APPLICATION NOTE

Liquid Chromatography/
Mass Spectrometry

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Analysis of Benzo[a]pyrene in Tobacco and Related Products by Ultra High-Performance Liquid Chromatography - Tandem Mass Spectrometry

Introduction

Benzo[a]pyrene (B[a]P) is a well-known carcinogenic compound found in tobacco products and tobacco smoke. The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) has recommended a gas chromatography/mass spectrometry (GC/MS) method to determine concentrations of B[a]P in tobacco products. In the proposed method, B[a]P is extracted from the tobacco with methanol, followed by a solid phase extraction (SPE) and subsequent concentration prior to GC/MS analysis.[1] The CORESTA recommended method is time-consuming, especially in sample preparation procedures, and showed low sensitivity. A recent study reported that B[a]P in tobacco filters could be analyzed utilizing liquid chromatography/atmospheric pressure photoionization paired with tandem mass spectrometry (LC/APPI-MS/MS).[2] APPI is an ionization process that uses a keypton lamp that produces UV light to charge gas phase analytes, but it is not a standard configuration on LC/MS/MS instruments. As such, it is not convenient to widely apply this method in tobacco testing labs performing routine analyses.

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In this application note, a simple, reliable and fast analytical method for the determination and quantification of B[a]P in tobacco products has been developed using a PerkinElmer QSight® LX50 UHPLC system, coupled to a PerkinElmer QSight 220 MS/MS detector. Tobacco cigarette leaf and filter (before smoking) were extracted with acetonitrile, and then analyzed directly by LC/MS/MS without SPE clean-up. The mass detection method was developed and optimized using an electrospray ionization (ESI) source. Two pairs of MRMs (m/z 253> 252 and 253>250) were monitored for all standards and unknown samples, and were used as quantitative and qualitative channels, respectively. Compared to the CORESTA recommended method, this study demonstrates a method that can significantly reduce sample preparation time and increase the specificity for target analysis.

Experimental

Hardware and Software

Chromatographic separation and subsequent detection were carried out using the PerkinElmer QSight LX50 UHPLC, coupled with the QSight 220 series tandem mass spectrometer. All instrument control, analysis and data processing were performed using the Simplicity 3Q™ software platform.

Solvents and Standards

All solvents and reagents used were LC/MS grade. A B[a]P standard was obtained from Dr. Ehrenstorfer GmbH. The stock solution was prepared by accurately weighing 1mg of standard dissolved in 10 mL of acetonitrile. The stock solution was kept in the refrigerator until usage. The stock standard was initially diluted to make an intermediate stock solution, which was subsequently used for the preparation of calibration standards. Calibration standard solutions (0.05, 0.1, 0.5, 1.0, 10.0, 50.0 and 100.0 ng/mL) were prepared daily in amber glass vials and sealed with polytetrafluoroethylene (PTFE) caps.

Sample Description

The cigarettes samples investigated in this study were purchased from a local store.

Sample Preparation

The method included the following steps:

a. Measure 1 g of cigarette leaf and 0.1 g filter (before smoking), and place them into 50 mL centrifuge tube.

b. Add 10 mL of acetonitrile to the tube and cap it.

c. Vortex for 1.5 minute.

d. Centrifuge extract in tube for 10 minutes at 6000 rpm.

e. Filter and transfer the supernatant into a 2 mL HPLC vial.

f. Inject 3 µL of sample for LC/MS/MS analysis.

Method Parameters

The LC method and MS parameters are presented in Table 1 and 2, respectively. Table 2 is further divided into 2a (MRM transitions with their respective optimized voltages) and 2b (MS source parameters). The MRM transitions, collision energy (CE), entrance voltage (EV) and collision cell lens 2 voltage (CCL2) were auto-optimized by software. MS source conditions, such as drying gas and nebulizer gas flow, as well as temperature settings, were optimized by flow injection analysis (FIA) method.

Results and Discussion

In this study, methanol and acetonitrile were compared as the extraction solvent. The results showed that acetonitrile gave higher extraction efficiency than methanol. In addition, when methanol was used as the extraction solvent, the repeatability of extraction results was poor. Thus, acetonitrile was selected as the extraction solvent for sample preparation.

Table 1. LC Method Parameters.

