Application brief | 000489

Software

Utilizing software in a Zero Pesticide Residue project to reduce matrix effects and improve extraction efficiency and quantitation

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Goal

To demonstrate a sensitive method to detect and quantitate pesticide residues to meet the Zero Pesticide labeling criteria for organic and baby food samples by reducing matrix effects and improving extraction efficiency utilizing the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS).

Introduction

Organic and baby foods are typically grown without the use of synthetic pesticides, with the expectation that they are “pesticide free”. This method describes a reliable and effective process to detect the potential presence of very low amounts of pesticide residues and is compliant with SANTE/12682/2019.

In the process of validating this procedure, the parameters of linearity, limit of quantification (LOQ), matrix effects, recovery, extraction efficiency, reproducibility, selectivity and specificity were measured and demonstrated.

The benefits of this method include an automated process to reduce matrix effects and improve extraction efficiency and target quantification within a

sample matrix through software customization and automatic control validation for both LC and GC workflows.

To maintain compliance with SANTE/12682/2019, NFT 90-210, LAB GTA-05, LAB GTA-26, XPT 90-214 and other regulatory requirements or good laboratory practices (GLP), the following parameters must be measured.

Calibration for quantitation

Bracketing calibration must be used unless the determination method has been shown to be free from significant drift. The lowest calibration level must be equal or lower than the Reporting Limit (RL). Single-level calibration provides accurate results if the sample extract is within ±30% of the calibration standard.

Reporting Limit

Control samples of all targeted analytes must be run with every batch of samples at or below the level corresponding to the RL. A measurable response at this level is required and should be checked to avoid false negatives.

Extraction conditions and efficiency

The extraction procedures used in the methods for the detection of residues in food/feed of plant and animal origin should be verified and results should be expressed as percent recoveries.

Matrix-effect assessment

Matrix effects frequently impact analysis in both GC and LC methods. Only in cases where the experiments clearly demonstrate that the matrix effects are not significant (≤±20%) can the calibration be done with standards in solvents. The standard addition procedure is designed to compensate for matrix effects and recovery losses. The amount of added analyte should be comparable to the target levels in the sample. In the standard addition approach, the concentration of the analyte in the test sample extract is derived by extrapolation, thus a linear response in the appropriate concentration range is essential for achieving accurate results.

On-going method performance verification during routine analysis for quantitative methods is necessary.

Routine recovery check

Where practicable, the recovery of all target analytes should be measured within each batch of samples. However, for practical considerations, the number of analytes may be reduced to 10% of the analytes (or minimum of 5) per detection system. All other analytes should be assessed every 6 months (or at a minimum, 12 months). Determination of recoveries should be checked at RL.

The recommended range of recovery is 60-140% in routine analysis. Recoveries outside this range would normally require re-analysis of the batch, e.g., NFT90-210 requires 70-120% recoveries when internal standard calibration is used.

Reporting Limit for screening methods

When using a screening method, the calibration standard solution corresponding to the RL should be measured at the beginning and at the end of the run to ensure that the analytes remain detectable throughout the entire batch of samples in the sequence.

Requirements for identification using selected ions

For the identification of analytes and confirmation of results using liquid chromatography with mass spectrometry (LC-MS), this requires the correct selection of ions. They must be sufficiently selective for each analyte in the matrix and in the relevant concentration range.

The relative intensities or ratios of the selected ions, expressed as a ratio to the most intense ion being used for identification, should match within ±30% (relative) with the reference ion ratio.

Internal standard (ISTD)

NFT90-210 requires a 30% recovery of the ISTD.

Limit of quantitation

NFT90-210 requires that the LQ must be checked for all ions with S/N > 3

Retention time deviation

XPT90-214 requires that the Relative Retention Time (RRT = RT (analyte)/RT(ISTD)) falls within ±0.5% (GC) and ±2.5% (LC) between the calibrant and the experimental.

Quantitation

As a practical approach, the residue results do not have to be adjusted for recovery when the mean recovery is within the range of 80-120%.

