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Original scientific paper

Development and validation of GC/MS method for determination of residual ethanol in Cannabis extract

Mirela Ristovska-Mladenovska[1*], Marija Darkovska Serafimovska[2], Marija Mitevska[3], Tijana Serafimovska[2], Zlatko Keskovski[3], Sasho Stefanoski[3], Aneta Dimitrovska[1]

[1]*Faculty of Pharmacy, University Ss. Cyril and Methodius, Majka Tereza 47, 1000 Skopje, R. North Macedonia*
[2]*Faculty of Medical sciences, University GoceDelcev, Krste Misirkov No.10-A, 2000 Stip, R. North Macedonia*
[3]*NYSK Holdings, 112 Str. No.217, Kjojlija, Municipality Petrovec, 1000 Skopje, R. North Macedonia*

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Abstract

Ethanol was found to be the most suitable solvent for optimal extraction of cannabinoids and terpenes from *Cannabis herba*. Since ethanol use can have a serious impact on human health, purification of pharmaceuticals after ethanol extraction is necessary. Ethanol belongs to Class 3 residual solvents and its concentration is limited to 5000 ppm (0.5%) in the final pharmaceutical dosage forms. Determination (quantification) of ethanol in the final extracts is crucial for the quality and safety of the product. A novel GC/MS method was developed and validated for the determination of residual ethanol in cannabis extracts. The developed method is rapid, specific, accurate and precise. The calibration curve was plotted between the obtained peak areas of ethanol (quantifier ion m/z=31) and its concentration at six different concentration levels. The response was linear over the range of 312.5 ppm – 10000 ppm (R^2 =0.9995). The limit of quantification, the limit of detection, system repeatability, method precision and recovery are demonstrated to evaluate the performance of the analytical procedure. The recovery range for added ethanol was 88.44% to 108.57%. The achieved results confirmed that the proposed method is suitable for monitoring the concentration of ethanol in cannabis extracts.

Key words: residual ethanol, cannabis extracts, gas chromatography-mass spectrometry

Introduction

Cannabis (*Cannabis sativa* and *Cannabis indica*) is a plant that has excited much interest throughout history especially over the last few years, as its medical purposes is becoming increasingly noticeable (Abuhasira et al., 2018; Beauchet et al., 2018; Blasco-Benito et al., 2018; Ebbert et al., 2018; Hutchison et al., 2019). Cannabis contains various groups of compounds like terpenes, flavonoids and probably the most characteristic among them cannabinoids (Boothet et al., 2019; Delgado-Povedano et al., 2020; Namdar et al., 2018; Nuutinen et al., 2018). All compounds, especially terpens and cannabinoids, can be extracted from the plant material by different approaches such as supercritical CO_2, or the most common classical extraction with a relatively apolar solvent - usually ethanol (Zekič et al., 2020). As demonstrated by Namdar et al. (2018), an optimum solvent for extraction of cannabinoids was found to be ethanol. The solvent (ethanol) can be detected as residue of such processing in the final products, and, in some cases, this impurity can be toxic (Le Daré et al., 2019). Ethanol is an organic chemical compound, volatile, flammable, colorless liquid with a slight characteristic odor. Ethanol belongs in Class 3 residual solvents, with low toxic potential and is limited to 5000 ppm or 0.5% (Ph.Eur, 2022).

The method of choice for measuring residual solvents

*email: mirela19_89@yahoo.com

in extracts is headspace (HS) gas chromatography (GC) coupled with mass spectrometry (MS) detection (Clifford et al., 2021; Marotta et al., 2018; Macherone et al., 2020; Shimadzu, 2022 Viviers et al., 2022).

The aim of this research was to develop and validate a new simple HS/GC/MS method for quantification of residual ethanol in cannabis extracts, without pretreatment of the sample. The purpose of this methodological procedure was to enable proper and accurate determination of residual ethanol, which is used as extraction solvent in Cannabis extracts.

Material and methods

Chemicals and reagents

The chemical standards for ethanol as reference material were supplied by CPA Chem (Table 1). Methylcellulose like sample inert carrier was supplied by Acros Organic. Helium as carrier gas was supplied by Messer.

