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Fast quantification of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) using microwave-accelerated derivatisation and gas chromatography–triple quadrupole mass spectrometry

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ABSTRACT

A rapid and sensitive determination of cannabinoids in urine is important in many fields, from workplace drug testing over toxicology to the fight against doping. The detection of cannabis abuse is normally based on the quantification of the most important metabolite 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THCA) in urine. In most fields THCA needs to be present at a concentration of exceeding 15 ng/mL before a positive result can be reported.

The method described in this paper, combines a 4 min GC–MS/MS method with a fast sample preparation procedure using microwave assisted derivatisation in order to complete the quantification of THCA in urine in 30 min, using only 1 mL of urine.

The method is selective, linear over the range 5–100 ng/mL and shows excellent precision and trueness and hence, the estimated measurement uncertainty at the threshold level is small. The method also complies with applicable criteria for mass spectrometry and chromatography. Therefore the method can be used for rapid screening and confirmatory purposes.

1. Introduction

The psychoactive substances from *Cannabis sativa* are amongst the most widely used illicit drugs in the world. Considering the various effects of cannabis [1], there is a need for rapid and sensitive detection methods in many fields: workplace drug testing [2], clinical and forensic toxicology [3], and the fight against doping [4]. Although they do not improve athletic performance, cannabinoids allow the athlete to relax and escape from pressure. Additionally, the use of cannabis reduces alertness and quick reflexes, making it dangerous in automobile and team sports [5,6]. Therefore natural (e.g. cannabis, hashish, marijuana) or synthetic Δ9-tetrahydrocannabinol (THC) are also prohibited in competition by the World Anti-Doping Agency (WADA) [7].

According to the guidelines of various scientific organizations, a two step approach is applied: after detecting the substance or its metabolites in a screening method, a confirmation procedure is performed in order to obtain additional information to support a possible positive result. This confirmation procedure needs to have equal (in case a hyphenated mass spectrometry method was used in the screening) or greater selectivity than the initial screening method [8,9].

In most fields, immunoassays are used as screening methods for the detection of cannabis metabolites in biological matrices. However, when using these techniques, there is a risk of reporting false-positive results, due to possible cross reactivity and potential interferences. Hence a chromatographic technique, combined with mass spectral identification is used for confirmation of the result [10,11]. In general chromatography-mass spectrometry needs to be used to comply with the stringent requirements to unequivocally identify a compound in most fields.

To differentiate active from passive users [12], a threshold concentration of 15 ng/mL for 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic a cid (THCA) in urine is set by WADA [13]. Similar as for the WADA-regulations, a threshold of 15 ng/mL is also commonly used in the fields of toxicology and in drugs at the workplace testing [9,14]. Of course any conclusion, whether or not a threshold concentration is exceeded, should take into account the measurement uncertainty associated with the result.

Both the limited reporting period and the limited amount of urine available are important factors in the development of a confirmation procedure. Traditionally, confirmatory methods for THCA use multiple sample preparation steps and are time-consuming [15].

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Table 1
Monitored transitions for THCA and THCA-d9 (internal standard), corresponding collision energies and transition ratio's (tested against WADA criteria for identification).

Compound name	Transition	Collision energy (eV)	WADA	Max. tolerance window	
			Identification criterium	Under limit	Upper limit
THCA	371 → 305	10			
THCA	371 → 289	10	10	88.8	108.8
THCA	371 → 265	10	10	76.8	96.8
THCA	371 → 95	10	10	68.4	88.4
THCA-d9	380 → 67	25			
THCA-d9	380 → 101	25	7.04	28.16	42.24
THCA-d9	380 → 84	25	10	40.5	60.5
THCA-d9	380 → 292	25	8.64	34.56	51.84

In this work a fast confirmation method is presented for the quantification of THCA in urine that not only fulfills all legal requirements (MS criteria, uncertainty, etc.), but which by using an accelerated sample pretreatment and a fast chromatographic method allows to quantify this metabolite rapidly and accurately in a low volume of urine.

2. Experimental

2.1. Instrumentation

An Agilent (Agilent Technologies, Palo Alto, USA) GC 7890 gas chromatograph coupled to an Agilent 7000B triple quadrupole mass spectrometer (Agilent Technologies) and a MPS2 autosampler and PTV-injector from Gerstel (Mülheim an der Ruhr, Germany) were used.

