How Appropriate Clean-Up Can Improve the Robustness of an LC-MS/MS Method for the Determination of Multiple Mycotoxins in a Range of Food Matrices

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ABSTRACT

The food and feed industries quest to balance quality and safety assurance with economy and efficiency has always posed challenges, but in the context of an increasingly globalized food supply, rising concern about foodborne illness, the need for mycotoxin test methods that help companies do more with less has taken on a new urgency. A variety of testing solutions exists for mycotoxin analysis, ranging from easy to use, rapid tests, which can be used at the point of production, to lab-based, reference methods that are more time-consuming but can be used to provide a more comprehensive view of the type and level of contamination. The co-occurrence of many mycotoxins in food and feed samples is a matter of concern. So, multi-toxin methods, using LC-MS/MS, are now commonly used for the determination of a wide range of regulated, "modified" and emerging mycotoxins in a variety of agricultural commodities and finished products.

In this work we conducted a series of tests to evaluate the robustness of two multi-mycotoxins methods based upon LC-MS/MS, which differed only by the inclusion of a clean-up step. Analyte response was monitored over a period of 35 days by plotting control charts for 14 compounds. The analysis was conducted non-stop 24h/day, every day for 35 days. A basic regression analysis was used to compare the intermediate precision of the two methods and to determine which of the two methods could maintain the required sensitivity for longer. We found that when an effective pass through SPE clean-up is implemented, the system can maintain the original signal for longer periods of time. Furthermore, the use of this clean-up step resulted in a reduction in matrix effects and isobaric interference, especially for late-eluting compounds, leading to lower variability and more accurate quantification. In this study we also present the results from additional experiments where the reduction of phospholipid content and overall matrix co-extractives is assessed.

An initial investment in a clean-up strategy, combined with the wise selection of a suitable LC-MS/MS system, can reduce failure rates and operational down time, which has a positive impact on the efficiency and productivity of the laboratory.

INTRODUCTION

In food testing laboratories, analytical requirements are often driven by regulations designed to protect consumers and trade alike but also the additional demands of the food industry to promote and protect their brands. Such analyses introduce challenges with maintaining instrument reliability and obtaining consistent performance across different analyses and over time. Modern, sensitive instruments can cope with the "dilute and shoot" approach that allows analysts to produce results in the most rapid manner. However, such continuous analysis of a wide range of different complex samples may necessitate implementing timely interventions for planned system maintenance or be faced with unexpected loss of performance requiring immediate attention and often repeat analysis.

System maintenance includes simple tasks such as cleaning of a cone, replacement of columns and consumables and flushing LC systems, to more complicated procedures involving venting the instrument to clean the MS ion optics and even quadrupole rods to mitigate charging effects. Charging occurs when the amount of charge being deposited onto a surface is greater than the rate at which the charge is dissipated. The contamination forms an insulation layer on the metal surface, which means it takes longer for any charge which lands on that surface to dissipate (quadrupole rods, differential apertures, etc.). This build-up of charge can take place on all metal components within the ion path (sampling/extractor cone, StepWave,™ ion optics, collision cells, quadrupoles, etc.) and it will cause a potential barrier which can affect the transmission of ions. Thus, charging can cause a drop in response over time. Making decisions that improve the robustness and ruggedness of methods, in a cost-effective way, has a massive impact on the operational efficiency and productivity of the food testing laboratory. In addition, analytical methods need to be robust so that they can be easily transferred for use in another laboratory if necessary.

The terms robustness and ruggedness refer to the ability of an analytical method to remain unaffected by small variations in the method parameters and influential environmental factors and characterize its reliability during normal usage.[1] Here we use the term robustness for expressing the stability of the method, in terms of response, against changes to the intrinsic method parameters (with and without clean-up) and the resulting variability of sample matrix.

We recently described a multi-toxin method for 31 regulated and emerging mycotoxins using a quick and simple pass-through SPE clean-up prior to LC-MS/MS analysis for three different food types (cereals, nuts, figs) and animal feeds.[2] In this white paper, we present the results of a series of tests to evaluate the robustness of two approaches, performed using the same UPLC™-MS/MS method, but with and without applying the SPE clean-up step.

