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METHOD DEVELOPMENT AND VALIDATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) RESIDUE IN COCOA AND COCOA PRODUCTS

Badrul H. Z., Salsazali S.
Cocoa Downstream Research Division, Cocoa Innovation and Technology Centre, Lot 12621 Kawasan Perindustrian Nilai, 71800 Nilai, Negeri Sembilan, Malaysia
Corresponding author: badrul@koko.gov.my

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ABSTRACT –*A quick and simple extraction method was developed and validated for polycyclic aromatic hydrocarbon residues in cocoa beans and cocoa powder using acetonitrile extraction and GC-MS/MS detection for benz[a]anthracene, chrysene, benzo[a]pyrene, and benzo[b]fluoranthene. Different extraction parameters such as extraction volume, extraction time and clean-up adsorbents were studied for their extraction efficiency and repeatability, assessed by recovery and relative standard deviation, respectively. The final optimized method was acetonitrile extraction with 20 mL extraction volume with 10 minutes extraction time. In this study, we found that the results for clean-up using primary secondary amine (PSA) adsorbent gave poor extraction efficiency. Hence clean-up step using only magnesium sulphate was used in the final method. The recoveries for cocoa beans samples spiked at 10 and 50 ng/g were in the ranged from 70-120 % for cocoa beans and cocoa powder with RSDs below 20 %.*

Keywords: cocoa beans, cocoa powder, GC-MS/MS, PAHs, *Theobroma cacao*

INTRODUCTION

PAHs are formed through pyrolysis during incomplete combustion of practically all organic substances. They can be defined as organic compounds consisting of condensed aromatic rings (Raters and Matissek, 2014). Most of the PAHs are carcinogenic and genotoxic and food can be contaminated from environmental sources, industrial food processing, and from certain home cooking practice (Alexander, et al., 2008). Likewise, contamination in cocoa can occur by drying cocoa on asphalt, on bitumen in the sun, or by using direct drying processes (Ziegenhals, et al., 2009).

Previous study on method development and determination of PAHs in cocoa and cocoa products deal with chocolate, cocoa butter, cocoa beans and cocoa drink, using a variety of techniques and instrumentations such as gas chromatography mass spectrometry (GC-MS), gas chromatography triple quadrupole mass spectrometry (GC-MS/MS), gas chromatography flame ionization detector (GC-FID), high performance liquid chromatography fluorescence detector, and HPLC-Orbitrap (Belo, *et al*., 2017; Ciecierska, 2020; Iwegbue, *et al*., 2015; Misnawi, 2012; Raters and Matissek, 2014; Rozentale, *et al*., 2019; Sess-Tchotch, *et al*., 2018; Ziegenhals, *et al*., 2009; Żyżelewicz, *et.al*., 2017). Most of the previous sample preparation procedures involve specific instrumentation with high volume of solvent used that could increase the cost and time of analysis.

The purpose of this work is to analyse four types of PAHs in dried cocoa beans and cocoa powder using a fast and easy to use solvent extraction technique and dispersive solid phase extraction (d-SPE) for clean-up purpose. The comprehensive workflow was validated and applied to cocoa beans and cocoa products and found to be highly reliable, precise, sensitive, and fit for purpose for PAHs analysis in cocoa and cocoa products.

MATERIAL AND METHODS

Reagents and materials

HPLC grade acetonitrile, anhydrous magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) were all obtained from Merck (Darmstadt, Germany). Water was purified through an ElgaPurelab Option-Q system (High Wycombe, UK). Two mL mini-centrifuge tube containing 150 mg $MgSO_4$, 50 mg C18, and 50 mg primary secondary amine (PSA) was purchased from Agilent Technologies (Palo Alto, USA).

PAHs reference standards of all analytes were purchased from Dr.Ehrenstorfer (Augsburg, Germany). Individual PAH stock solutions (~1000 µg mL^{-1}) were prepared in acetonitrile for benzo[b]fluoranthene, benzo(a)pyrene, benz[a]anthracene, and dichloromethane for chrysene and kept at -20 °C in the dark. Mixed intermediate standard solutions (10 µg mL^{-1}, 1 µg mL^{-1} and 0.1 µg mL^{-1}) of multiple PAHs were prepared by diluting an appropriate volume of each individual stock standard solution in acetonitrile. All working solutions containing the target PAHs were prepared freshly by dilutions of the intermediate standard solution in acetonitrile and kept in scintillation vials at 4 °C in the refrigerator.

