Supporting Information

Optimization and validation of a cheaper, safer, and more sustainable methodology for aflatoxins determination in rich-lipidic matrices (pistachio nuts) using deep eutectic solvent extraction and UHPLC-FLD analysis

Andrea Schincaglia[a,b], Luisa Pasti[c], Alberto Cavazzini [a], Giorgia Purcaro *[b], Marco Beccaria *[a,d]

[a]*University of Ferrara, Department of Chemical Pharmaceutical, and Agricultural Sciences, Via Luigi Borsari 46, 44121, Ferrara, Italy.*

[b]*University of Liège, Gembloux Agro-Bio Tech, Passage des Déportés 2, 5030, Gembloux, Belgium.*

[c]*University of Ferrara, Department of Environmental and Prevention Sciences, Via L. Borsari 46, 44121 Ferrara, Italy.*

[d]*University of Liège, Organic and Biological Analytical Chemistry Group, MolSys Research Unit, 4000 Liège, Belgium*

*Corresponding authors:

E-mail address: gpurcaro@uliege.be (G. Purcaro)

Email address: marco.beccaria@unife.it (M. Beccaria)

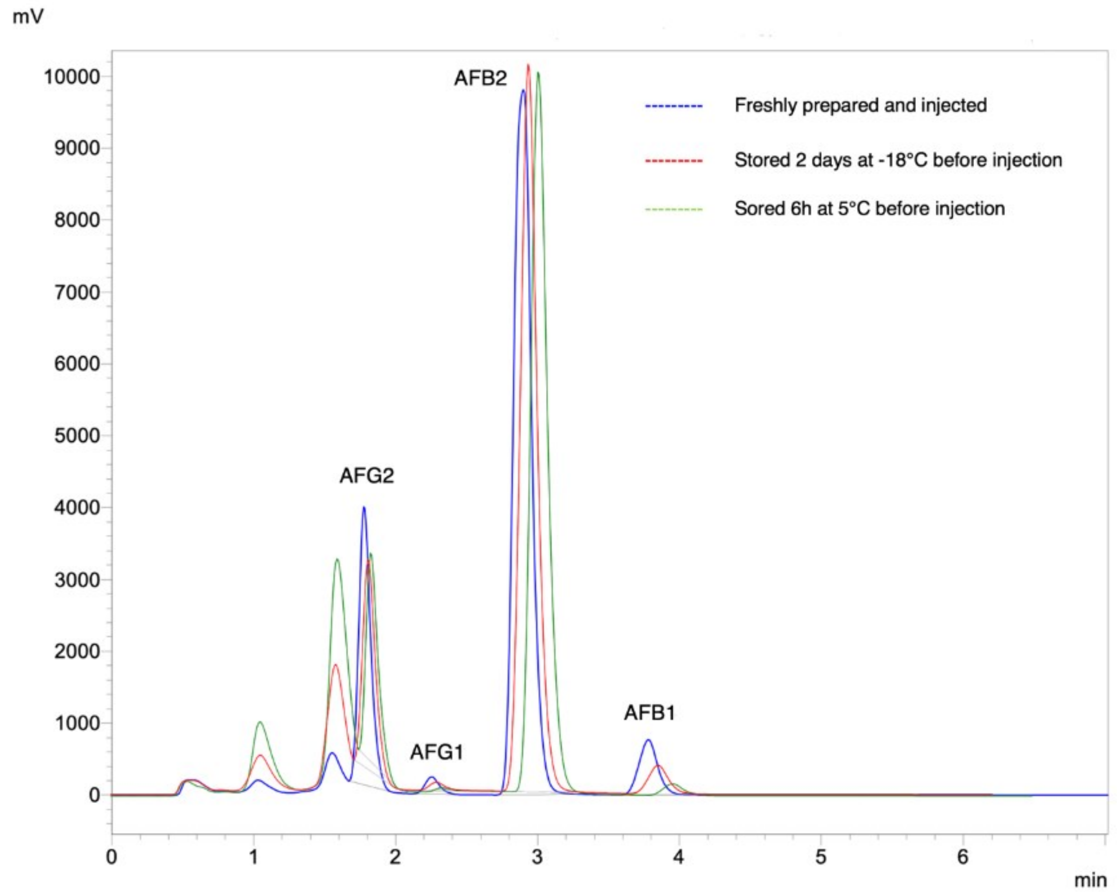

Figure S1. Chromatograms showing the stability of a 20 ng/mL AF-TOT standard solution in DES-4 under different storage conditions over time. Notable decreases in peak areas, particularly for AFB1 and AFG2, are observed with delayed injections. Chromatograms were acquired using an initial isocratic elution program with 60% H_2O and 40% MeOH over 6.5 min. The injection volume was 10 µL, with detection at 365 nm excitation and 430 nm emission wavelengths.