Per SANTE/12682/2019, NFT 90-210, LAB GTA-05, LAB GTA-26, XPT 90-214 and many other regulations, or to adhere to good laboratory practices, the following steps must be achieved while considering analyzing samples.

Strategy

To meet the criteria for a Zero Pesticide Residues label, the tested matrix should show no measurable pesticide residues or detection at or below 10 ppb, thus representing both the minimum acceptable value and required limit of quantitation. The simplest method to measure the analytes of interest is to set the standard addition to the sample at 10 ppb and calculate extraction recovery with every single sample also at the exact 10 ppb value. This encompasses the 'on-the-fly' matrix-effect, recovery check, and performance validation parameters while providing the most accurate quantification results. The process described below is also shown in Figure 1.

Quantitation with matrix effect

To enable quantitation that addresses the matrix effect, take an aliquot of the sample following extraction and divide into two portions for injection:

- Portion 1 — label as pure sample (PS) and assign as a "0 (zero) value" calibration standard type.
- Portion 2 — add 10 ppb of the standard, label as the spiked sample (SS) and assign to the "10 ppb value" 'calibration standard' type.

Measurement of extraction recovery

To measure the extraction recovery, add 10 ppb of the standard to the sample before extraction and divide into two portions for injection:

- Portion 1 — the fortified sample (FS) will show 10 ppb extracted peaks; assign as the "10 ppb value" 'check standard' type
- Portion 2 — add an additional 10 ppb of standard and label as the spiked fortified sample (SFS) with a total quantity of 20 ppb of standard; assign as the "20 ppb value" 'calibration standard' type.

If recovery is 100% then a signal twice higher than fortified sample will be shown. The deviation will allow to evaluate exact extraction recovery.

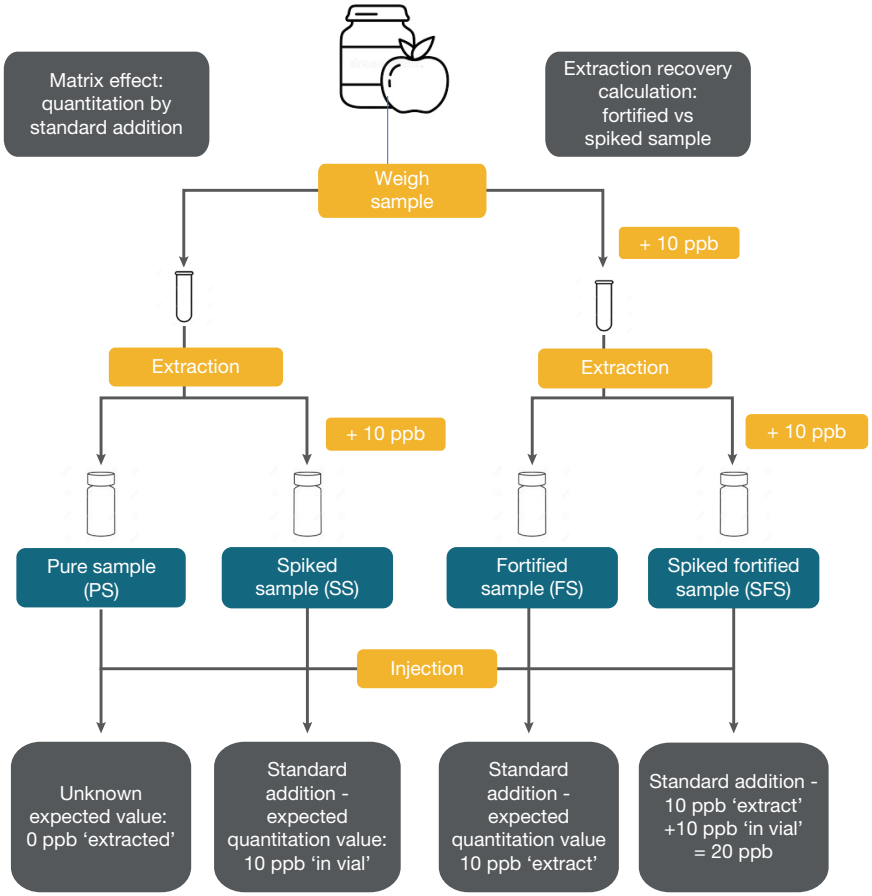


Figure 1. Preparation process to measure matrix effects and extraction recovery

The calibration type is set to ‘group’ mode, requesting a ‘blank’ between set of samples. The blank can be a ‘fake’ (i.e., no injection) or an injection of solvent.