The GC column – 12 m × 250 μm and 0.25 μm film thickness – was a HP-1MS from J&W Scientific (Agilent Technologies, Palo Alto, USA). The temperature program was as follows: the initial temperature was 110 °C (0.15 min), increased at 70 °C/min to 310 °C (held for 1 min). The transfer line was set at 310 °C. Helium (Air Liquide, Desteldonk, Belgium) was used as carrier gas at a flow rate of 3 mL/min.

Injection was performed using the following PTV setting: in the solvent vent mode 10 μL was injected (0.5 μL/s injection speed) and the PTV-temperature settings were: 110 °C (0.15 min), 12 °C/s to 310 °C (2 min) and 12 °C/s to 380 °C (1 min). The vent flow was 60 mL/min and the vent pressure 5 psi until 0.1 min.

In the collision cell of the triple quadrupole mass spectrometer, He was used as a quench gas at 2.25 mL/min and N_2 as collision gas at 1.5 mL/min.

Quantification and confirmation were performed in selected reaction monitoring (SRM) mode. The monitored transitions and corresponding collision energies are given in Table 1.

For derivatisation a domestic microwave oven (Samsung M643) was used in this study, which has a total capacity of 750 W. The effective capacity was verified with every batch.

2.2. Reagents

THCA and THCA-d9 were purchased from Cerriliant. NaOH and acetic acid were from Merck (Overijse, Belgium). N-methy-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was obtained from Chem. Fabrik Karl Bucher (Waldstetten, Germany). Ethanethiol and ethylacetate were purchased from Acros (Geel, Belgium) and acetonitrile (ACN) was from Biosolve (The Netherlands). Ammoniumiodide (NH_4I) from Sigma–Aldrich (Bornem, Belgium) and n-hexane from Biosolve (Valkenswaard, The Netherlands). All reagents were analytical grade.

2.3. Sample preparation

1 mL of urine was spiked with 50 μL of the internal standard containing 0.5 μg/mL THCA-d9. Hydrolysis was performed by incubation for 7 min in an oven at 56 ± 5 °C after addition of 100 μL of 6 M NaOH solution. After briefly cooling down 1.5 mL acetic acid and 3 mL n-hexane/ethyl acetate (9/1, v/v) were added. For the liquid–liquid extraction, this mixture was mixed by vortexing for 1 min, then the organic phase was transferred and evaporated under oxygen free nitrogen at 40 ± 5 °C.

Table 2
Relative standard deviation and bias calculated out of 6 replicates at 3 concentration levels by 3 persons.

Conc (ng/mL)	2/3 RSD_{max}	RSD (%)	Bias (%)
5	23.68	6.73	3.30
15	20.07	4.05	5.26
100	15.08	1.71	0.66

The dried residue was derivatised using 20 μL acetonitrile, 50 μL MSTFA and 50 μL of MSTFA/ethanethiol/NH_4I (500:4:2) in a microwave reactor at 750 W during 1.5 min.

2.4. Validation

2.4.1. Microwave calibration

The microwave calibration procedure was based upon a calorimetric methodology. In a glass beaker, 1 L distilled water was heated in the microwave for 90 s at 100% power. The microwave power absorbed by the water that was used for heating the volume was estimated from the measured temperature rise ΔT [16].

$$\Delta T = \frac{P \cdot t}{V \cdot C_p \cdot \rho}$$

In this formula P is the microwave power in Watt (W), t the time of heating in seconds, V the volume of heated water (m^3), C_p the heat capacity (J/(kg K)) and ρ the density (kg/m^3).

2.4.2. Quantification of THCA

To construct calibration curves, blank urine samples were spiked at 6 concentration levels. The least squares method was used for fitting the calibration curves and controlling linearity of the method. For the calculation of accuracy and precision negative urine samples were spiked with THCA at 3 different concentration levels (1, 15 and 100 ng/mL). On every level 6 replicates were analyzed by 3 different persons. The tolerances for precision were calculated following the Horwitz equation ($RSD_{max} = 2(1 - 0.5 \log C)$) and should not exceed 2/3 RSD_{max}. The accuracy, expressed as bias, may not exceed 15%.