Instrumentation and chromatographic conditions

The analysis was performed on a GC/MS QP2010SE single quadrupole mass spectrometer with static headspace (HS-20) with loop and autosampler for sample introduction, column Rxi-624, Sil MS, 30.0m x 0.25 mm x 1.40 µm and helium as a carrier gas.

The mass spectrometer was operated in the electron-impact mode (70 eV). Data acquisition was previously in scan mode with scan range of m/z 30-50, which allows to detect the retention time of ethanol peak and its ion masses. After detecting the retention time of ethanol peak in scan mode, SIM mode was created, according to the specific ions monitored and the peak retention time.

The chromatographic conditions are shown in Table 2.

Table 1. Standards for Ethanol used as reference material

Substance name	Manufacturer / Lot No:	Supplier	Expiration date/retest date
Ethanol 5 µg/mL	731568	CPA Chem	02.2022
Ethanol 2500 µg/mL	731569	CPA Chem	02.2022
Ethanol 20000 mg/L	745860	CPA Chem	05.2022

Table 2. Chromatographic conditions for determination of residual ethanol

Headspace parameters	
Equilibration	15 minutes
Vial pressure	350 kP; pressurizing time-1.5 min
Pressurizing equilibration time	0.1 min
Loop load time	0.20 min, equilibration: 0.20 min
Injection mode	split
Injection time	0.20 min
Oven temperature	120ºC
Sample line temperature	150ºC
Transfer line temperature	150ºC
GC-parameters	
Column	Rxi-624, Sil MS, 30.0m x 0.25 mm x 1.40 µm
Column temperature	30ºC
Carrier gas	Helium
Constant linear velocity, pressure	65.2Kp
Total flow	94.2 cm/sec
Column flow	1.24 mL/min
Purge flow	0.0 mL/min
Split ratio	75
Oven program	
30.0ºC	hold 3.0 min
10ºC/min to 55ºC	hold 0.0 min
45ºC/min to 200ºC	hold 2.0 min
Total GC run time:	10.72 min
Detector	
Operation mode	Selected Ion monitoring mode (SIM)
Ion source temperature	200ºC, EI mode, 70 eV
Interface temperature	300ºC
Solvent cut time	0.1 min

Table 3. The chromatogram characteristics, identification, and quantification ions of ethanol

	Peak RT (min)	Referent Ion m/z	Identification Ion 1 m/z	Identification Ion 2 m/z	Identification Ion 3 m/z
Ethanol	~3,4	31	45	46	43

Chromatogram characteristics, identification, and quantification ions of ethanol

The chromatogram characteristics, retention time, identification and quantification ions are present in the Table 3.

Preparation of stock solutions and working standards

Working calibration solutions of Ethanol were prepared in 6 different concentration levels:

- Std 1 = 312.5 ppm ethanol corresponding to 39.0625 µg on the column
- Std 2 = 625 ppm ethanol corresponding to 78.125 µg on the column
- Std 3 = 1.250 ppm ethanol corresponding to 156.25 µg on the column
- Std 4 = 2.500 ppm ethanol corresponding to 312.5 µg on the column
- Std 5 = 5.000 ppm ethanol corresponding to 625 µg on the column
- Std 6 = 10.000 ppm ethanol corresponding to 1250 µg on the column

In each 20-mL headspace vials were measured around 125 mg methyl cellulose like sample inert carrier and were added 125 µL of each standard in triplicate. The vials were closed tight.

Sample preparation

125 mg of sample (precise weight) were measured in triplicate in a 20-mL headspace vial and the vials were closed tight.