Column	PerkinElmer Brownlee C18, 100×2.1 mm, 2.7 µm (PN: N9308404)					
Mobile Phase	*Solvent A:* 5 mM ammonium acetate in water with 0.1 % formic acid *Solvent B:* Acetonitrile with 0.1 % formic acid					
	Step	Time (min)	Flow Rate (mL/min)	%A	%B	Curve
	1	Initial	0.3	20	80	
	2	4.00	0.3	20	80	Linear
Analysis Time	4.0 min					
Oven Temp.	45 °C					
Injection Volume	3 µL					

Table 2. MS Method Parameters.

a. MRM Transitions

Compound	Precursor Ion (m/z)	Product Ion (m/z)	EV(V)	CCL2(V)	CE (eV)
B[a]P	252.90	252.00	30	-93	-40
	252.90	250.00	30	-120	-75

b. MS Source Parameters

Parameter	Value
Ionization Mode	ESI; Positive
Drying Gas	75
HSID Temperature (°C)	320
Nebulizer Gas Setting	220
Electrospray Voltage (V)	5500
Source Temperature	500

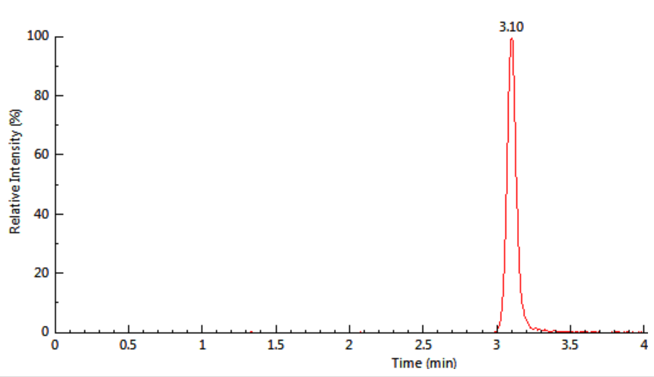


Figure 1. Typical TIC for B[a]P of 10 µg/L (m/z 253> 252 and 253>250 were used as quantitative and qualitative channels, respectively).

Linearity and Limit of Quantification

Good linearity was observed over a concentration range from 0.05 to 100 ng/mL, with a regression coefficient (r^2) ≥ 0.999 in a neat solution. Figure 2 shows the calibration curve for B[a]P.

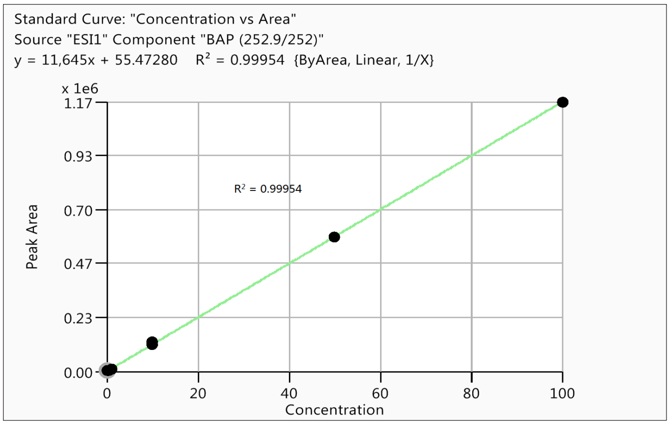


Figure 2. Calibration curve for B[a]P over range from 0.05 to 100 ng/mL.

The limit of quantification (LOQ) was calculated based on a signal-to-noise ratio ≥10 for the quantifier MRM transition. As described above, the LOQ of B[a]P was ≤ 0.05 ng/mL.

Recovery

An internal standard was not used in this test to evaluate the method recovery. The absolute recovery was studied by spiking known amounts of a reference standard into matrix blank samples.

A brand of cigarette was used as leaf and filter matrix blank separately.

Table 3 summarizes the precision and recovery for B[a]P in cigarette leaf. Recoveries of the whole analytical procedure at low and medium levels were 70.10 - 72.00%. The precision in terms of %RSD was less than 20%. The use of an isotope labeled B[a]P internal standard could significantly improve recovery and repeatability for the method.

Table 3. Average recoveries and % RSD at spiking level 1 and 10 ng/mL in cigarette leaf.

Analyte	Matrix Sample (ng/mL)	Spiked (ng/mL)	Detected (ng/mL)	% Recovery	% RSD (n=5)
B[a]P	0.78	1.00	1.50	72.00	17.50
	0.78	10.00	7.79	70.10	6.40

Table 4 summarizes the precision and recovery for B[a]P in the cigarette filter (before smoking). Recoveries of the whole analytical procedure at low and medium levels were 91.00 - 96.60%. The precision in terms of %RSD was less than 5%. The results demonstrated a good method for the analysis of B[a]P in cigarette filters. The method evaluation using filter samples (after smoking) will be described in a future study.