For correct identification of the compound, WADA criteria [13] state that retention times (RT) of the analyte shall not differ more than ±0.1 min or 2% from that of the same substance in a spiked urine sample. Further the transition ratios are calculated by dividing the area of the given qualifier by the quantifier. Maximum tolerance windows for these relative ion intensities are determined by WADA [17]. For the least intense diagnostic transition, signal-to-noise (S/N) ratio shall be greater than 3.

To verify to what extent the method can determine THCA and THCA-d9 in matrices without interferences from other compounds, several mixtures of possible interfering compounds (narcotics, anabolic steroids, beta-blockers, diuretics and stimulants [18]) were analyzed.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the IUPAC standard approach SA1, which uses the mean blank signal as the basis for the calculation of the LOD and LOQ values [19].

The standard deviation, calculated over 40 control samples, was used to determine tolerance windows for quality control and measurement uncertainty for THCA.

2.4.3. Cross validation

To verify the applicability of the method for the analysis of routine samples, the method was compared to the GC–MS method which was formerly used in our lab [15]. Therefore 20 urines, positive for THCA, were analyzed with both methods and the obtained concentrations were compared.

3. Results and discussion

3.1. Sample preparation procedure

In previous described methods using gas chromatography–mass spectrometry, the quantity of urine used per analysis varied between 2 and 3 mL [10,15,20–24]. Since the amount of urine available is limited and for confirmation procedures usually 3 aliquots are analyzed, it is important to lower the volume of urine

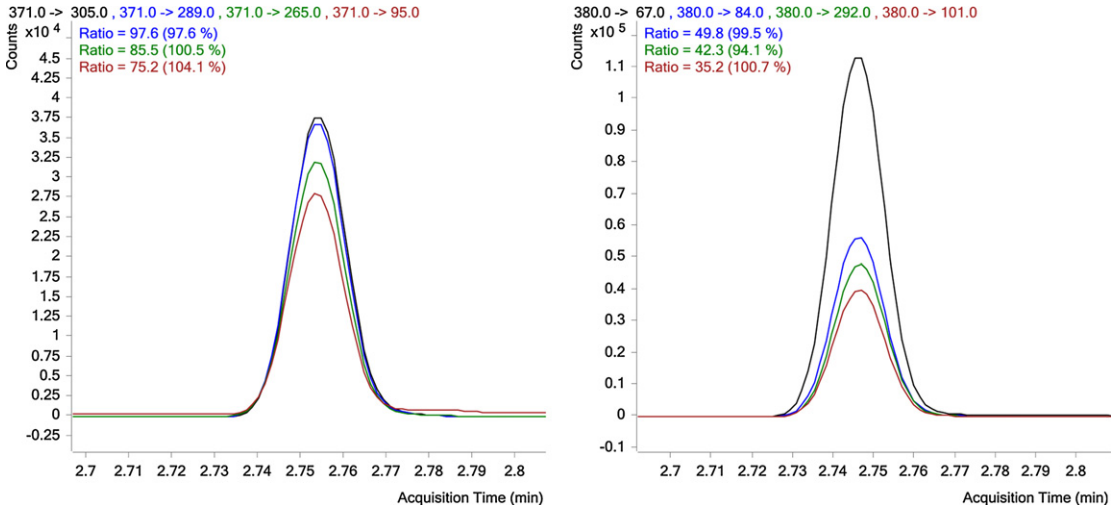

Fig. 1. THCA and THCA-d9 ion traces in a urine sample, spiked with THCA at 15 ng/mL (left) and THCA-d9 at 50 ng/mL (right).

consumed per analysis. The combination of large volume injection using a PTV injector and tandem mass spectrometry for higher selectivity and sensitivity (Fig. 1), allowed for using only 1 mL of urine.

Since THCA is mostly excreted in urine in a conjugated form, first a hydrolysis step is performed. In previous described methods this is either done with an enzymatic hydrolysis using β-glucuronidase (incubation up to 60 min) [24] or an alkaline hydrolysis using NaOH or KOH (usually between 15 min and 30 min incubation) [10,20,21]. THCA however is connected to the glucuronide using an ester bond, which allows the use of a faster alkaline hydrolysis. Indeed, previous research showed that incubation for 7 min provides complete hydrolysis [25]. Hence, after verification of this procedure the hydrolysis time in this study was set to 7 min as well.