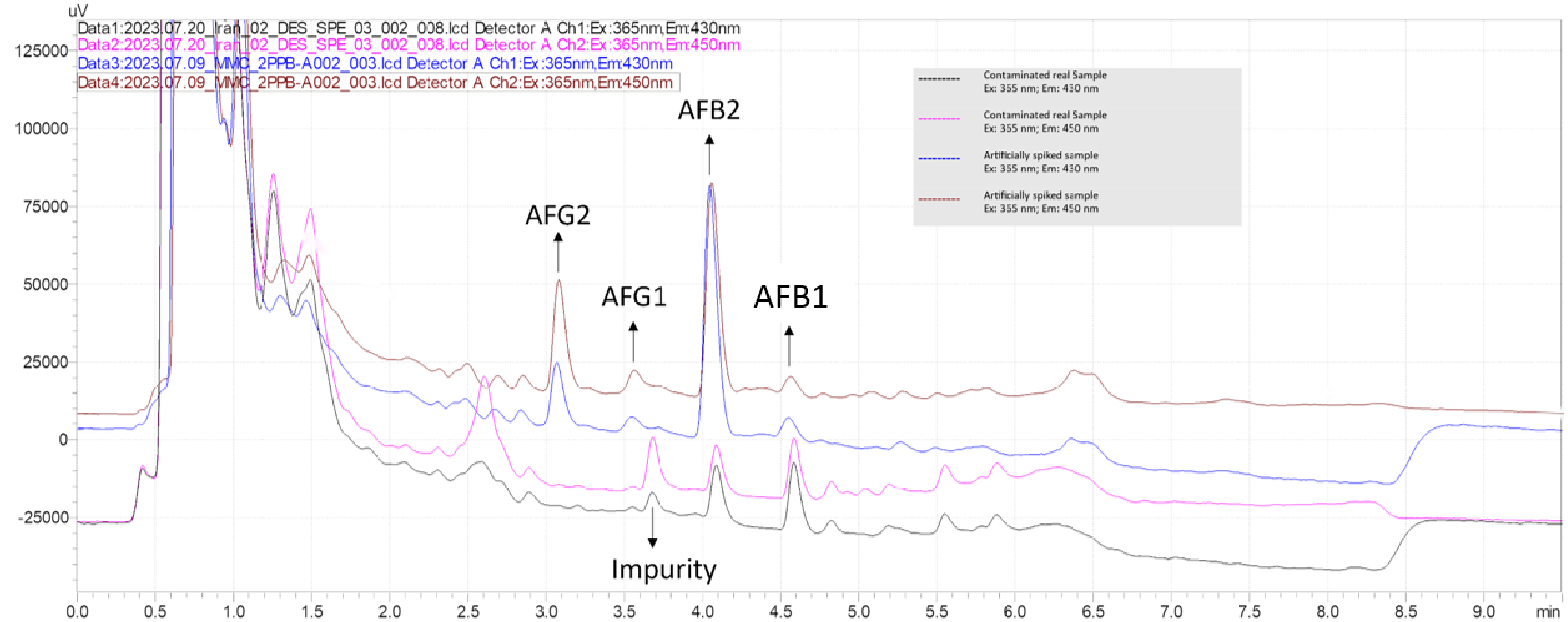

Figure S2. Comparison of different chromatograms: black) contaminated real-life sample from Iran (named Iran-2), acquired at 365 nm excitation wavelength and 430 nm emission wavelength; pink) contaminated real-life sample from Iran (named Iran-2), acquired at 365 nm excitation wavelength and 450 nm emission wavelength; blue) artificially contaminated sample used for the matrix-matched calibration curve, acquired at 365 nm excitation wavelength and 430 nm emission wavelength; brown) artificially contaminated sample used for the matrix-matched calibration curve, acquired at 365 nm excitation wavelength and 450 nm emission wavelength.