Results and discussion

Gas chromatography (GC) is the main analytical technique that is utilized in residual ethanol determination since it is suitable for the analysis of volatile compounds and is essential for the separating capability of capillary columns. GC coupled with a headspace (HS) sampling system is usually recommended for qualitative and quantitative determinations of organic volatile impurities in pharmacopoeias general chapters. The flame ionization detector (FID) is most commonly used for the detection of residual ethanol on GC techniques. It has good selectivity, linearity and higher upper values of the working range as the main advantages of GC-FID. But when using a complex matrix, such as cannabis extracts and the identity of other volatile compounds needs to be confirmed, the method of choice for identification and quantification of residual solvents is headspace (HS) gas chromatography (GC) coupled with mass spectrometry (MS) detector. Therefore, an HS/GC//MS method for residual ethanol in cannabis extracts was developed. There are many different organic volatile compounds (terpenes) in the cannabis matrix that elute (co-elute) with ethanol. So, we use the unique mass spectrum of each of the eluted compounds to optimally separate and detect them without using additional detectors. Identifying and quantifying the residual ethanol in complex matrix using HS/GC/MS method results in a simple and faster analysis, enhanced productivity, and quicker release of the product (Aparicio Ruiz et al., 2018; Marotta et al., 2018).

The initial chromatographic conditions were based on the compendial method on identification and control of residual solvents (Ph. Eur. chapter 2.4.24.). The method was further modified to better meet the intended use for determination of ethanol in Cannabis extracts. Thus, the temperature program was adjusted for faster elution of all volatile compounds expected in the extract based on their corresponding boiling points, considering suitable selectivity of the method to enable the determination of ethanol.

Method validation

Analytical methods should be developed, optimized, and validated to provide that method is adequate for its intended use (Swartz et al., 2018). The developed method was validated according to the guidelines of the International Conference of Harmonization for validation of analytical procedures (ICH Q2R1, 2022; ICH Q2(R2)/Q14 EWG, 2022). Various aspects of performance characteristics were assessed during the method validation as: Specificity/Selectivity, Linearity, Limit of Quantification, Limit of Detection and Recovery.

Specificity and selectivity

A mixture of potential solvents that might co-elute with ethanol has been made. They were released in scan mode in order to identify and determine whether they interfere with the peak of ethanol. Good resolution was achieved between ethanol and co-eluting peaks of methanol and acetone (resolution above 2.0 for both critical pairs of peaks). After detecting the retention time of ethanol peak in scan mode and its ion masses, SIM mode was created, according to the specific ions monitored and the peak retention time.

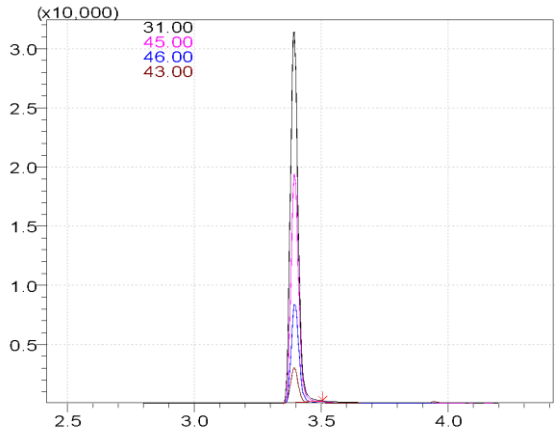

Fig. 1. Chromatogram characteristics, retention time, identification and quantification ions of ethanol .