Cocoa beans samples for fortification

Organic dried cocoa beans were obtained from Cocoa Research and Development Centre, Tawau. The samples were used for blanks, fortified shaken using 1500 ShaQer for 10 min, then centrifuged at 12000 rpm for 5 min at 4 °C. Hereafter, 1 mL of the supernatant was transferred into d-SPE tube. The tube was vortexed for 30 s. After centrifugation at 12000 rpm for 5 min, an aliquot of 0.5 mL extract was filtered through 0.2 µm PVDF filter into autosampler vial. This 0.5 mL extract was then diluted with 0.5 mL acetonitrile and injected into GC-MS/MS.

Gas chromatography-triple quadrupole mass spectrometry analysis

GC-MS/MS analysis was performed using an Agilent 7890A GC equipped with an Agilent 7693B autosampler and an Agilent 7000B triple quadrupole mass spectrometry system (Agilent Technologies, Palo Alto, USA). HP-5MS 30 m x 0.25 mm i.d. x 0.25 µm film thickness was used for the chromatographic separation of the compounds. Five µL injection volume was performed using a 7890A GC multimode inlet system operated in a PTV solvent vent injection mode. In this mode, injector temperature was ramped from 70 °C to 325 °C at 600 °C/min. Helium (99.999%) was used as carrier gas and quenching gas at a flow rate of 1.2 mL/min (constant flow) and 2.25 mL/min, respectively. Nitrogen (99.999%) was used as the collision gas at a flow rate of 1.5 mL/min. The initial oven temperature was 70 °C, with an initial time of 0.1 min. The oven was heated to 170 °C at

samples for recovery assays and matrix-matched standards for calibration in the experiments. The whole laboratory samples were ground using Retsch ZM 200 ultra-centrifugal mill (Haan, Germany) so that the greatest dimension of the particles does not exceed 1 mm, while avoiding the formation of paste. Subsequently, representative portions of previously homogenised samples were weighed and transferred into 50 mL screw cap centrifuge tubes and fortified with 200 µL and 100 µL from 0.1 µg mL^{-1} and 1 µg mL^{-1} intermediate standard solution respectively. The samples were then allowed to stand at room temperature for 1 hour until analysis to give final spiking concentration levels of 10 µg/kg and 50 µg/kg.

Extraction and clean-up procedure

The samples were extracted according to the original unbuffered QuEChERS method (Anastassiades et al., 2003) with some modification. After homogenization, 2 g of samples were weighed in a 50 mL screw cap centrifuge tubes and fortified with intermediate standard solution to give final spiking concentration of 10 and 50µg/kg. Deionised water was added and the mixtures were homogenised using a vortex mixer for 30 seconds and left to stand at room temperature formatrix swelling (hydration). Then, acetonitrile was added to the samples. The tubes were shaken using SPEX SamplePrep 1500 ShaQer (New Jersey, USA) for 1 min. After that, 4 g $MgSO_4$ and 1 g NaCl were added and the mixtures were immediately

50 °C/min and then to 300 °C at 15°C/min. The final temperature was held for 4 min and the total run time was 14.767 min. The mass spectrometer was operated in electron impact ionization (EI) mode. The temperatures of the transfer line, ion source, quadrupole 1 and quadrupole 2 were 280 °C, 300 °C, 180 °C and 180 °C respectively. Agilent MassHunter B.05.00 software was used for instrument control and data analysis.

Analytical method validation

Validation of the analytical method for cocoa beans and cocoa powder was performed as described in SANTE/11945/2015 (SANTE/EU, 2015). The method was tested to assess for validation parameters and criteria in terms of linearity, method detection limit (MDL), limit of quantification (LOQ), specificity, accuracy, and precision. The calibration curves were plotted to obtain the linearity of the system at six calibration levels ranging between 0.5 and 10 ng/mL (data not shown). Matrix-matched calibration standards were used for quantification of the analytes. Method detection limit (MDL) was defined as 3 x standard deviation of at least 7 low concentration replicates (10 ng/g) analyzed over 3 days. Limit of quantification (LOQ) was defined as 3 x standard deviation obtained in MDL study. Specificity of the proposed method was assessed by analyzing the response in both blank and control samples. The accuracy of the method was expressed in terms of average recoveries of spiked blank matrix at 10 and 50 ng/g concentration levels. Precision of the method was represented as relative standard deviation (RSD %) of within-laboratory repeatability (same day) and reproducibility (different days) analyses.