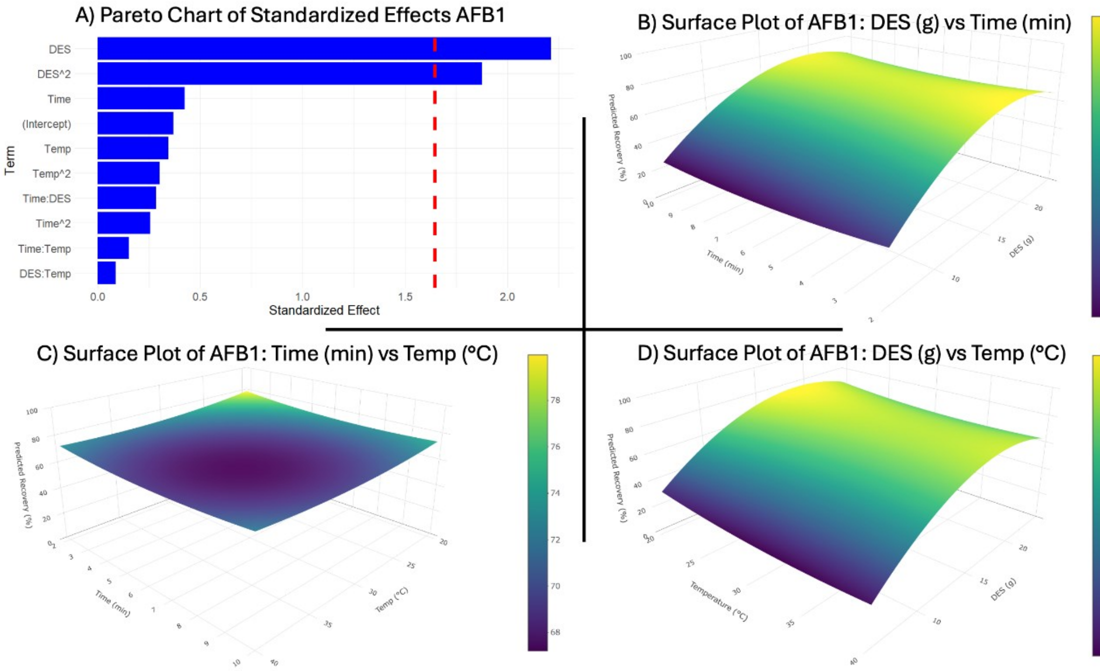

Figure S2. Results summary for **AFB$_1$** from the DoE (k=3). A) Pareto Chart of the Standardized Effects; B) Response Surface AFB$_1$: Predicted Recovery vs DES (g) vs Time (min); C) Response Surface AFB$_1$: Predicted Recovery vs Time (min) vs Temperature (°C); D) Response Surface AFB$_1$: Predicted Recovery vs Temperature (°C) vs DES (g).