INTERMEDIATE PRECISION

THE EXPERIMENT

A test to evaluate the intermediate precision, which is the precision obtained within a single laboratory over time, was performed for the multi-toxin LC-MS/MS method after preparing spiked wheat samples by following two approaches:

- Dilute and shoot method (no clean-up), herein referred to as "D&S";
- Pass-through SPE clean-up method, herein referred to as "SPE"

The SPE approach has been described in detail in a previous application note.[2] Briefly, 5 g of homogenized blank wheat flour was extracted with 20 mL of 80:20 MeCN:H_2O containing 0.75% of acetic acid and 0.2% of formic acid (v/v). After centrifugation, 0.4 mL of supernatant were loaded onto the Oasis™ PRiME HLB Cartridge (3cc, 150 mg, p/n 186008717) and discarded, a second portion of the supernatant (1.2 mL) were loaded and collected. The purified extract was diluted 1:5 with water prior to analysis by UPLC-MS/MS. The D&S approach differed only in that no clean-up was applied, so the supernatant after centrifugation was diluted 1:5 with water prior to UPLC-MS/MS. Internal standards were omitted on this occasion as the objective was to compare absolute response over time. Both methods resulted in an overall dilution factor of 20.

After dilution with water, test extracts were spiked with a standard mix of 14 mycotoxins.

At day zero, two sets of 20 LC vials were prepared for each experiment, the first series contained the spiked extract, whilst the second series contained the blank extract.

Analyses were carried out on a ACQUITY™ UPLC I-Class PLUS coupled with a Xevo™ TQ-S micro. The system ran the sequence, shown in Table 1, repeatedly non-stop 24h/day, every day for 35 days, resulting in a total of 2900 injections, of which 708 were from the spiked extracts (representing 708 data points) and 2047 from the matrix blank extracts. Mobile phase bottles were re-filled as and when required, and the sequence was only paused once (day 19) for cleaning of the sampling cone, mimicking the approach to routine maintenance typical of a routine food testing laboratory. The injection volume was set at 8 µL, equivalent to ~1.1 g of solid wheat extract.

RESULTS

Control charts play an important role in a performance-based program of quality assurance because they provide an easy to interpret picture of the statistical state of an analysis. ISO/IEC 17025 requires quality control procedures and, where practicable, the use of statistical techniques to monitor the validity of tests and detect possible trends. For monitoring ongoing quality control, when values from control samples fall outside the maximum acceptable limits, no analytical results are reported, and remedial actions must be taken to identify the sources of error, to remove such errors and repeat the analysis. The more reliable the analytical method, the longer the method will stay within control without manual intervention. Control charts were prepared, for the peak areas of the mycotoxins of interest, from the analyses of the spiked extracts. In the control charts, mean ±2*std. dev. was used as warning limit, while mean ±3*std. dev. was used as maximum acceptable limit[3,4] (mean and std. dev. were obtained from a previous test). Figure 1 shows the control charts for the response (peak area) for two representative mycotoxins using both D&S and SPE approaches. Values for the control sample fall outside the warning limits more often when the D&S approach is used.

N.	Name
1	Spiked sample
2	Blank sample
3	Blank sample
4	Blank sample
5	Spiked sample
6	Blank sample
7	Blank sample
8	Blank sample
9	Spiked sample
10	Blank sample
11	Blank sample
12	Blank sample
13	Spiked sample
14	Blank sample
15	Blank sample
16	Blank sample
17	Blank solvent
n	Repeat above sequence

Table 1. Repeating unit of the analytical sequence.

