

Rapid and Sensitive Quantitation of Synthetic Cannabinoids in Serum by UHPLC-Triple Quadrupole Mass Spectrometry



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Introduction

Synthetic cannabinoids first appeared on the recreational drug scene in 2004. The term synthetic cannabinoid refers to an increasing number of manmade mind-altering chemicals that are either sprayed on dried, shredded plant material so they can be smoked, or sold as liquids to be vaporized and inhaled in e-cigarettes and other devices. Many synthetic cannabinoids are full agonists of the cannabinoid receptors CB₁ and CB₂, and therefore their psychoactive effects are similar to those of cannabis. They include compounds such as e.g. naphthoyl-, phenylacetyl-, benzoyl- and cyclopropyl-indoles, cyclohexylphenoles, and various other indazole and indole derivatives.

In an attempt to circumvent current legislation synthetic cannabinoids are misleadingly marketed via the internet or in headshops as “bath salts, plant food or research chemicals” and are often sold in colorful small bags with creative names, such as “Unicorn Magic Dust”, “Be Happy”, or “Aliens”. They are then judged by consumers to be safe and legal, despite the fact that their effects can lead to high blood pressure, nausea, hallucination, psychosis, physical addiction and even life-threatening conditions. In order to keep ahead of evolving drug prohibition laws and modern detection methods, new or slightly modified variants (analogues) of synthetic cannabinoids are regularly synthesized and rapidly marketed to the recreational drug user market.

Within the field of forensic toxicology, there is therefore a growing need to rapidly detect and quantify these compounds. Unlike UHPLC-triple quadrupole mass spectrometry, traditional detection methods such as immunoassay and LC-UV do not have the required specificity, sensitivity or flexibility to cope with this ever-growing analytical challenge. This manuscript

describes the technology, sample preparation and the application of UHPLC-triple quadrupole mass spectrometry using multiple reaction monitoring (MRM) to rapidly and reliably quantify synthetic cannabinoids in human serum at the sub ng/mL level.

Figure 1: The EVOQ LC-TQ



The EVOQ Ion Source,
comprising:

1. VIP Heated-ESI
2. Active Exhaust
3. Orifice Interface
4. IQ Dual Ion Funnel

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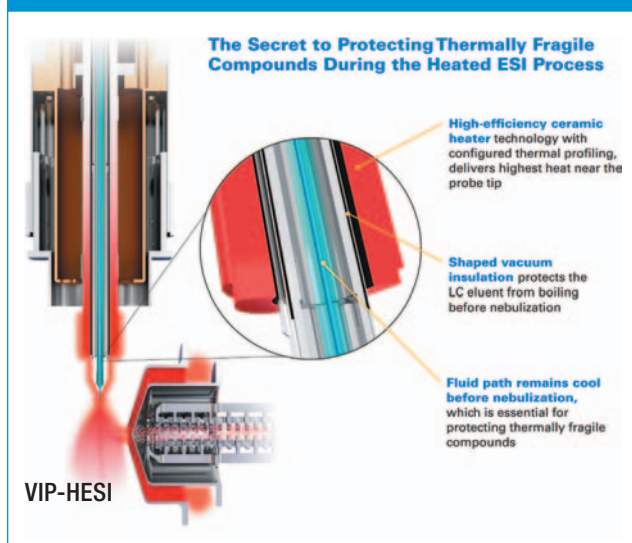
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The EVOQ LC-TQ

Synthetic cannabinoid serum concentrations are typically well below 1 ng/mL, therefore any method used for their analysis must be very sensitive. Frequent structural modification of these substances also means that analytical methods need to be regularly updated to cover newly emergent analytes. Analyses using the Bruker EVOQ LC Triple Quad system, as illustrated in **Figure 1** deliver, quantitative assays, with the sensitivity and flexibility required. The EVOQ is available in two versions, the EVOQ Qube for standard applications and the EVOQ Elite for the highest sensitivity applications. Both systems are supported with the Bruker Advance UHPLC system as well as the optional Bruker On-Line Extraction (OLE) module for automating sample clean up and extraction.

The EVOQ triple quadrupole electrospray ionization (ESI) ion source uses a heated vacuum insulated probe referred to as the VIP-HESI to assist nebulization. The high-efficiency ceramic heater technology delivers the highest heat near the probe tip and the vacuum insulation protects the LC eluent from boiling inside the probe prior to nebulization. As a result, the fluid path remains cool which is essential for protecting thermally labile compounds. The ion source is depicted in **Figure 2**; on the right side in red, is the high efficiency ceramic heater, in the middle, in blue, the capillary, and in between is the vacuum insulation that protects the LC eluent. The source housing is equipped with an active exhaust that consists of a nozzle through which a high amount of compressed air is expanded, working like a water jet pump. The expanded air is then routed to the exhaust, thus minimizing recirculation of gases within the source housing minimizing contamination and delivering maximum robustness.