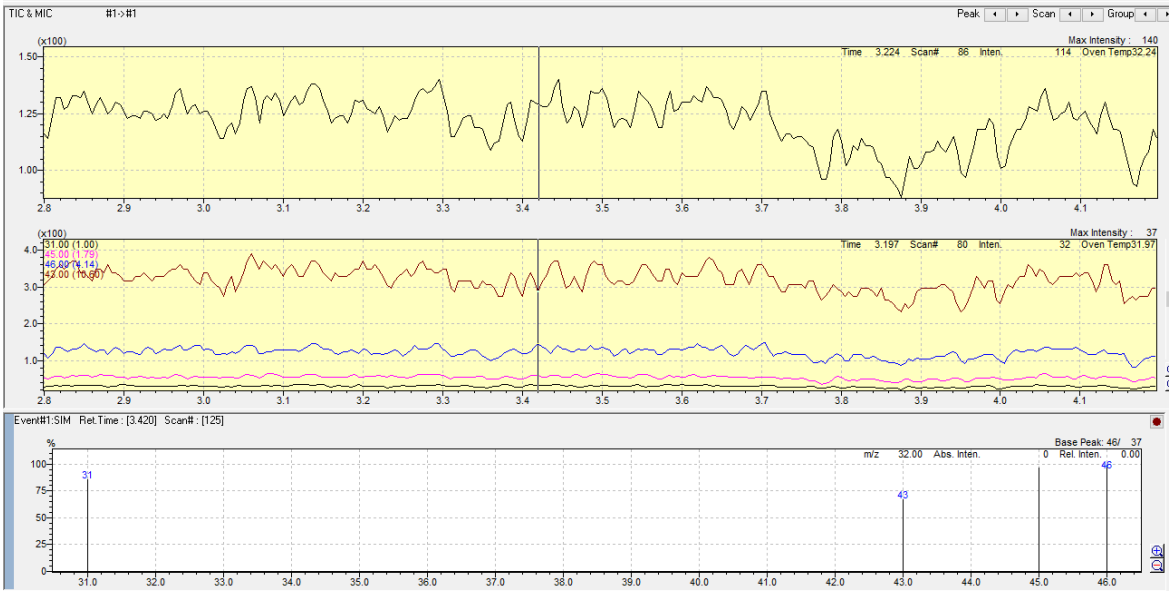

Fig. 2a. Chromatogram and Ion Tranzition of blank solution

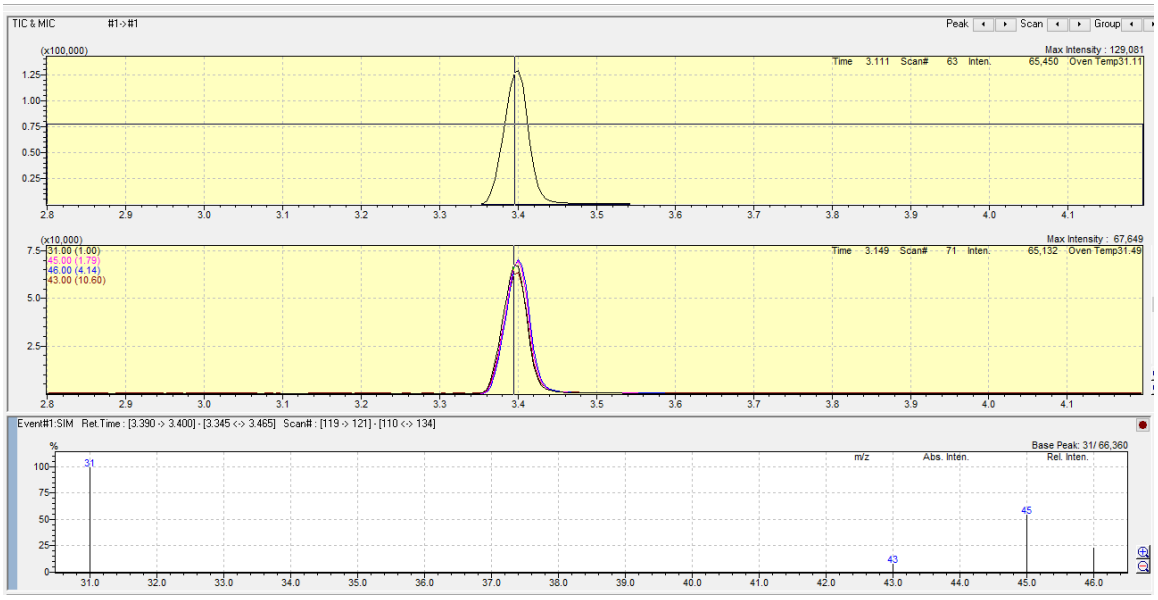

Fig. 2b. Chromatogram of Ethanol and ion transition (Retention time- 3.365 min; Referent ion-31m/z)