Table 4. Average recoveries and % RSD at spiking level 1 and 10 ng/mL in cigarette filter (before smoke).

Analyte	Matrix Sample (ng/mL)	Spiked (ng/mL)	Detected (ng/mL)	% Recovery	% RSD (n=5)
B[a]P	0.00	1.00	0.91	91.00	3.2
	0.00	10.00	9.66	96.60	2.6

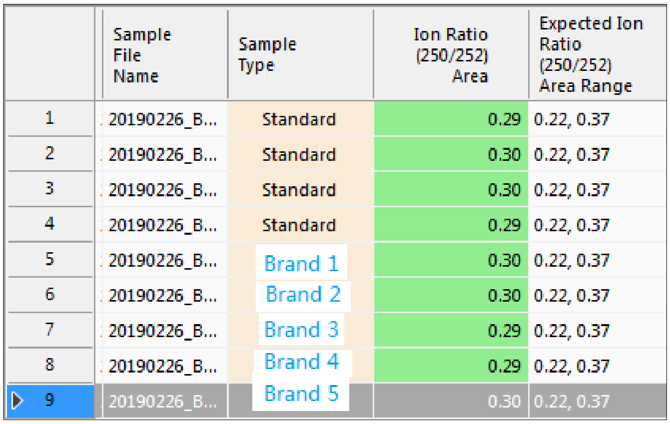
Sample Analysis

Five brands of cigarette samples were prepared, as shown in the sample preparation section, and subsequently analyzed. Table 5 shows the results of B[a]P in the five brands of cigarette leaf samples. Different quantities of B[a]P were detected in each of the cigarette leaf samples.

Table 5. Detected amounts of B[a]P present in the five brands of cigarette leaf.

Analyte	Brand 1 (ng/g)	Brand 2 (ng/g)	Brand 3 (ng/g)	Brand 4 (ng/g)	Brand 5 (ng/g)
B[a]P	7.8	12.5	5.2	36.4	3.1

To avoid false results, Simplicity 3Q software automatically calculated ion ratios, compared them against the reference values, and provided identification for all unknown samples in accordance with the criteria specified in the European Commission SANTE document 11945/2015.[3] Figure 3 shows the automated identification of results in the software based on the criteria of ion ratio.



	Sample File Name	Sample Type	Ion Ratio (250/252) Area	Expected Ion Ratio (250/252) Area Range
1	20190226_B...	Standard	0.29	0.22, 0.37
2	20190226_B...	Standard	0.30	0.22, 0.37
3	20190226_B...	Standard	0.30	0.22, 0.37
4	20190226_B...	Standard	0.29	0.22, 0.37
5	20190226_B...	Brand 1	0.30	0.22, 0.37
6	20190226_B...	Brand 2	0.30	0.22, 0.37
7	20190226_B...	Brand 3	0.29	0.22, 0.37
8	20190226_B...	Brand 4	0.29	0.22, 0.37
9	20190226_B...	Brand 5	0.30	0.22, 0.37

Figure 3. B[a]P identified by Simplicity 3Q software in the five brands of cigarette leaf.

Figures 4 and 5 show the MRM chromatograms of the quantifier and qualifier transition for B[a]P in the reference standard sample (1.0 ng/mL), as well as the cigarette leaf sample Brand 3, which was used to calculate ion ratios. In this study, the ion ratios are consistent between the reference standard samples and unknown samples, confirming B[a]P positively in the detected samples.