RESULTS AND DISCUSSION

Optimization of extraction and clean-up procedure

In this study, four extraction and clean-up procedures were compared to evaluate the extraction efficiency and clean-up effectiveness of cocoa matrices (**Figure 1**). The hydration of samples was studied by adding appropriate volume of water into the samples prior to solvent extraction. According to previous study, matrix hydration is an important step in order to achieve maximum extraction efficiency in dry and high fat cocoa matrices (Zainudin and Salleh, 2017). Acetonitrile was chosen as the solvent of choice for PAHs extraction based on the original QuEChERS method (Anastassiades, et al., 2003). In this study, extraction volume was varied between 10, 15 and 20 mL to study the extraction efficiency of acetonitrile in cocoa matrices for PAHs. Finally, the clean-up effect was studied by varying the adsorbent used such as $MgSO_4$, C18, and primary secondary amine (PSA). Comparison was also made between clean-up procedure with no clean-up procedure.

Recoveries and RSD values were evaluated for each procedure and results were tabulated in **Table 1**. Method 1 and Method 2, which deals with the extraction of PAHs using 10 and 15 mL acetonitrile respectively and d-SPE clean-up (150 mg $MgSO_4$ + 50 mg C_{18} + 50 mg PSA), resulted in acceptable recoveries for both chrysene and benz[a]anthracene at 75.77 to 100.28 %. However, recoveries for benzo(a)pyrene and benzo(b)fluoranthene were rather poor and were ranged from 42.94 to 57.93 %. When the d-SPE clean-up step was omitted, we found that recoveries for chrysene and benz[a]anthracene decreased while recoveries for benzo(a)pyrene and benzo(b)fluoranthene increased slightly. Increasing the extraction volume to 20 mL and removing C18 and PSA in the clean-up step bring about Method 3. Results showed that recoveries for all four PAHs greatly improved compared to previous methods. Recoveries for Method 3 were ranged from 71.39 to 88.24 %. As in the case for Method 1 and 2, omitting the clean-up step gave poor recoveries where most of the PAHs studied presented recoveries below 70 %, except for benzo(b)fluoranthene. The application of PSA as adsorbent in d-SPE clean-up was known to decrease the recoveries of certain pesticides (Muhamad, et al., 2012; Zainudin, et al., 2015). In this case, PSA affected the extraction efficiency by not only adsorbing the interferences, but also adsorb benzo(a)pyrene and benzo(b)fluoranthene, hence resulted in poor recoveries for both

compounds. Finally, to study the effect of C18 in the clean-up step, the combination of $MgSO_4$ and C18 was proposed as in Method 4. Results showed that most of PAHs gave good recoveries except for benz[a]anthracene, in which recovery of 64.24 % was observed. Nevertheless, recoveries of some of the PAHs were still below the acceptable range of 70-120 % according to (SANTE/EU, 2015).

Method validation

20 mL acetonitrile with $MgSO_4$ d-SPE clean-up (Method 3) was chose as the optimized method for validation study in cocoa beans and cocoa powder. Good linearity was achieved for all four PAH studied in cocoa beans and cocoa powder using GC-MS/MS with correlation coefficients better than 0.990 and most of the residuals were below 20 % (data not shown). The selectivity of the analytical method and the instrumentation used was determined by comparing the chromatograms of a blank matrix solution with the spiked matrix solution as can be seen in **Figure 2 – 5**. In the blank matrix, interferences were less at the analytes' retention times. As a result, in the spiked samples, we can see that the analytes of interest were well separated from the other components present in the matrix and hence allowed the differentiation and quantification of the analytes. This shows that the method developed could remove most of the interferences in matrices and thus exhibited its specificity. Recoveries obtained for PAHs in cocoa beans and powder were in the 70-120% ranged with RSD values less than 20 % (**Table 2 – 5**). Both the recovery and RSD values met the method performance criteria and indicate the good precision and accuracy of the proposed method. The MDL obtained were ranged between 1.15 to 5.6 ng/g while the LOQ were ranged between 3.44 to 16.8 ng/g. Therefore, it can be concluded that the method is sensitive enough to quantify PAHs in cocoa beans and cocoa powder.