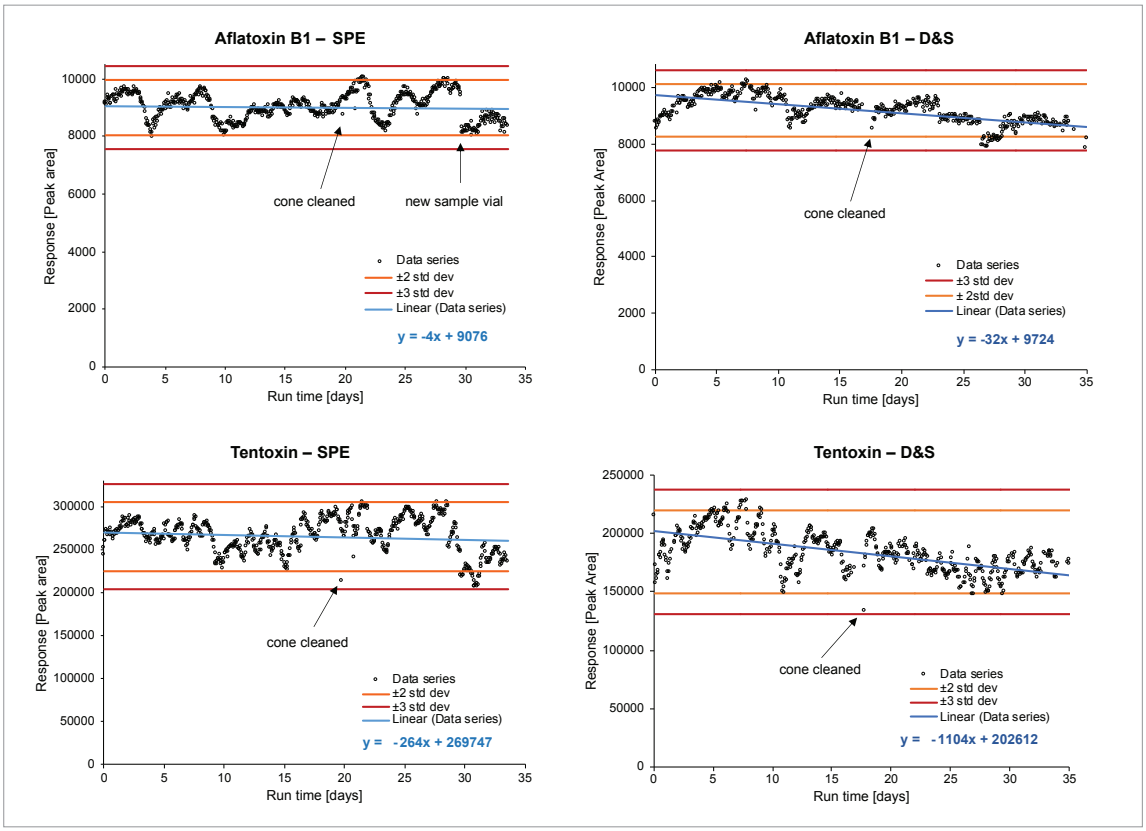

Figure 1. Control charts representing the response of aflatoxin B1 and tentoxin as a function of analysis time in SPE and D&S experiments. The linear regression equation is shown at the bottom right corner of each chart.

It should be noted that the control charts presented a stepped-profile, and each gap between two steps corresponded to manual intervention (i.e., mobile phase refilling, change of sample vial, cone clean). Cleaning the sampling cone involves turning the instrument into "standby" mode. After switching the instrument back into "operate" it usually takes a few injections to stabilize the response.

The best fit (linear regression) of the peak area considering the entire data points series was plotted on each chart, which shows a decrease in response over time in all cases, represented by the negative slope of the curves. The graphs show that the D&S data presented steeper negative slopes compared to the data after SPE pass-through. One can investigate these trends in more detail by using the linear regression equation to calculate t_{10}, which is the estimated time at which the response decreased by 10% from the initial value. The percentage relative difference of the slopes (Δ_b%) for the two experiments was also calculated (data reported in Table 2). Δ_b% ranged from -1 to -89%, meaning that the SPE approach provided improved intermediate precision when compared to the D&S approach. Values for t10 were greater when applying the SPE clean-up (mean = 94 days, median = 68 days, range: 16–263 days) compared to the D&S method (mean = 25 days, median = 22 days, range: 9–42 days). This represents additional evidence of the improved robustness when an effective SPE clean-up is implemented within the sample preparation protocol and suggests periods between planned maintenance could be longer.