Figure 2: EVOQ LC-TQ Ion Source



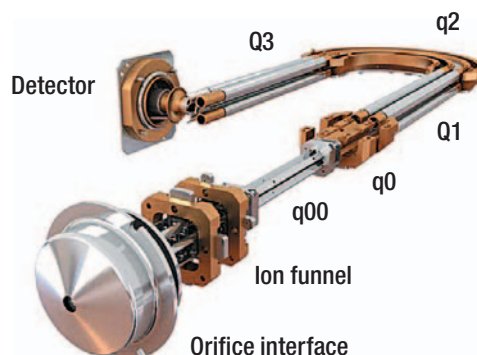
After ionization, the analytes pass through an orifice into the vacuum region of the dual ion funnel. The ion beam is fo-

cused through the ion funnel that consists of four quadrupoles wrapped with an interlaced chip.

Figure 3 shows the actual ion path with the mass filters Q1 and Q3 and the collision cell q2. The collision cell is bent in a 180-degree angle making it more compact (contributing to the smaller instrument footprint) compared to a linear one.

Figure 3: EVOQ LC-TQ Ion Path

Ion Optics and Quadrupoles



Also, since neutral particles are not focused by the electric field, they cannot travel around the 180-degree bend, resulting in less chemical noise, a higher signal to noise ratio and consequently higher sensitivity. The 90-degree angle from Q3 to the detector has the same effect.

MRM Method Development and Sample Preparation

The Bruker EVOQ LC Triple Quad system includes a comprehensive MRM compound library with more than 500 analytes with optimized transitions and collision energies. Analytes can be selected from the MRM library and saved to the method. For analytes not contained in the library, the MRM Builder tool can be used to determine the best transitions and their optimal collision energy. Optimization is performed by infusing a standard solution of the analyte or a mixture of analytes, identifying the precursor ion and the number of products desired as well as specifying the polarity in the software. The optimized transitions and collision energy can then be exported to the method and also added to a user library for future reference. For each analyte, a PDF report with the results is automatically generated and stored. The retention time and the retention time window for each analyte is then added to the method, along with the scan time determined automatically by the software according to the average peak width and the required number of data points per peak.

A typical synthetic cannabinoid testing regime may consist of many different types of analytes. The method must also be easy to update to accommodate newly emergent substances at the required sensitivity to be able to detect them in serum.

The sample preparation method and accompanying UHPLC gradient for synthetic cannabinoid testing was developed by

forensic scientists at the Institute of Legal Medicine in Freiburg, Germany, and is illustrated in **Figure 4**. The UHPLC and MS conditions are shown in **Figures 5**.

Figure 6 is an example of the resulting chromatogram showing the first ten eluting compounds of a 99-compound screen including some of the newest synthetic cannabinoids at a concentration of 50 pg/mL.

Method Validation

The synthetic cannabinoid method, once developed, must be validated to show that it is suitable for its intended use. The presented method has been validated according to the guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh). Validation parameters included selectivity, matrix effects and recovery, limits of detection and quantitation (LOD and LOQ), linearity, precision and accuracy.

To determine selectivity, blank serum from 10 individual donors was analyzed without the addition of analytes or internal

standards and two blank serum samples with the addition of internal standards. As there were no interfering signals for