While in the past, when using single MS technology, a preliminary extraction was needed for sample clean-up in order to obtain sufficient selectivity [15], this is not necessary when using MS/MS. The elimination of this step reduces the sample preparation time. Hence, sample pretreatment for GC–MS/MS is less time-consuming and becomes comparable to the fastest recently published LC–MS procedures [25–28].

While the free compound is usually isolated out of the urine matrix using labor-intensive solid-phase extraction (SPE) [22] or time consuming liquid–liquid extraction (up to 20 min mixing/rolling) [15,23,24], this step is now optimized. Therefore blank urine samples spiked at 15 ng/mL THCA were analyzed, using different extraction times (3 replicates each). From the recoveries given in Fig. 2 can be concluded that the extraction can be shortened to 1 min of vortexing without compromising on efficiency, resulting in a drastic reduction of analysis time. This

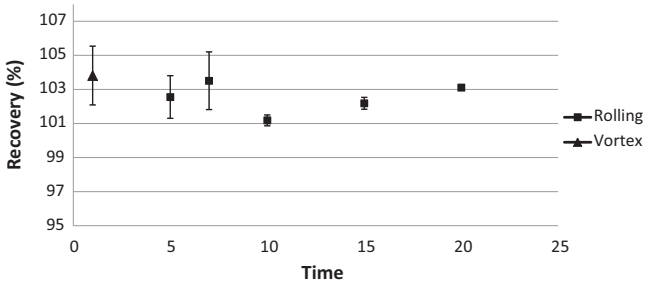

Fig. 2. Optimization extraction procedure: recoveries as a function of the mixing time for samples spiked at 15 ng/mL THCA ($n = 3$).

can be explained by the addition of the deuterated internal standard THCA-d9 which compensates very well for any losses during this and other sample preparation steps. Further the changes in peak area of THCA in this experiment are showing that the equilibrium is set quickly. Indeed, compared to the traditional 20 min of rolling where an average peak area of 30,877 with a RSD of 4.33% was found ($n = 3$), all other peak areas did not differ more than 0.14% from this value.

Recently, most of the fast quantification methods for THCA have been based on LC–MS analysis. One of the primary reasons for this is that GC–MS requires a time-consuming derivatisation step to improve the compounds characteristics in terms of chromatography. This derivatisation is usually performed with silylation reagents and the energy needed to complete the silylation reaction is traditionally added by conventional heating, which requires long reactions times up to 1 h [29,30]. Supplying the energy transfer by microwave irradiation instead of thermal heating can reduce this derivatisation time to a few minutes [31]. When using microwave-assisted derivatisation (MAD), the reaction mixture is heated rapidly from the inside out, in contrast to conventional heating which is slow and enters the sample from the surface, heating also the reaction tube [32]. Reducing the time required for this and other sample preparation steps prior to GC analysis greatly improves the competitiveness of GC–MS. Indeed, GC–MS is less costly than LC–MS and shows better separation power.

The GC–MS method described here allows for a perfect separation of the target compound from any matrix interferences in less than 4 min. To the best of our knowledge, this is faster than previously described GC [15,23,24] and LC [25–27] method run times, greatly improving sample turn-around and throughput.

Hence, the optimized sample preparation procedure together with a GC run of only 4 min allows for a quantification of THCA in 1 mL of urine in less than 30 min.

3.2. Derivatization procedure

The domestic microwave system used in this experiment has a nominal power of 750 W. Since quality control is of great importance in doping control, a procedure is needed to monitor the performance of the microwave through time. When using the absorbed power as a parameter, the stability of the microwave can easily be monitored on a daily base [33]. Therefore 1 L of distilled water is heated for 90 s at 100% nominal power. Using the abovementioned formula, one can calculate the effective absorbed power out of the temperature rise of the water. From different

Table 3
Comparing microwave assisted derivatisation (90 s at 750 W) to derivatisation using conventional heating (30 min at 80 °C), 2 sets of 6 control samples spiked at 25 ng/mL THCA.