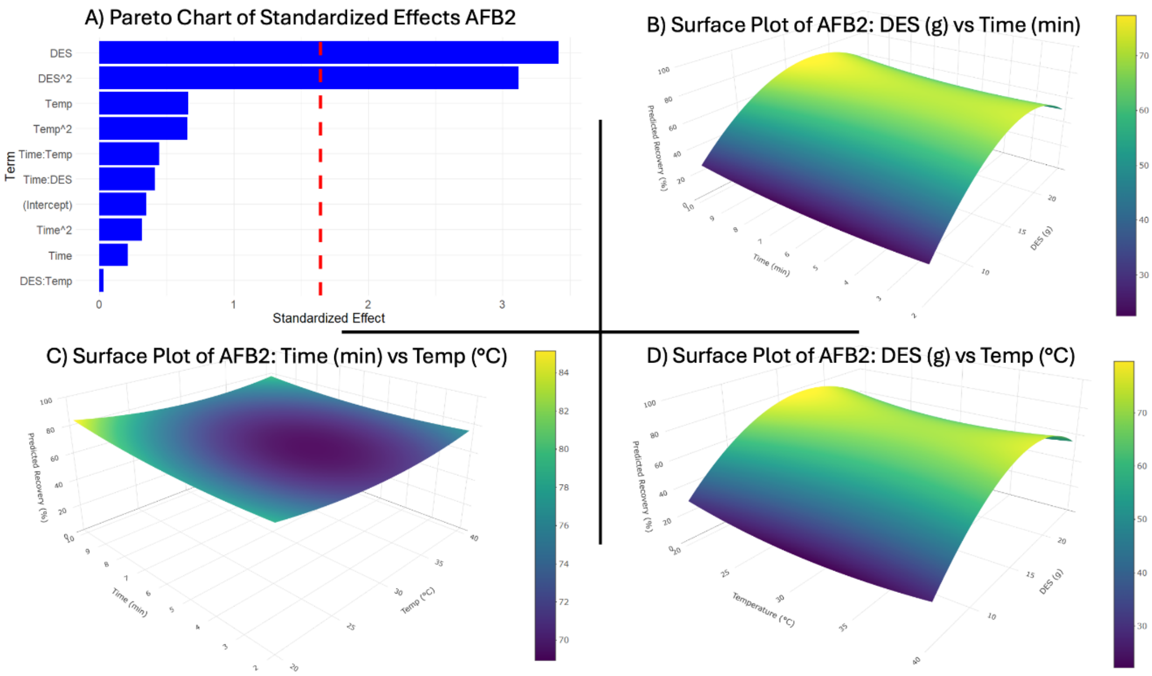

Figure S3. Results summary for **AFB$_2$** from the DoE (k=3). A) Pareto Chart of the Standardized Effects; B) Response Surface AFB$_2$: Predicted Recovery vs DES (g) vs Time (min); C) Response Surface AFB$_2$: Predicted Recovery vs Time (min) vs Temperature (°C); D) Response Surface AFB$_2$: Predicted Recovery vs Temperature (°C) vs DES (g).

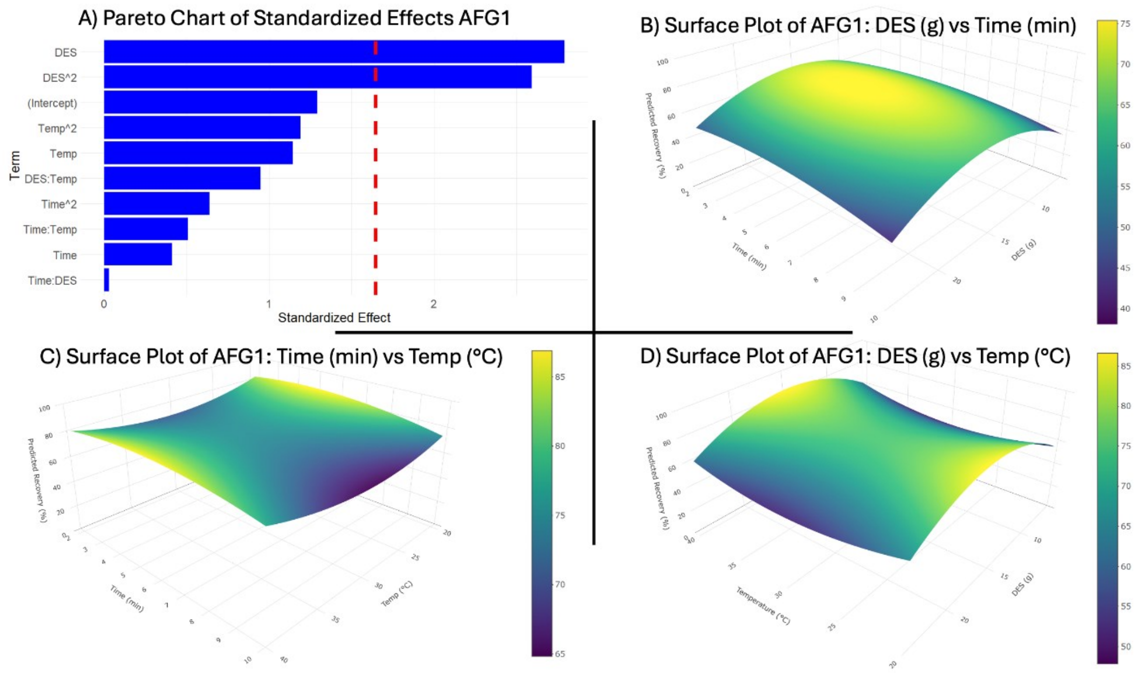

Figure S4. Results summary for **AFG$_1$** from the DoE (k=3). A) Pareto Chart of the Standardized Effects; B) Response Surface AFG$_1$: Predicted Recovery vs DES (g) vs Time (min); C) Response Surface AFG$_1$: Predicted Recovery vs Time (min) vs Temperature (°C); D) Response Surface AFG$_1$: Predicted Recovery vs Temperature (°C) vs DES (g).