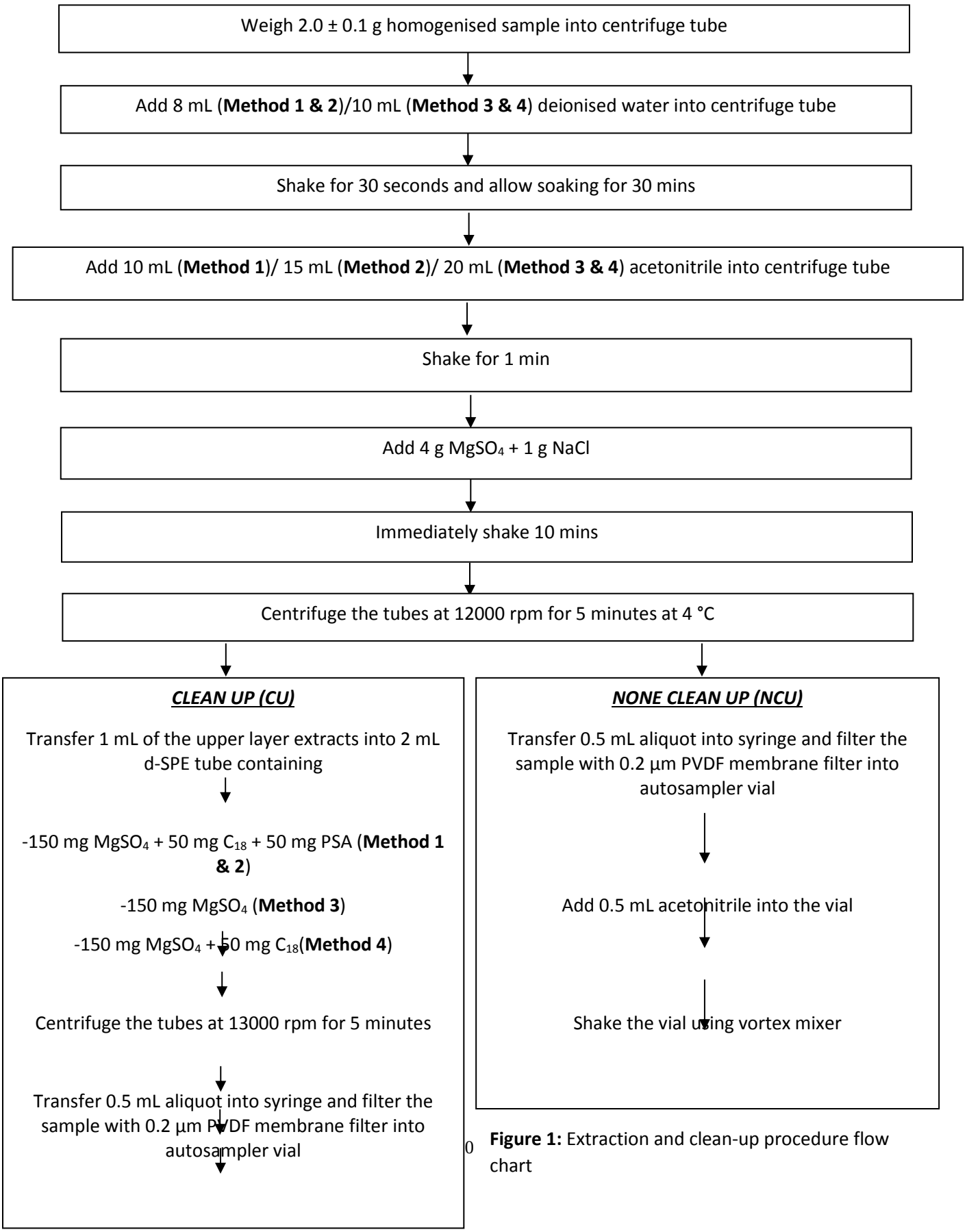

Figure 1: Extraction and clean-up procedure flow chart

Table 1: Recovery values (%) and RSD (%) for different extraction and clean-up procedures