	Oven	Microwave
Calibration curve eq.	$y = 0.128580x - 0.020451$ $R^2 = 0.99880$	$y = 0.129871x - 0.028104$ $R^2 = 0.99953$
Mean concentration control samples (ng/mL)	24.3	24.41
Standard deviation on concentration control samples	0.392	0.493
t-Test	0.7716	

The significance level: $\alpha = 0.05$.

measurements over a period of 2 months, an absorbed power of 576 ± 39 W was found.

When using microwave assisted derivatisation, reaction solvent, microwave power and reaction time are important parameters [34,35].

In contrast to conventional heating, the amount of heat transferred to the reaction mixture depends on the dielectric properties of the molecules [32]. Because polar molecules have a permanent dipole moment, they can absorb the microwave energy and convert it into heat. ACN has a high polarity and is therefore very suitable for absorbing electromagnetic energy [36]. This means that the derivatisation mixture for silylation commonly known in the doping control field [37] can still be used.

To investigate the yield of the derivatisation reaction when using microwave energy, comparison was made with conventional heating in an oven. Therefore 2 calibration curves were made by spiking 6 blank urines with THCA at different concentration levels (5, 10, 20, 50, 75 and 100 ng/mL). For derivatisation the first is heated for 90 s in the microwave, while for the second conventional heating for 30 min at 80 °C is used. The equations of both calibration curves are showed in Table 3, coefficients of correlation were both higher than 99%.

Subsequently 2 sets of 6 THCA control samples spiked at 25 ng/mL were prepared using both heating methods to compare their performance. A *t*-test was used to determine whether there is a significant difference between the means of both groups. The calculated P-value was 0.7716, which indicates no difference between both groups.

This proves that the derivatisation yield after 90 s of irradiation at 750 W is comparable to the yield of the derivatisation with 30 min of conventional heating at 80 °C.

3.3. Validation

3.3.1. Quantitative

For the quantification of THCA calibration curves over 6 concentration levels (5, 10, 20, 50, 75 and 100 ng/mL) were constructed, using the method of least squares. Hereby the coefficient of correlation R^2 was above 99%.

Precision and bias were determined out of 6 replicates at 3 concentration levels (5, 15 and 100 ng/mL), This experiment was repeated by 3 different persons, leading to 18 replicates at each level. The Horwitz equation was used to calculate the tolerance levels for precision. For every calibration level the obtained relative standard deviations (RSD) were lower than 2/3rd of the calculated RSD_{max}. The bias was always below 15% (Table 2).

Retention times did not differ more than 2% between all samples, thereby complying with WADA's criteria [17].

To ensure correct identification in tandem mass spectrometric detection, two or more precursor-product ion transitions are monitored. The relative abundance of each transition is calculated relative to the most abundant transition (quantifier). The allowed tolerance windows for the relative abundances are traditionally imposed by governing bodies in the given field. Here the tolerance levels were calculated according to WADA technical document [17]. For THCA all calculated transition ratios (Fig. 1) are between the calculated lower and upper limits (Table 1). Additionally the S/N ratio of the least intense diagnostic transition is calculated. On every concentration level of the calibration curve, this ratio was higher than 3–1 for the least abundant transition.

Selectivity of the method was tested by analyzing ten blank urines and urines spiked with mixtures of other WADA prohibited drugs and/or metabolites (50 anabolic steroids, 15 beta-blockers, 30 diuretics, 17 narcotics and 35 stimulants) [18]. These results showed that there are no interferences of the urine matrix and spiked compounds with the target analytes.

The calculation of the LOD, described by IUPAC, is based on the mean value of the blank signal [19]. Therefore 10 blank urine samples were analyzed and the standard deviation on the blank signal was calculated as 0.0178. With a slope of the calibration curve of 0.9399 and a coverage factor *k* of 3 for a 99.9% probability, the calculated LOD was 0.0567 ng/mL. For the quantification limit IUPAC uses a coverage factor of 10. Using the same calculation the LOQ was found at 0.1889 ng/mL. Although this is a low detection limit, it is of little importance in doping control since THCA is a threshold substance, but it does show the possibility to detect trace amounts of this compound in a biological matrix with limited sample preparation using GC–MS/MS.