#	Name	Level	Type
6	SS_APPLE	SPK_10	Calibration Standard
7	PS_APPLE	NS	Calibration Standard
8	ISOOCTANE4		Blank
9	SFS_APPLE	dope_spike_10	Calibration Standard
10	FS_APPLE	dopé_10	Check Standard
11	ISOOCTANE3		Blank
12	SS_BANANA	SPK_10	Calibration Standard
13	PS_BANANA	NS	Calibration Standard
14	ISOOCTANE3		Blank
15	SFS_BANANA	dope_spike_10	Calibration Standard
16	FS_BANANA	dopé_10	Check Standard

Figure 2. Sample batch entry into Chromeleon software

Conducting the experiment at the expected quantitation level ensures the most accurate results. The initial fortified and spiked value are made at 10 ppb, because no sample will be accepted at higher value. However, if an accurate quantitation is needed, a new spiking experiment at the estimated quantitation value is necessary while using the standard addition method.

We will demonstrate this strategy with three workflow examples: the first two utilize a GC-MS system and the third used an LC-MS platform.

Workflow 1: GC-MS with a routine recovery check

ISTD review

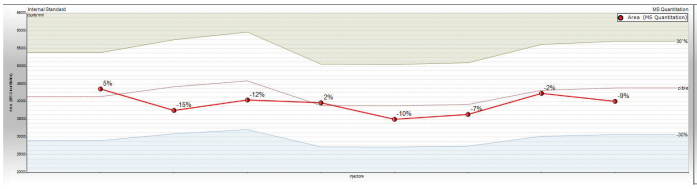
The first step is to assess the internal standard data. If it does not pass both the recovery and retention time criteria, the peaks and quantification of the individual targets will not be accurate.

The criteria can be adjusted according to lab requirements; however, most regulations require that the ISTD peak area in the unknown sample must be within 30% of the ISTD peak area in the calibration standard sample. The retention time in the sample must not shift to more than 0.5% from the retention time value in calibrant.

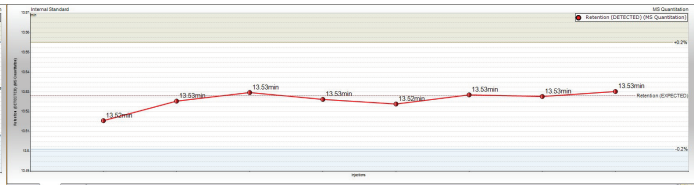
The Chromeleon CDS will automatically measure and validate those points (Figure 3), showing visual alerts if failure is detected, as well as offering several ways of reviewing results according to user’s preference:

- A deviations value of ISTD area is shown in the top left interactive chart along with the acceptable limits.
- RT Values are shown in the top right interactive chart as well as the acceptable limits.
- An interactive table can also show values and a pass or fail signal.

If ISTD validation failure occurs, reinject the corresponding samples. If all tests pass, the next step is to start the results review.