Experiment details	Clean Up (CU)/None Clean Up (NCU)	Recovery (%) ± RSD (%)			
		Chrysene	**Benz[a]anthracene**	**Benzo[a]pyrene**	**Benzo[b]fluoranthene**
Method 1	CU	*83.81 ± 18.287*	*75.77 ± 0.674*	*45.04 ± 4.731*	*48.05 ± 0.172*
	NCU	*65.31 ± 0.504*	*73.37 ± 1.654*	*55.39 ± 0.384*	*50.7 ± 1.186*
Method 2	CU	*100.28 ± 4.666*	*88.42 ± 6.569*	*42.94 ± 0.508*	*57.93 ± 2.758*
	NCU	*77.19 ± 1.937*	*63.58 ± 0.366*	*65.43 ± 2.930*	*56.97 ± 1.214*
Method 3	CU	*71.39 ± 2.374*	*88.24 ± 7.077*	*87.44 ± 4.484*	*76.23 ± 7.139*
	NCU	*61.79 ± 1.948*	*68.52 ± 3.388*	*56.20 ± 0.264*	*92.26 ± 5.997*
Method 4	CU	*71.87 ± 1.177*	*64.24 ± 0.038*	*76.41 ± 2.445*	*72.31 ± 0.561*

Method 1: Extraction (8 mL H_2O + 10 mL acetonitrile); Clean-up (150 mg $MgSO_4$ + 50 mg C_{18} + 50 mg PSA)

Method 2: Extraction (8 mL H_2O + 15 mL acetonitrile); Clean-up (150 mg $MgSO_4$ + 50 mg C_{18} + 50 mg PSA)

Method 3: Extraction (10 mL H_2O + 20 mL acetonitrile); Clean-up (150 mg $MgSO_4$)

Method 4: Extraction (10 mL H_2O + 20 mL acetonitrile); Clean-up (150 mg $MgSO_4$ + 50 mg C_{18})