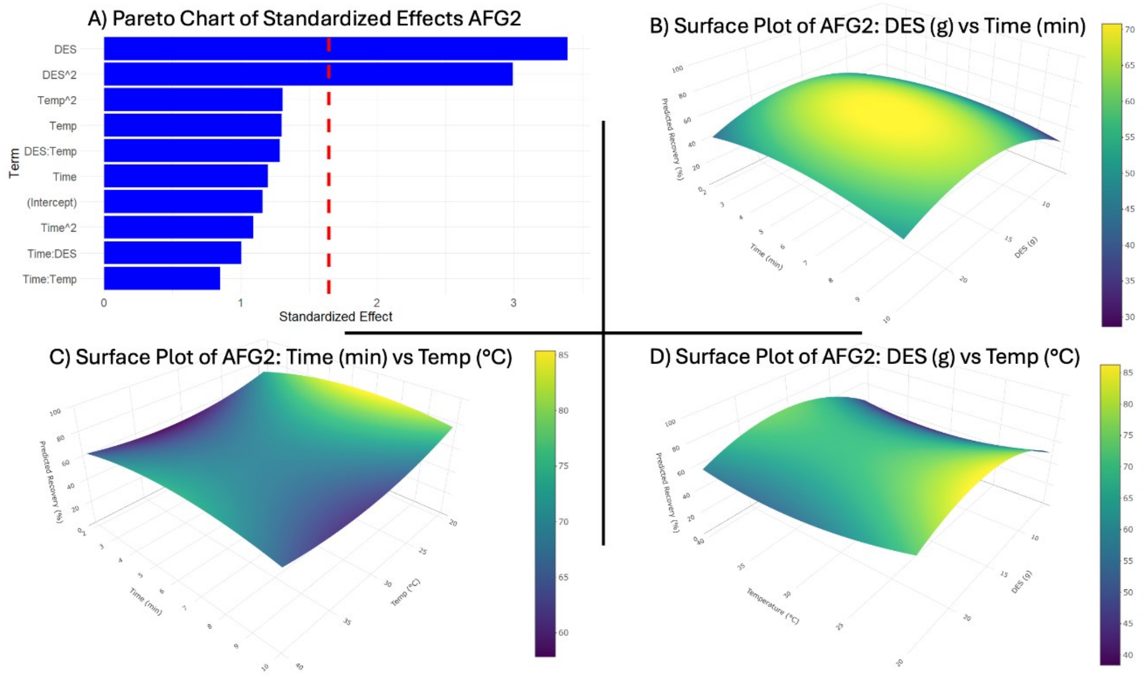

Figure S5. Results summary for **AFG$_2$** from the DoE (k=3). A) Pareto Chart of the Standardized Effects; B) Response Surface AFG$_2$: Predicted Recovery vs DES (g) vs Time (min); C) Response Surface AFG$_2$: Predicted Recovery vs Time (min) vs Temperature (°C); D) Response Surface AFG$_2$: Predicted Recovery vs Temperature (°C) vs DES (g).

Table S1. Red Principles of the RGB algorithm: performance comparison between the reference and proposed method, scores were given on a scale from 0 to 100.

RED PRINCIPLES (analytical performance)	Method name	R1: Scope of application	R2: LOD and LOQ			R3: Precision			R4: Accuracy		
		Score	LOD (ng/g)	LOQ (ng/g)	Score	RSD% (repeatability)[d]	RSD% (reproducibility)[e]	Score	Trueness (bias%)	Recovery (%)	Score
	AOAC 991.31	100	AFB1 - 0.15[a] AFB2 - 0.06 AFG1 - 0.13 AFG2 - 0.05	AFB1 - 0.52[a] AFB2 - 0.21 AFG1 - 0.43 AFG2 - 0.16	100	AFB1 - 0.1[a] AFB2 - 0.5 AFG1 - 0.4 AFG2 - 2.3	AFB1 - 3.1[a] AFB2 - 6.8 AFG1 - 1.1 AFG2 - 6.3	100	AFB1 - 2.9[b] AFB2 - 2.3 AFG1 - 2.2 AFG2 - 2.8	AFB1 – 103[c] AFB2 - 100 AFG1 - 95 AFG2 - 101	100
	DES-SPE-UHPLC-FLD	100	AFB1 - 0.22[b] AFB2 - 0.02 AFG1 - 0.12 AFG2 - 0.03	AFB1 - 0.72[b] AFB2 - 0.05 AFG1 - 0.41 AFG2 - 0.11	100	AFB1 - 1.2[b] AFB2 - 1.6 AFG1 - 2.2 AFG2 - 1.9	AFB1 – 2.1[b] AFB2 – 2.9 AFG1 – 1.6 AFG2 – 1.9	100	AFB1 – 2.7[b] AFB2 – 3.4 AFG1 – 5.3 AFG2 – 2.8	AFB1 – 86.8[b] AFB2 – 83.0 AFG1 – 99.1 AFG2 – 89.1	100