Analyte	Conc [µg/kg]	Sample prep approach	[Area/day]	t_{10} [day]	Δ_b [%]
Aflatoxin B1	50	SPE	-4	263	-89
		D&S	-32	29	
Aflatoxin B2	50	SPE	-21	30	-69
		D&S	-68	9	
Aflatoxin G1	50	SPE	-6	203	-79
		D&S	-27	42	
Aflatoxin G2	50	SPE	-3	184	-81
		D&S	-15	35	
Ochratoxin A	200	SPE	-59	39	-45
		D&S	-108	21	
Zearalenone	3000	SPE	-234	16	-1
		D&S	-237	16	
Deoxynivalenol	1200	SPE	-11	122	-83
		D&S	-65	21	
T-2 toxin	600	SPE	-96	64	-65
		D&S	-272	23	
HT-2 toxin	600	SPE	-5	42	-32
		D&S	-7	28	
Beauvericin	1200	SPE	-6234	32	-67
		D&S	-18693	11	
Enniatin B	1200	SPE	-28	43	-59
		D&S	-69	17	
15-Acetyl-Deoxynivalenol	1200	SPE	-7	113	-73
		D&S	-26	31	
Fusarenon X	1200	SPE	-7	73	-47
		D&S	-13	38	
Tentoxin	1200	SPE	-264	89	-76
		D&S	-1104	21	

Table 2. Concentrations of the 14 mycotoxins in the spiked wheat sample; sample preparation approach (SPE = Solid Phase Extraction, D&S = Dilute and Shoot); slope of the best-fit linear curve (b); time at which the response dropped by 10% from the initial value (t_{10}); and percentage relative slope difference between the two methods (Δ_b).

REDUCTION OF MATRIX EFFECTS

MATRIX EFFECTS

Matrix effects are often observed when using LC-MS/MS for mycotoxin analysis. The response of target analytes is impacted by the presence of co-eluting compounds coming from the matrix.[5] There are several ways of calculation matrix effect;[6,7] in the present study we derived the percentage matrix effect (%ME) as follows:

$$\%ME = \left(\frac{b_M}{b_S} - 1\right) * 100$$

Where b_M and b_S are the slope of the matrix-matched and solvent calibration curves, respectively. Negative *%ME* values are a quantitative measurement of ion suppression, positive *%ME* values are a quantitative measurement of ion enhancement, while *%ME* close to zero are indication of low to absent matrix effects. Matrix effects introduce a bias which can negatively impact the accuracy of an analytical method. Three common strategies to compensate for the bias caused by these effects are:

1. Use of stable isotopically labelled internal standards;
2. Use of matrix-matched calibration graphs;
3. Use of standard addition.

However, significant ion suppression/enhancement factors and compromised method robustness go hand in hand because they are associated by a common factor, that is, the presence of unwanted co-extractive material in the sample extract that is injected into an LC-MS system. For this reason, although matrix effects can be compensated by one of the approaches listed above, it is always good practice to reduce matrix effects as much as possible, in a cost-effective way, to improve method robustness.

After plotting the linear calibration curves for 32 mycotoxins in solvent and sample extracts with and without the Oasis PRiME HLB SPE clean-up, we calculated the matrix effect for each method in wheat, peanuts, figs, and animal feeds. We then calculated the difference, *%ΔME*, as follows:

$$\%\Delta ME = |\%ME_{D\&S}| - |\%ME_{SPE}|$$

Where $|\%ME_{D\&S}|$ and $|\%ME_{SPE}|$ are the absolute value of the percentage matrix effect of D&S and SPE methods, respectively. *%ΔME* is the percentage difference of matrix effects between the two method. Positive *%ΔME* values indicate a higher matrix effect in the D&S approach, while negative *%ΔME* values indicate higher matrix effects in the SPE method. Figure 2 represents a heat-map showing the *%ΔME* for each analyte in the four food and feeds tested and Figure 3 illustrates *%ΔME* in the form of bar-plot. Both show that the SPE method provides overall lower matrix effects for most analytes. The greatest impact of the clean-up was found to be on fumonisins (with a matrix effect reduction of up to 325% in wheat and 293% in peanuts for fumonisin B_2) and enniatins (up to 86% matrix effect reduction in peanuts for enniatin A).