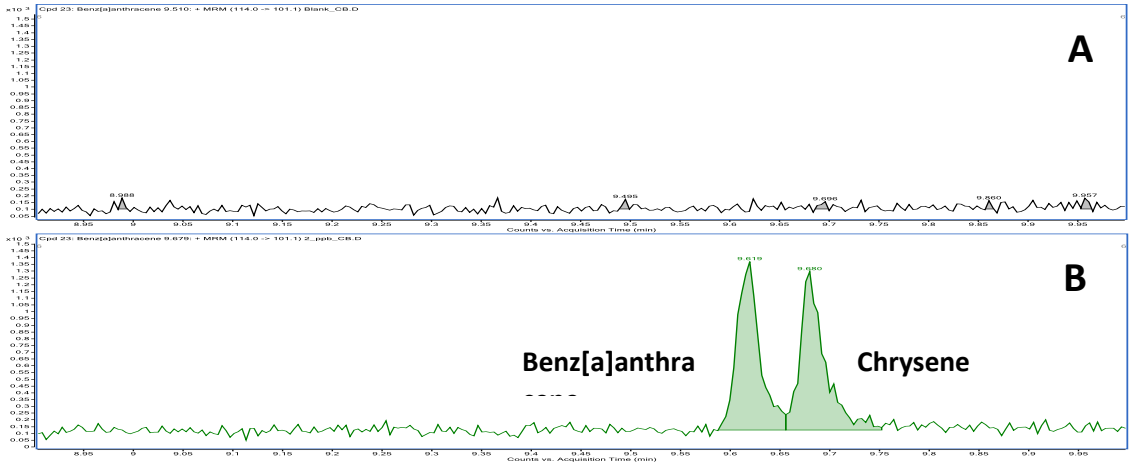

Figure 2: (A) Blank and (B) spiked cocoa beans at 2 ng/g

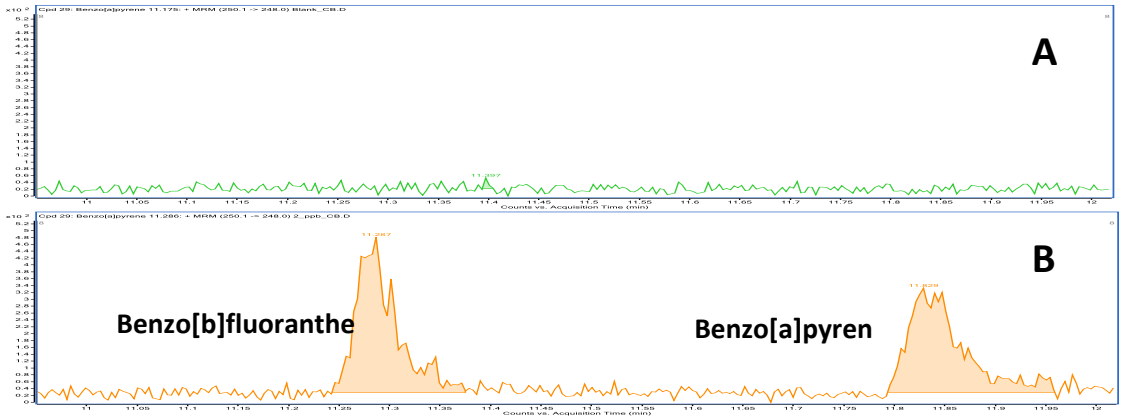

Figure 3: (A) Blank and (B) spiked cocoa beans at 2 ng/g

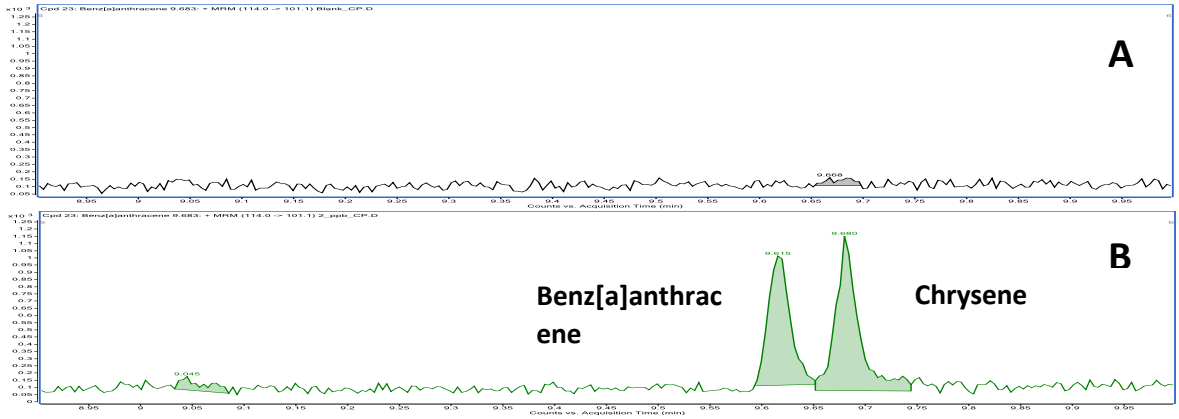

Figure 4: (A) Blank and (B) spiked cocoa powder at 2 ng/g

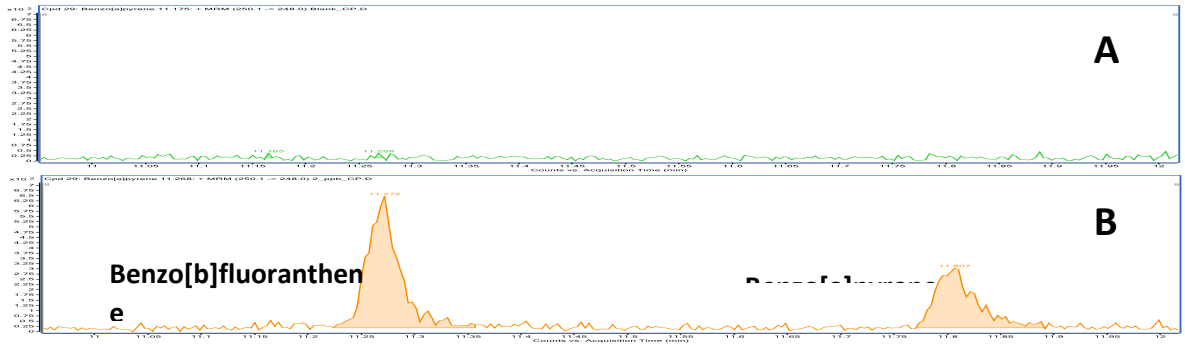

Figure 5: (A) Blank and (B) spiked cocoa powder at 2 ng/g

Table 2: Recovery and RSD, MDL, and LOQ values for benz[a]anthracene in cocoa beans and cocoa powder