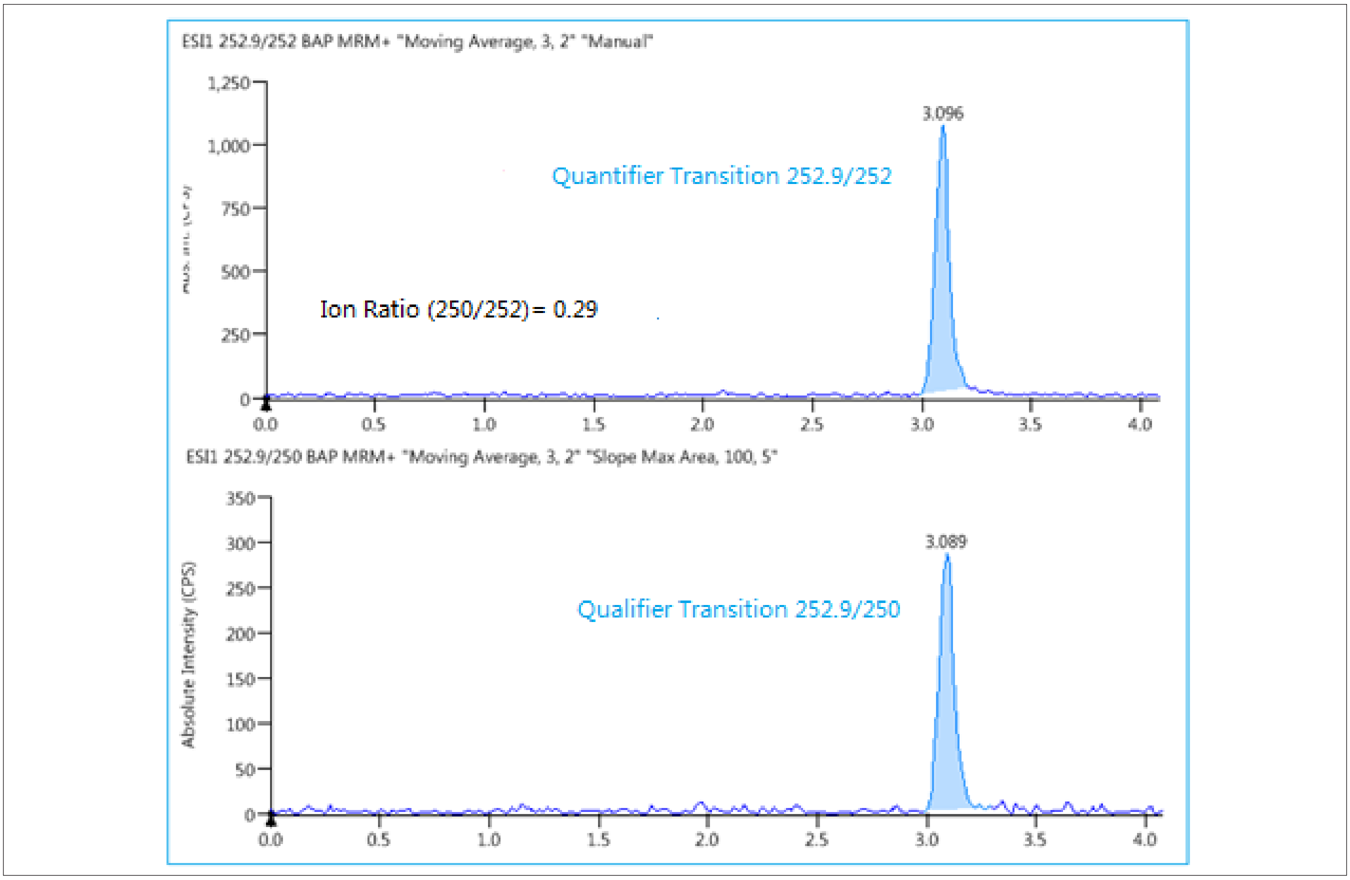


Figure 4. MRM chromatograms of quantifier and qualifier transition for B[a]P in the reference standard sample (1.0 ng/mL), which were used to calculate ion ratio (Ion Ration = 0.29).