Analyte	Wheat	Peanuts	Figs	Feeds
AFB_1	0	-3	-2	7
AFB_2	1	-6	-4	4
AFG_1	1	-3	-3	5
AFG_2	-1	-5	-2	6
FB_1	52	34	15	41
FB_2	325	293	36	41
FB_3	89	55	26	49
OTA	15	16	3	-6
ZEA	2	-9	-2	10
DON	7	3	9	7
DON-3-Glu	6	-3	7	1
NIV	-11	3	8	3
HT-2	0	-10	-7	0
T-2	0	-3	4	7
3-Ac-DON	2	-3	8	15
15-Ac-DON	2	-3	3	7
DAS	7	1	1	-10
NEO	7	0	2	-9
FUS-X	2	-4	-13	24
Deepoxy - DON	-6	-9	0	-2
Citrinin	20	40	-4	61
Sterigmatocystin	0	-11	-7	15
Beauvericin	5	28	19	-53
Enniatin A	38	86	20	7
Enniatin B	-2	17	3	14
Enniatin A_1	23	56	20	34
Enniatin B_1	20	36	9	34
AOH	-5	-18	5	6
AOH-Me-Ether	6	8	-5	15
Tentoxin	6	-4	1	13
Tenuazonic acid	20	-6	13	-13
Altenuene	12	9	1	-3

Figure 2. Heat-map showing the %ΔME for 32 mycotoxins in different foods and feed products.

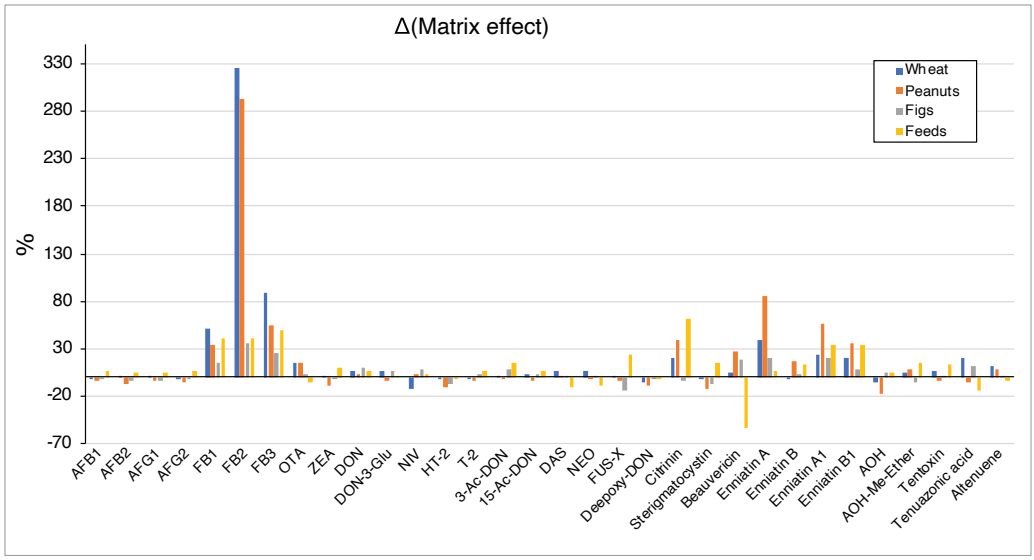

Figure 3. Bar-plot showing the %ΔME for 32 mycotoxins in different foods and feed products.