Figure 4: Sample preparation

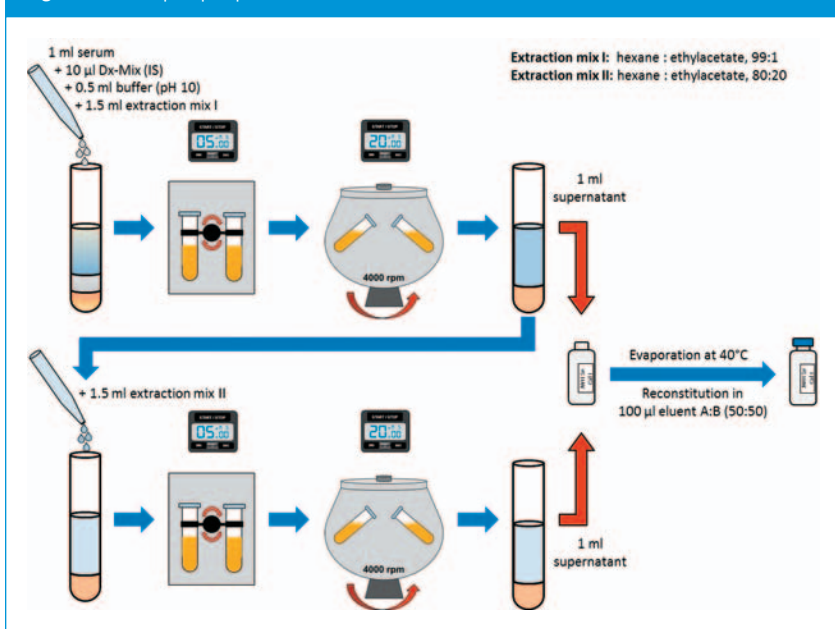


Figure 5: Conditions

LC system	Bruker Advance™ UHPLC
Eluent A	Water, 2 mM ammonium formate, 0.1% formic acid, 1% acetonitrile
Eluent B	Acetonitrile, 2 mM ammonium formate, 0.1% formic acid, 1% H ₂ O
Analytical column	Kinetex® C18 100A, 2.6µm (100 x 2.1mm)
Flow rate	0.5 ml/min
Injection volume	10 µl
Gradient:	<p>0.0 to 1.0 min: 20% B 1.0 to 2.5 min: 20% B to 60% B, linear 2.5 to 4.0 min: 60% B to 65% B, linear 4.0 to 5.5 min: 65% B 5.5 to 8.0 min: 65% B to 90% B, linear 8.0 to 10.0 min: 90% B 10.0 to 10.1 min: 90% B to 20% B, linear 10.1 to 12.0 min: 20% B</p>
Mass Spectrometer	Bruker EVOQ Elite™ Triple Quadrupol MS
Source	VIP-HESI, positive mode, 4700 V
Probe gas	50 units at 400°C
Cone gas	25 units at 350°C
Nebulizing gas	50 units
Collision gas	Argon, 1.5 mTorr
Mode	Multi Reaction Monitoring (MRM), 2 transitions per analyte, 1 transition per IS

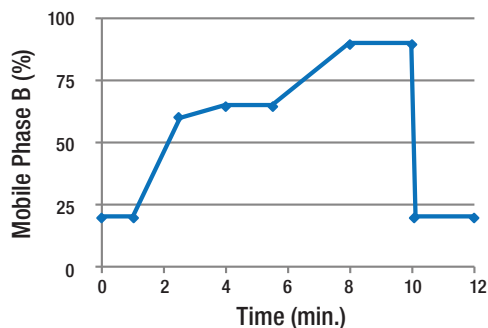
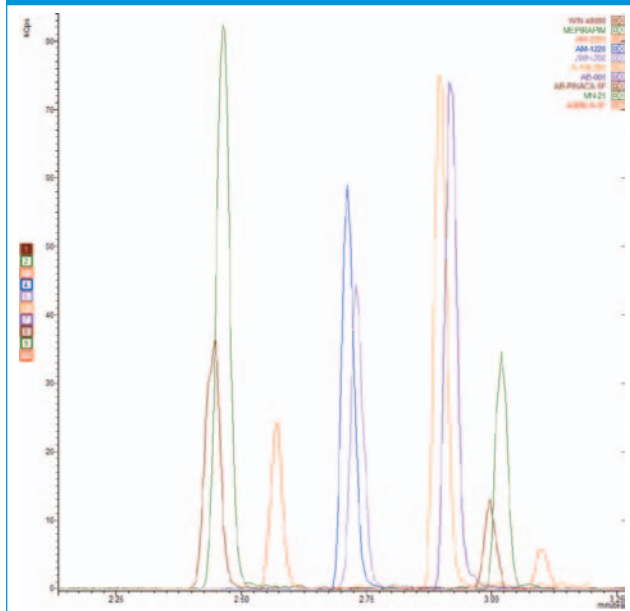


Figure 6: Chromatographic separation. First ten eluting compounds of lowest calibrator 50 pg/ml



the ion transitions of the analytes, the method was deemed to be selective. To examine matrix effects and recovery, five different serum samples from five different individuals spiked before extraction, five additional serum samples spiked after extraction and five neat standards were measured at a low (0.1 pg/mL) and a high concentration (1 ng/mL) and compared. The matrix effects and the recovery were then calculated according to Matuszewski et al (Strategies for the Assessment of Matrix Effects in Quantitative Bioanalytical Methods Based

on HPLC-MS/MS, B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, *Anal. Chem.*, 2003, 75 (13), pp 3019-3030). Matrix effects were within $\pm 25\%$ for 89 of the 99 analytes. Higher matrix effects can be compensated by the use of an appropriate internal standard. Recovery was $>50\%$ for 79 analytes.

LOD and LOQ were evaluated from calibration curves constructed with equidistant calibrators in the range of the expected LOD, (5 to 50 pg/mL) and calculated according to DIN 32645 (Deutsches Institut für Normung. DIN 32 645 Nachweis-, Erfassungs- und Bestimmungsgrenze, Beuth Verlag; Berlin, 1994). The LOD was ≤ 10 pg/mL for 89 analytes with the highest LOD being 50 pg/mL. The calculated LOQ was ≤ 30 pg/mL for 94 of the 99 analytes and was finally defined as the lowest calibrator concentration of 50 pg/mL.

To demonstrate linearity, six calibration curves with seven calibrators each were constructed, ranging from 50 pg/mL up to 1.25 ng/mL. A weighting factor of $1/x^2$ was used. Precision and accuracy were determined by the analysis of two replicates of low (0.05 ng/mL), medium (0.25 ng/mL) and high (1 ng/mL) QC samples on eight consecutive days. A bias (accuracy) within $\pm 15\%$ and RSD's of $<15\%$ were obtained.

Conclusion

A method for the sensitive quantitation and/or the semi-quantitative determination of synthetic cannabinoids including recently emerged substances has been developed using the Bruker EVOQ LC Triple Quad system. The method is easily customizable using the MRM builder to add new compounds to the user library. The low limits of detection obtained provide the necessary capability to prove the recent use of synthetic cannabinoids in forensic cases such as driving under the influence of drugs or in post-mortem cases.