-propanol values in combination with high 2- and 3-methyl-1-butanol concentrations characteristic for fruit distillates), thus contributing to the interdisciplinary evaluation of suspicious death cases in forensic medicine [18]. Anethole is a congener characteristic of certain alcoholic beverages like ouzo and raki, therefore detection in serum samples is useful to check the plausibility of post-offence drinking claims in forensic toxicology [19]. Congeners identification and quantitation is essential in modeling and simulation of alcoholic distillation, while methanol content in wine distillates is stringently limited in some legislations and distillers must improve their operational policies in order to comply with legal requirements [20, 21].

Conclusions

We have developed a reliable gas-chromatographic method for the qualitative and quantitative determination of ethanol congeners, which was validated on the basis of precision, linearity, accuracy, repeatability, limit of detection and quantification.

Our method is useful for both forensic toxicology laboratories aiming to verify drivers post-offence statements concerning the types of alcoholic beverages consumption and also for chemical analysis laboratories asked to determine alcoholic beverages genuine feature.

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