Benz[a]anthracene

Sample	**Spiking level: 10 ng/g**			**Spiking level: 50 ng/g**		
	Recovery (%)	SD	RSD (%)	Recovery (%)	SD	RSD (%)
Cocoa beans (n = 15)	73.32	3.82	5.21	75.12 (n = 13)	2.20	2.92
Cocoa powder (n = 9)	72.18	5.72	7.92	78.96 (n = 15)	6.35	8.04

Sample	**Spiking level: 10 ng/g**			**MDL (ng/g)**	**LOQ (ng/g)**
	Concentration (ng/g)	SD	RSD (%)		
Cocoa beans (n = 15)	7.33	0.38	5.21	1.15	3.44
Cocoa powder (n = 9)	7.22	0.57	7.92	1.72	5.15

Table 3: Recovery and RSD, MDL, and LOQ values for chrysene in cocoa beans and cocoa powder

Chrysene

Sample	**Spiking level: 10 ng/g**			**Spiking level: 50 ng/g**		
	Recovery (%)	SD	RSD (%)	Recovery (%)	SD	RSD (%)
Cocoa beans (n = 15)	87.80	11.69	13.31	80.06 (n = 13)	2.73	3.41
Cocoa powder (n = 9)	104.11	17.46	16.77	80.12 (n = 15)	4.37	5.46

Sample	**Spiking level: 10 ng/g**			**MDL (ng/g)**	**LOQ (ng/g)**
	Concentration (ng/g)	SD	RSD (%)		
Cocoa beans (n = 15)	8.78	1.17	13.31	3.51	10.52
Cocoa powder (n = 9)	10.41	1.75	16.77	5.24	15.71

Table 4: Recovery and RSD, MDL, and LOQ values for benzo(b)fluoranthene in cocoa beans and cocoa powder

Benzo[b]fluoranthene

Sample	Spiking level: 10 ng/g			Spiking level: 50 ng/g		
	Recovery (%)	SD	RSD (%)	Recovery (%)	SD	RSD (%)
Cocoa beans (n = 15)	87.74	13.16	15.00	74.37 (n = 14)	5.62	7.56
Cocoa powder (n = 15)	93.93	18.67	19.88	80.43 (n = 15)	8.69	10.81

Sample	Spiking level: 10 ng/g			MDL (ng/g)	LOQ (ng/g)
	Concentration (ng/g)	SD	RSD (%)		
Cocoa beans (n = 15)	8.77	1.32	15.00	3.95	11.85
Cocoa powder (n = 15)	9.39	1.87	19.88	5.60	16.80

Table 5: Recovery and RSD, MDL, and LOQ values for benzo(a)pyrene in cocoa beans and cocoa powder

Benzo[a]pyrene

Sample	Spiking level: 10 ng/g			Spiking level: 50 ng/g		
	Recovery (%)	SD	RSD (%)	Recovery (%)	SD	RSD (%)
Cocoa beans (n = 15)	85.43	7.63	8.93	76.10 (n = 14)	5.25	6.89
Cocoa powder (n = 10)	83.37	16.48	19.77	76.92 (n = 15)	5.68	7.38

Sample	Spiking level: 10 ng/g			MDL (ng/g)	LOQ (ng/g)
	Concentration (ng/g)	SD	RSD (%)		
Cocoa beans (n = 15)	8.54	0.76	8.93	2.29	6.86
Cocoa powder (n = 10)	8.34	1.65	19.77	4.94	14.83

CONCLUSIONS

Efficient and rapid sample extraction and clean-up procedure based on acetonitrile extraction and d-SPE clean using $MgSO_4$ was successfully developed and validated for benz[a]anthracene, chrysene, benzo[a]pyrene, and benzo[b]fluoranthene in cocoa beans and cocoa powder. The clean-up procedure plays an important part in the overall procedure where without it matrix interferences could interfere with the analysis and hence preventing the correct analyte quantitation. Finally, the method validation data demonstrated that the proposed optimized method is selective, sensitive, accurate, and precise within the established linearity range.

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REFERENCES

Alexander, J., Benford, D., Cockburn, A., Cravedi, J., Dogliotti, E., Domenico, A. Di, Verger, P. (2008). Polycyclic Aromatic Hydrocarbons in Food - Scientific Opinion of the Panel on Contaminants in the Food Chain. *EFSA Journal*, *6*(8), 1–114. https://doi.org/10.2903/j.efsa.2008.724

Anastassiades, M., Lehotay, S. J., Stajnbaher, D., & Schenck, F. J. (2003). Fast and Easy Multiresidue Method Employing Acetonitrile. *Journal of AOAC International*, *86*(2), 412–431.