PHOSPHOLIPIDS REMOVAL

The presence of co-extracted substances in the extract, including phospholipids, can lead to interference in the UPLC-MS analysis, contamination of the analytical column and other components of the UPLC system, contamination of the mass spectrometer itself and ion suppression due to matrix effects. The Oasis PRiME HLB sorbent is highly effective in removing fats and phospholipids. Figure 4 shows a chromatogram from the analysis of a wheat extract (top), acquired by monitoring MRM transitions of common lecithins, whilst the middle trace is a chromatogram of the same sample acquired after passing through SPE, using the same LC-MS/MS acquisition method. The peaks for mycotoxins that elute in the same region of the chromatogram are shown for reference (bottom chromatogram in Figure 4). The removal of phospholipids is beneficial for the co-elunting analytes as it will decrease matrix effects. It is evident that almost no phospholipid signal is present in the cleaned-up extract.

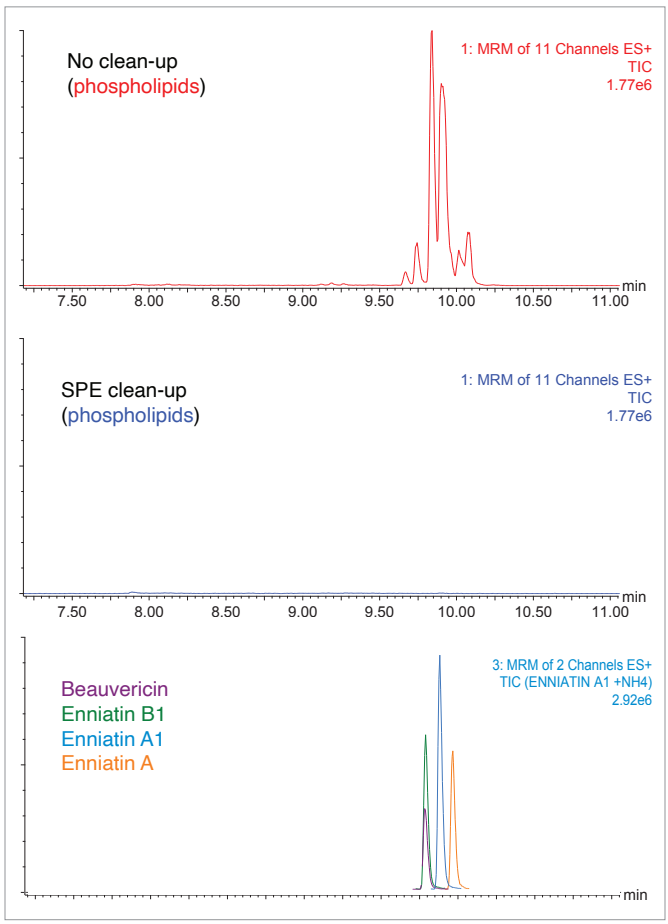

Figure 4. TIC chromatograms of crude wheat extract (top) and wheat extract after SPE clean-up (middle) monitoring the phospholipids MRM. The bottom trace shows the chromatograms of enniatins and beauvericin which coelute with phospholipids.

MS SCAN ANALYSIS

Another way to assess the amount of co-extracted material injected into the LC-MS/MS system is to record chromatograms in SCAN mode over the entire LC gradient window and compare the profiles generated by the D&S and SPE methods.

Representative wheat, peanuts, figs and animal feeds samples were processed using D&S and SPE protocols, and 8µL of each extract were injected onto an LC-MS/MS system comprising ACQUITY UPLC I-Class PLUS coupled with a Xevo TQ-S micro. Chromatograms were acquired in ESI+ SCAN mode (mass range: 50–800 *m/z*, scan time: 0.1 s, cone voltage: 10 V) from 0 to 14 min.

Figure 5 shows the total ion current (TIC) chromatograms and total area from integrating the whole trace. The percentage decrease of total area when applying the SPE clean-up was 18% for animal feeds, 13% for peanuts, 7% for figs, and 6% for wheat. The red colour in each chromatogram highlights the features that have been removed by the SPE pass-through step.