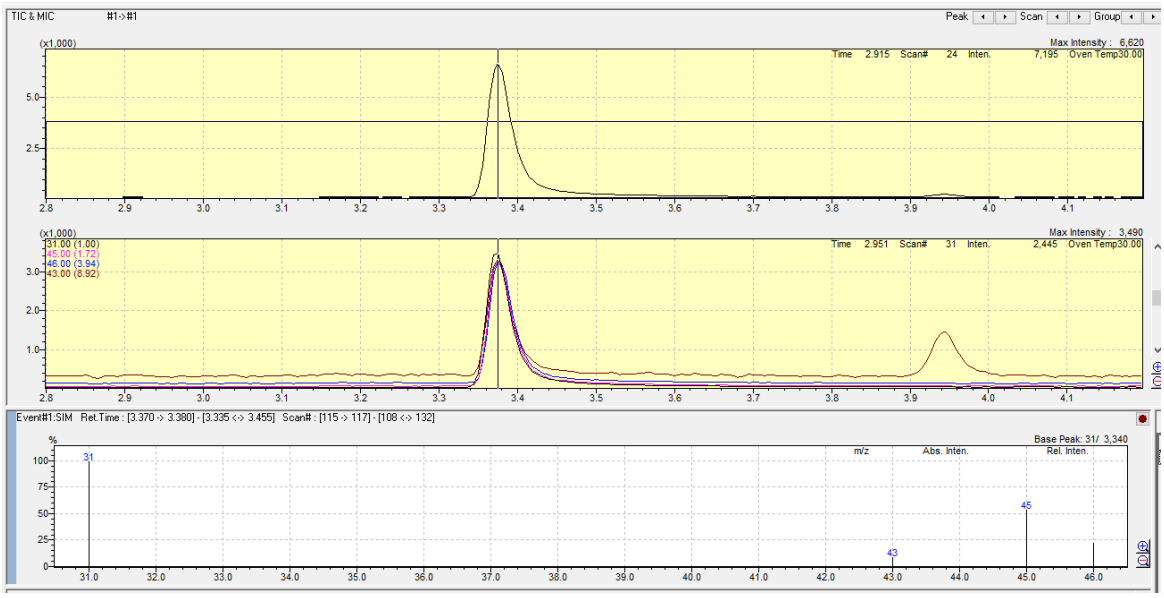

Fig. 3. Chromatogram of cannabis extract.

Blank solution, standard solution of ethanol and cannabis extract were injected in SIM mode. (Figure 1, Figure 2a, Figure 2b and Figure 3, respectively). The method showed good chromatographic specificity and selectivity, without interference.

Linearity

Linearity was performed using ethanol standard solutions (quantifier ion m/z=31) in six different concentrations in the concentration range of 312.5 ppm – 10000 ppm, which presents 0.0625 - 200% of the maximum tolerated presence of ethanol in Cannabis extracts.

Correlations between the concentration of ethanol, in ppm and detector response i.e., peak area, expressed as the average value of three separate injections of standard solutions of ethanol in the same concentration level are shown in Table 4 and Figure 4.

Relative standard deviation of the six concentration levels is less than 15% (Council Directive 96/23/EC). The response was linear over the range (R^2 =0.9995).

Limit of detection and Limit of quantification

Determination of Quantitation limit (QL) and Detection limit (DL) is based on the standard deviation of the response and the Slope calculations. The quantitation limit was determined to be 491.42 ppm and the detection limit was determined to be 162.17 ppm.

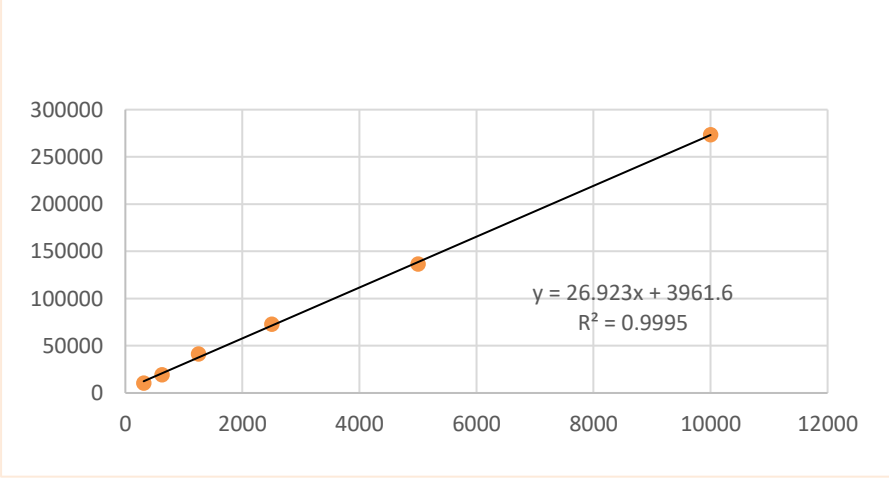

Fig. 4. Linearity of ethanol standard solutions in six different concentrations.