Belo, R. F. C., Figueiredo, J. P., Nunes, C. M., Pissinatti, R., Souza, S. V. C. de, & Junqueira, R. G. (2017). Accelerated solvent extraction method for the quantification of polycyclic aromatic hydrocarbons in cocoa beans by gas chromatography–mass spectrometry. *Journal of Chromatography B*, *1053*, 87–100. https://doi.org/10.1016/j.jchromb.2017.03.017

Ciecierska, M. (2020). Cocoa beans of different origins and varieties and their derived products contamination with polycyclic aromatic hydrocarbons. *Food Chemistry*, *317*(September 2019), 126408. https://doi.org/10.1016/j.foodchem.2020.126408

Iwegbue, C. M. A., Agadaga, H., Bassey, F. I., Overah, L. C., Tesi, G. O., & Nwajei, G. E. (2015). Concentrations and profiles of polycyclic aromatic hydrocarbons in some commercial brands of tea-, coffee-, and cocoa-based food drinks in Nigeria. *International Journal of Food Properties*, *18*(10), 2124–2133. https://doi.org/10.1080/10942912.2014.908906

Misnawi. (2012). Effect of cocoa bean drying methods on polycyclic aromatic hydrocarbons contamination in cocoa butter. *International Food Research Journal*, *19*(4), 1589–1594.

Muhamad, H., Zainudin, B. H., & Abu Bakar, N. K. (2012). Comparative study of different clean-up techniques for the determination of λ-cyhalothrin and cypermethrin in palm oil matrices by gas chromatography with electron capture detection. *Food Chemistry*, *134*(4), 2489–2496. https://doi.org/10.1016/j.foodchem.2012.04.095

Raters, M., & Matissek, R. (2014). Quantitation of polycyclic aromatic hydrocarbons (PAH4) in cocoa and chocolate samples by an HPLC-FD method. *Journal of Agricultural and Food Chemistry*, *62*(44), 10666–10671. https://doi.org/10.1021/jf5028729

Rozentale, I., Zacs, D., & Bartkevics, V. (2019). Application of Dopant-Assisted Atmospheric Pressure Photoionisation HPLC-MS Method for the Sensitive Determination of Polycyclic Aromatic Hydrocarbons in Dark Chocolate. *Journal of Chromatographic Science*, *57*(3), 220–229. https://doi.org/10.1093/chromsci/bmy103

SANTE/EU. (2015). *Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed* (Vol. 11945).

Sess-Tchotch, D. A., Kedjebo, K. B. D., Faulet, B. M., Fontana-Tachon, A., Alter, P., Durand, N., … Guehi, T. S. (2018). Analytical Method Validation and Rapid Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Cocoa Butter Using HPLC-FLD. *Food Analytical Methods*, *11*(11), 3138–3146. https://doi.org/10.1007/s12161-018-1282-2

Zainudin, B. H., & Salleh, S. (2017). Method Development, Optimization and Validation of Matrix Hydration Effect on Pesticide

Residues in Cocoa Beans Using Modified QuEChERS Method and Gas Chromatography Tandem Mass Spectrometry. *Food Analytical Methods*, 1–12. https://doi.org/10.1007/s12161-017-0954-7

Zainudin, B. H., Salleh, S., Mohamed, R., Yap, K. C., & Muhamad, H. (2015). Development, validation and determination of multiclass pesticide residues in cocoa beans using gas chromatography and liquid chromatography tandem mass spectrometry. *Food Chemistry*, *172*, 585–595. https://doi.org/10.1016/j.foodchem.2014.09.123

Ziegenhals, K., Speer, K., & Jira, W. (2009). Polycyclic aromatic hydrocarbons (PAH) in chocolate on the German market. *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit*, *4*(2), 128–135. https://doi.org/10.1007/s00003-009-0478-1

Żyżelewicz, D., Oracz, J., Krysiak, W., Budryn, G., & Nebesny, E. (2017). Effects of various roasting conditions on acrylamide, acrolein, and polycyclic aromatic hydrocarbons content in cocoa bean and the derived chocolates. *Drying Technology*, *35*(3), 363–374. https://doi.org/10.1080/07373937.2016.1175470