It should be noticed that the portion of the chromatogram where there is a higher reduction of background peaks is between 7.5 and 11 minutes. This is in accordance with the matrix effect experiments, as well as with the fact that most lipophilic interferences, including phospholipids, are late eluters, and are effectively removed by the Oasis PRiME HLB. We observed overall a lower matrix effect for the compounds eluting later in the chromatogram, at a high proportion of organic mobile phase.

DRIED RESIDUE ANALYSIS

A simple experiment was performed to quantify the amount of solid residue remaining after evaporation of the sample extracts, with and without applying the SPE clean-up. The clearness of the extracts after reconstitution was also evaluated.

Initially, empty glass tubes were weighted on an analytical balance (sensitivity = ±0.1 mg). After extracting different food matrices following the procedure described in the first section, 1.2 mL of the crude sample extract were transferred into different glass tubes. A second series of the same extracts were passed through the Oasis PRiME HLB SPE cartridge and 1.2 mL of the resulting clean-up extract were transferred into different glass tubes. After drying at 40 °C under a gentle nitrogen stream, each glass tube was weighed again. The weight difference (weight of tube after evaporation – initial weight of empty tube) is equivalent to the weight of the solid residue in each extract for each experiment.

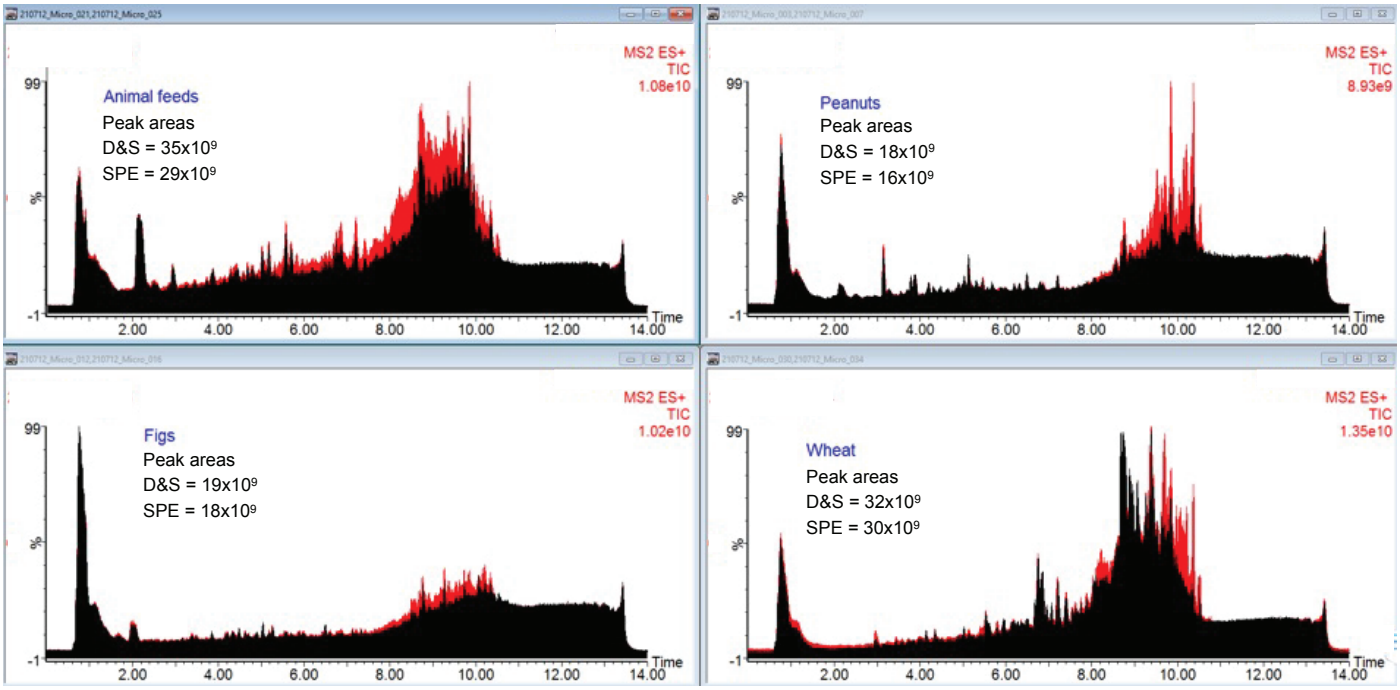

Figure 5. ESI+ SCAN chromatograms (red traces = D&S; black traces = SPE). Traces were filled with the respected color, and total areas are shown on each chromatogram.