[a]Data in this cell are reported from Karapinar et al., Measurement Food 13 (2024) 100124, the authors used the reference method AOAC 991.31.
[b]This work.
[c]Recoveries reported by the manufacturer of the immunoaffinity column, following the reference method AOAC 991.31.
[d]Repeatability is intended ass the variability in results when a measurement is performed by a single analyst over a short time scale, in both methods this definition was meant as intra-day repeatability.
[e]Reproducibility is usually intended as measure of the variability in results between laboratories, since for the present work an inter-laboratory evaluation was not the goal, only inter-day precision was evaluated.

Table S2. Green principles of the RGB algorithm: greenness comparison between the reference and proposed method, scores were given on a scale from 0 to 100.

GREEN PRINCIPLES (green chemistry)	Method name	G1: Toxicity of reagents (impact and biodegradation)		G2: Amount of reagents and waste			G3: Consumption of energy and other media	G4: Direct impacts (safety, use of animals and GMOs)			
		Pictograms	Score	Reagents	Waste	Score	Score	Occupational hazards	Safety of users (0-100)	Use of animals (0 if no, 1 if yes)	Use of GMO (0 if no, 1 if yes)
	AOAC 991.31	7	30	125 mL	150 g	15	75	3	30	0	0
	DES-SPE-UHPLC-FLD	3	70	6.2 mL	11.63 g	90	75	1	90	0	0

Table S3. Blue principles of the RGB algorithm: practicality comparison between the reference and proposed method, scores were given on a scale from 0 to 100.

BLUE PRINCIPLES (practical side)	Method name	B1: Cost-efficiency		B2: Time-efficiency		B3: Requirements			B4: Operational simplicity		
		Total cost	**Score**	**Speed of analysis**	**Score**	**Sample consumption**	**Sample consumption (score)**	**Other needs: advanced instruments, skills, facilities (score)**	**Miniaturization (score)**	**Integration and automation (score)**	**Portability (score)**
	AOAC 991.31	€20.6	30	4 samples/h	75	25g	10	75	5	25	0
	DES-SPE-UHPLC-FLD	€7.8	90	4 samples/h	75	2.5g	100	75	100	25	0

Table S4. Matrix effect (%) of analytes in Procedural Blank Spiked (PBS) compared to External Calibration (EC). The matrix effect was calculated as follows: ME(%)=[($slope_{PBS}$/$slope_{EC}$) -1]*100.

Analyte	Matrix Effect %
	PBS / EC
AFB_1	-23.36
AFB_2	-21.66
AFG_1	-23.79
AFG_2	-36.72

Table S5. Results of the lack-of-fit tests conducted to assess the linearity of the calibration curves for each analyte (AFB_1, AFB_2, AFG_1, and AFG_2). The table presents the F-values and corresponding *p*-values (Pr(>F)) for each analyte. All the *p*-values are greater than 0.05, which means there is no significant lack of fit. This supports the adequacy of the linear calibration models.

	Matrix-matched calibration		External calibration		Procedural blank calibration	
Analyte	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
AFB_1	0.120	0.888	0.458	0.765	0.353	0.788
AFB_2	0.464	0.639	0.565	0.693	0.777	0.518
AFG_1	0.012	0.988	0.579	0.684	0.689	0.567
AFG_2	0.147	0.865	0.172	0.949	0.875	0.470

Table S6. Recoveries calculated at five concentration levels for each analyte, comparing slopes of matrix-matched and procedural blank calibration curves.

	Concentration levels				
	1 ng/g	2 ng/g	4 ng/g	6 ng/g	8 ng/g
Analyte	Recovery (%) ± SD (n=4)				
AFB_1	92.3 ± 0.6	91.7 ± 0.9	82.7 ± 1.0	86.1 ± 0.4	83.0 ± 0.6
AFB_2	86.3 ± 0.7	88.9 ± 0.8	81.0 ± 0.7	80.0 ± 0.6	79.6 ± 0.8
AFG_1	99.1 ± 2.4	100.5 ± 6.7	93.8 ± 2.1	101.6 ± 3.7	103.2 ± 1.9
AFG_2	88.9 ± 1.5	92.0 ± 2.8	85.0 ± 0.5	90.0 ± 3.0	91.9 ± 1.3