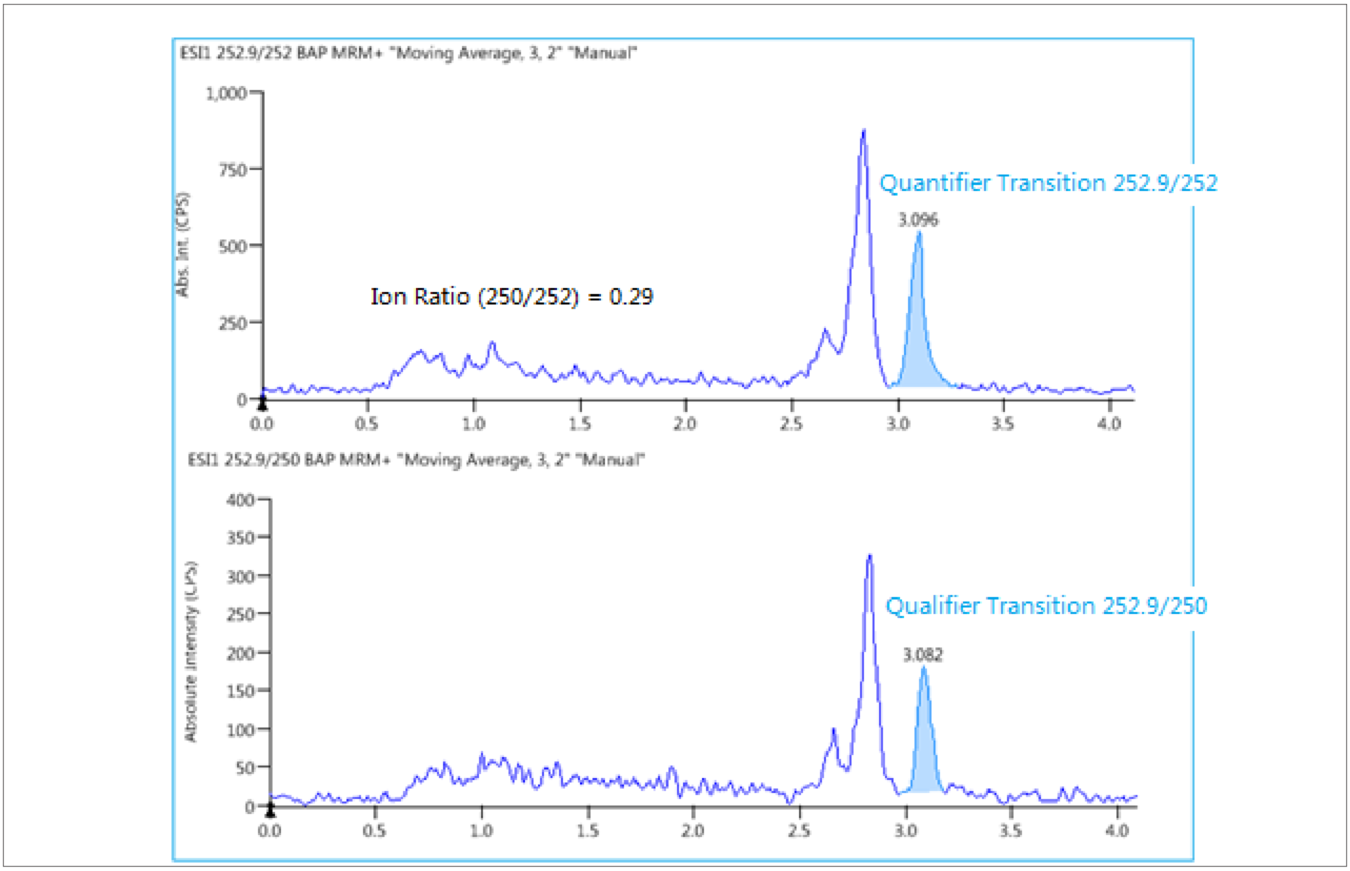


Figure 5. MRM chromatograms of quantifier and qualifier transition for B[a]P in Brand 3 cigarette leaf sample, which were used to calculate ion ratio (Ion Ratio = 0.29).

Conclusions

An easy and fast LC/MS/MS method for Benzo[a]pyrene analysis in tobacco products was developed by coupling a UHPLC system to a triple quadrupole mass spectrometer. The proposed method adopts a more approachable ESI technology, and avoids SPE and concentration steps for sample preparation, thus allowing for efficient and effective detection of B[a]P analysis in routine laboratory settings. Owing to the high sensitivity of the method, a small sample volume was used to reduce the potential for matrix effect and contamination.

Remark

For future work, a collaborator will be involved to investigate the B[a]P data of smoked cigarette filters utilizing this analytical method, which would demonstrate the human exposure to cigarette smoke.

References

1. Determination of Benzo[a]pyrene in tobacco products by GC – MS, CORESTA Recommended Method No. 82, March 2018.
2. Xiaotao Zhang, Hongwei, Hou. Novel method to analysis benzo[a]pyrene in filter by liquid chromatography/tandem mass spectrometry: Application to assess mouth level benzo[a]pyrene exposure. *Rapid Commun. Mass Spectrom.* 2014, 28, 1468-1472.
3. Guidance document on the analytical quality control and method validation procedures for pesticides residues analysis in food and feed. European Commission SANTE document 11945/2015.

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