The percentage difference of dried weight between the D&S and SPE experiments was calculated for each matrix, and the results are reported in Table 3. Extracts were then reconstituted in 0.5 mL of 95:5 water:methanol.

It can be observed that when the SPE clean-up is applied, there is a reduction in the mass of unwanted co-extracted sample material ranging from 16 to 66%, meaning that the amount of sample matrix co-extractives introduced into the LC-MS/MS system at each injection is reduced by between -16 and -66%. After reconstitution, extracts subjected to the Oasis PRiME HLB pass-through clean-up, appeared to be much clearer (Figure 6).

Matrix	Dried weight crude extract [mg]	Dried weight SPE cleaned-up extract [mg]	Weight % difference
Wheat	**7.6**	**3.6**	-53
Peanuts	4.5	3.8	-16
Figs	8.9	3.0	-66
Animal feed	1.3	1.0	-23

Table 3. Dried weight of crude extracts, dried weight of extract after SPE clean-up, percentage weight difference.

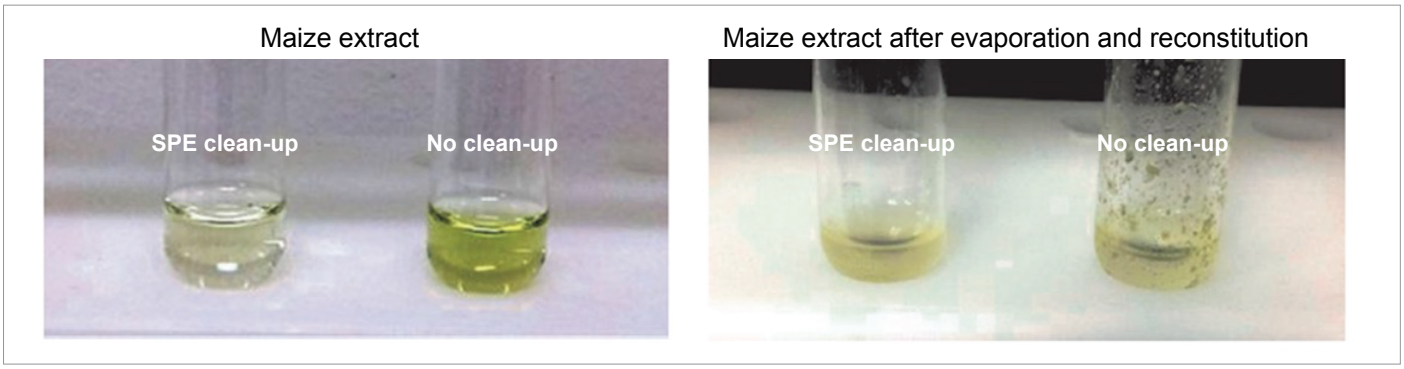

Figure 6. Maize extract with and without the Oasis PRiME HLB pass-through (left); maize extract after evaporation and reconstitution with 95:5 water:methanol (right).

CONCLUSIONS

When developing an analytical method, sensitivity, speed, and ease-of-use are usually considered the most important factors to assess. However, robustness is equally as important, as robust methods lead to fewer system failures and associated downtime, and less frequent system maintenance. Consequently, in a food testing lab, better robustness will ultimately result in improvements in efficiency and productivity.

We have seen in the previous sections that the incorporation of an effective clean-up step into the sample preparation protocol will reduce the amount of unwanted co-extractives introduced into the UPLC-MS/MS system. This is important because clean-up is one of the options used to mitigate the impact of matrix effects, but also, and most importantly, because it will reduce the rate at which charging effects occur, and reduces the frequency of intervention for maintenance thus enhancing the robustness of the method.

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