Table 4. Linearity on ethanol standard solutions in six different concentrations

Conc (ppm)	Peak area	Average area	Response Factor (RF)
312.5	10330	10309	33.06
	10311		33.00
	10286		32.92
625	18728	19399	29.96
	19342		30.95
	20128		32.20
1250	40458	41248	32.37
	41178		32.94
	42109		33.69
2500	69656	72768	27.86
	74089		29.64
	74560		29.82
5000	131028	136581	26.21
	137043		27.41
	141671		28.33
10000	286350	273515	28.64
	264901		26.49
	269294		26.93
		AVE	30.13
		STD	2.57
		RSD (%)	8.53

Precision

The system precision (repeatability) and method precision (repeatability of the method) were evaluated by performing replicate analysis of the six subsequent injections of the standard solution ethanol in a final concentration of 625 µg, representing 5000 ppm. Relative standard deviations were calculated to obtain the precision of the method. The results are shown in Table 5.

The results of repeatability of the method demonstrates good precision (RSD < 15 %) (Council Directive 96/23/EC, 2002).

Accuracy

For evaluation of parameter accuracy samples of Cannabis extract were spiked with known quantities of ethanol on three different concentration levels. Each spiked sample was injected six times separately.

The accuracy is expressed as percent of recovery; calculated from the ratio of determined and added quantity of analyzed substance. Results are shown in Table 6 and Figure 5.

The method shows good recovery in the range of 88.44% to 108.57%, with linear response (R^2 =0.9924).

Table 5. Results from determination of repeatability of the system (system precision) and repeatability of the method

System repeatability		Method repeatability	
-inj.	Peak area		Conc. of ethanol
1	145615	Assay 1	643.339 µg (5146.712 ppm)
2	131028	Assay 2	578.02 µg (4624.16 ppm)
3	137043	Assay 3	604.954 µg (4839.632 ppm)
4	146919	Assay 4	649.178 µg (5193.424)
5	141671	Assay 5	625.678 µg (5005.424 ppm)
6	138388	Assay 6	610.977 µg (4887.816 ppm)
AVE	140111	AVE	618.691
STD	5898.45	STD	26.41265
RSD (%)	4.21	RSD (%)	4.27

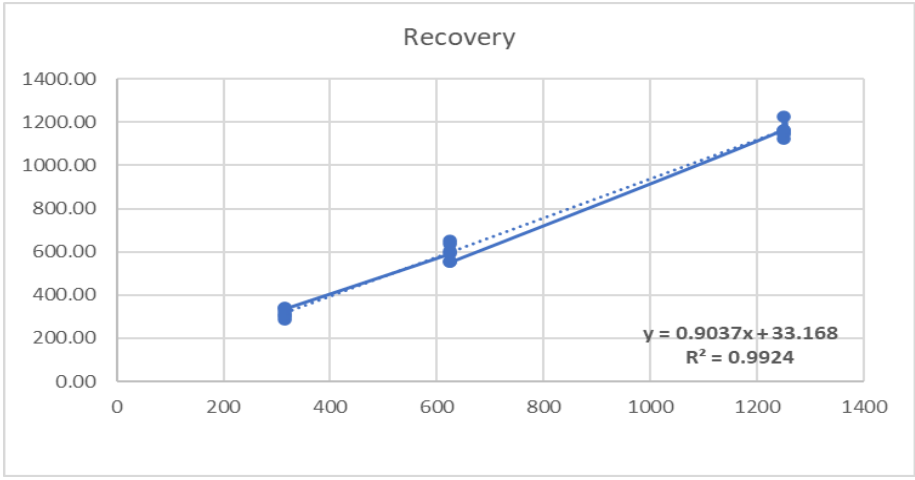

Fig. 5. The recovery range (linearity response) for added ethanol.