In an effort to harmonize the results amongst all laboratories world-wide, WADA has defined the maximum combined uncertainties ($u_{c\ max}$) for all threshold compounds and calculated a decision limit (DL) that needs to be used when reporting results. This DL is the sum of the original threshold value and a guard band. The guard band is based upon the maximum acceptable value of the combined standard uncertainty ($u_{c\ max}$) multiplied by an appropriate expansion factor *k* (1.645; representing a 95% one-sided confidence level). In the case of THCA, a urine sample can be reported as 'positive' if the concentration of THCA exceeds the DL of 18 ng/mL. Consequently, the standard uncertainty u_c of any methodology used should be less than the $u_{c\ max}$ of 1.5 ng/mL. This protocol is in agreement with the Eurachem guidelines on the use of uncertainty data in compliance testing [38].

Over 40 quality control samples, spiked at 15 ng/mL THCA, the RSD and bias were found to be 5.88% and 2.50% respectively. Using these values the calculation of the combined standard uncertainty u_c (by taking the square root out of the sum of squares of both parameters) resulted in 0.632 ng/mL. This is well below the maximal combined standard uncertainty $u_{c\ max}$ of 1.5 ng/mL imposed by WADA. The expanded uncertainty for a 95% confidence level based on a 2-tailed distribution is calculated using a coverage factor *k* of 2.

3.3.2. Application to routine samples

20 urines samples, with concentrations of THCA above the WADA decision limit of 18 ng/mL, were analyzed. The results were compared with concentrations obtained when analyzing these urine samples with the GC–MS quantification method of De Cock et al. [15]. To check the correlation between the 2 methods a

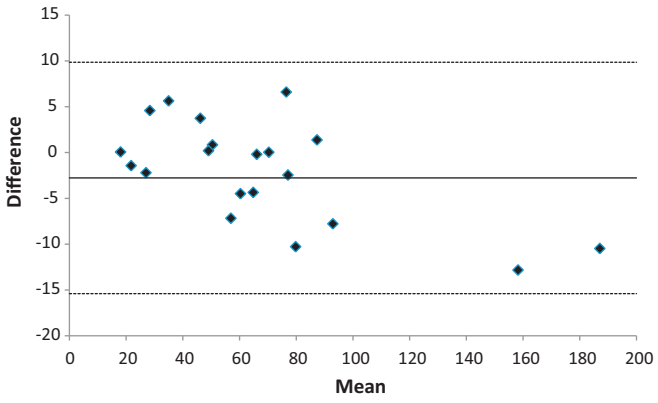

Fig. 3. Bland–Altman plot for the correlation between the GC–MS method of De Cock et al. [15] and the developed GC–QqQ-MS method for 20 urine samples positive for THCA. The 95% confidence interval (dotted lines) around the mean difference (solid line) is showed.

Bland–Altman plot is used. As showed in Fig. 3 all values are in the 95% confidence interval, which proves the agreement between the GC–MS and the newly developed GC–QqQ-MS method.

3.4. Additional considerations

Sample preparation is probably the most polluting part of an analytical method because of the high quantities of organic solvents used. Special attention was paid to the impact on the environment and the associated overall cost per analysis.

In order to speed up the sample preparation time, methods using (automated) SPE for the extraction of THCA out of urine were developed [20,27]. The SPE protocol normally requires a conditioning step using 2–4 mL of solvents [26,39]. After loading the sample, unwanted interferences are removed by successive washes using several mL of solvents like methanol, acetic acid and ACN [20,26,27]. Finally some more MeOH and ACN is added to elute the compounds of interest from the cartridge.

The LLE methodology proposed here, only consumes 3 mL of organic solvents to extract the free THCA out of urine and there is no need for SPE cartridges. Such procedure is more environmentally friendly and reduces the overall cost of the procedure.

4. Conclusions

A selective method for the quantification of THCA in urine was developed and validated. Special attention was paid to the limited volume of urine available and the total analysis time.

The use of microwave assisted derivatisation allows for a fast and complete silylation of THCA in 90 s, whereas this step would take at least 30 min using conventional heating in an oven. In order to monitor the performance of the domestic microwave a calorimetric methodology was used.

This method can be applied as a confirmation procedure after a positive finding of THCA in a screening method and allows for the quantification in less than 30 min.

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