Table 6. Recovery of ethanol, expressed as a percentage

Spiked conc. of Ethanol (ppm)	Determined conc. Ethanol (ppm)	recovery, %	Average	SD	RSD	Confidence level (95.0%)
2500	2483	99.31	101.63	6.70	6.59	±7.03
	2301	92.05				
	2416	96.65				
	2625	104.99				
	2714	108.57				
	2705	108.19				
5000	4739	94.79	95.84	6.68	6.97	±7.02
	4809	96.17				
	5229	104.59				
	5113	102.27				
	4440	88.80				
	4422	88.44				
10000	9314	93.14	92.99	2.80	3.01	±2.93
	9818	98.18				
	8992	89.92				
	9182	91.82				
	9184	91.84				
	9306	93.06				

Conclusion

A new method for identification and quantification of residual ethanol using GC/MS QP2010SE single quadrupole mass spectrometer with static headspace (HS-20) with loop and autosampler for sample introduction has been developed and validated.

For identifying and measuring residual solvents in cannabis concentrates, headspace technology combined with GC/MS is clearly the most effective method. Compared with other methods, headspace sample introduction requires minimal sample preparation and requires very little operator interaction. By combining headspace sampling with GC/MS, rapid, unambiguous, and interference-free integrations are possible, with little likelihood of false positives. This method provides the ability to identify unknown compounds in a sample that are not the target compounds.

The proposed method is simple, rapid, accurate, precise and specific, and can detect and quantify low concentrations of ethanol (LD =162.17 ppm, LQ =491.42 ppm). Our results confirmed the aim of our study and showed the possibility of monitoring the concentration of ethanol in cannabis extracts.

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Резиме

Развој и валидација на GC/MS метод за определување на резидуален етанол во екстракти од канабис

Мирела Ристовска-Младеновска[1], Марија Дарковска Серафимовска[2], Марија Митевска[3], Тијана Серафимовска[2], Златко Кесковски[3], Сашо Стефаноски[3], Анета Димитровска[1]

[1]Фармацевтски факултет, Универзитет „Св. Кирил и Методиј“, Мајка Тереза бр. 47, 1000 Скопје, Р. Северна Македонија
[2]Факултет за медицински науки, Универзитет „Гоце Делчев“, Крсте Мисирков бр.10-А, 2000 Штип, Р. Северна Македонија
[3]НЈСК Холдингс, ул. 112 Бр.217, Ќојлија, општина Петровец, 1000 Скопје, Р. Северна Македонија

Клучни зборови: резидуален етанол, екстракти од канабис, гасна хроматографија-масена спектрометрија

Етанолот се има докажано како најсоодветен растворувач за оптимална екстракција на канабиноиди и терпени од канабис растение. Бидејќи употребата на етанол може да има сериозно влијание врз здравјето на луѓето, неопходно е прочистување на фармацевтските производи по екстракцијата со етанол. Етанолот припаѓа во класа 3 резидуални растворувачи и неговата концентрација е ограничена на 5000 ppm (0,5%) во крајните фармацевтски дозирани форми. Определувањето (квантификацијата) на етанолот во крајните екстракти е клучно за квалитетот и безбедноста на производот. За та цел е развиен и валидиран нов GC/MS метод за определување на резидуалниот етанол во екстрактите од канабис. Развиениот метод е брз, специфичен, точен и прецизен. Калибрационата крива е добиена од максималните површини на етанол (јон за квантификација, m/z=31) и неговата концентрација, на шест различни нивоа на концентрација. Добиениот одговор е линеарен во опсег од 312,5 ppm – 10000 ppm (R^2 = 0,9995). Лимитот на квантификација, лимитот на детекција, повторливоста на системот, прецизноста на методот и точноста се прикажани за да се оценат перформансите на аналитичкиот метод. Опсегот за точност на додаден етанол е добиен од 88,44% до 108,57%. Добиените резултати потврдија дека предложениот метод е погоден за следење на концентрацијата на етанол во екстрактите од канабис.