ISTD area deviation (non spike) from moving reference (spike)



ISTD retention time deviation from reference

Injection Name	Ret.Time min Internal Standard	Area counts*min Internal Standard	Variability% Aire SS-SFS/PS-FS Spiked vs non-Spiked Internal Standard	Recovery CHeck test Internal Standard	Rel.RT.Dev. % Internal Standard	RT check test Internal Standard
SS_APPLE	*13.52*	*41427*	0.0		*-0.06*	
PS_APPLE	13.52	43593	5.2	Pass	-0.09	Pass
SFS_APPLE	13.53	44216	0.0		0.01	
FS_APPLE	13.53	37529	-15.1	Pass	-0.02	Pass
SS_BANANA	13.53	45874	0.0		0.00	
PS_BANANA	13.53	40480	-11.8	Pass	0.01	Pass
SFS_BANANA	13.53	38915	0.0		-0.02	
FS_BANANA	13.53	39690	2.0	Pass	-0.01	Pass
SS_CAROTS	13.53	38840	0.0		-0.02	
PS_CAROTS	13.52	35031	-9.8	Pass	-0.03	Pass
SFS_CAROTS	13.53	39223	0.0		0.02	
FS_CAROTS	13.53	36399	-7.2	Pass	0.00	Pass
SS_EGGPLANT	13.53	43194	0.0		-0.02	
PS_EGGPLANT	13.53	42343	-2.0	Pass	0.00	Pass

Figure 3. Reprocessing – step 1: checking ISTD Area and RRT

Sequence and results review

The next step is to review all the sample results to confirm that all the targets are below acceptable limit (i.e., free of pesticides or below the regulated value of 10 ppb).

If a peak is detected, Chromeleon CDS will automatically quantitate the amount present. A red flag will help the operator spot the positive results which can only be confirmed if the relative retention time and relative ion ratio are within the tolerance ranges. Chromeleon software will automatically create a red flag for any failure.

Chromeleon CDS offers several view settings that are fully customizable, including an interactive results table that will red-flag quantitation values above 10 ppb: percent recovery in every matrices (outside 80-120%), relative retention time value (above 0.5%), and the ion ratio test (outside 30% tolerance) for one target in all samples.

The user can choose to visualize the corresponding chromatograms of the set of four injections related to the sample analyzed (SS, PS, FS, and SFS) shown at the top of interactive table in Figure 4. By using the feature that allows the spiked sample to be normalized in all the chromatograms, it can be shown that the peak quantitation is far from the maximum allowable quantitation (e.g., the spiked peak shadow is overlaid in all chromatograms).

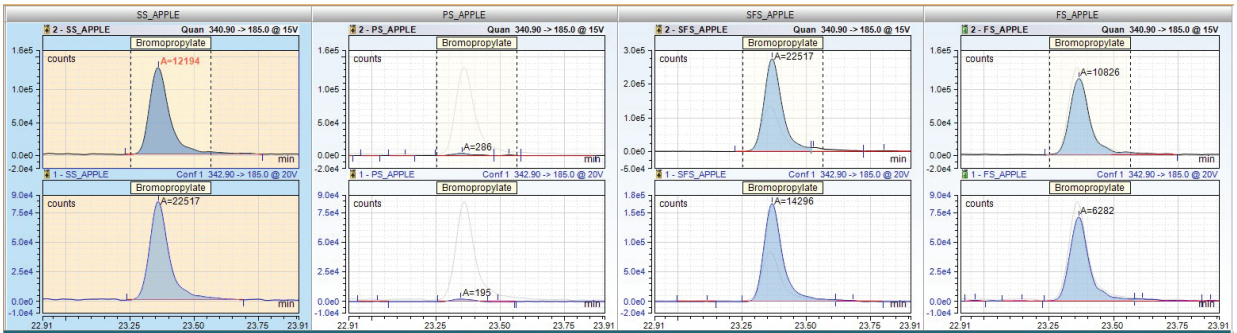
In this example, the compound bromopropylate is detected below the 10-ppb level in all matrices. The ion ratio is correct in the apple sample, but below the reporting limit. The concentration is very low in other matrices and the ion ratios are not within the expected range, therefore, no reporting will occur with this batch.

Workflow 2. GC with routine recovery check alternative

Customized view settings

Alternatively, all recoveries can be checked to determine if they are within acceptable range. A table can be generated that shows the recoveries in all matrices simultaneously (Figure 5). From there, the decision can be made to repeat the extraction before reviewing all the compounds. This provides a significant time savings by knowing immediately if the sample preparation step is valid.

In the example below, the compound cyanophos has a significantly higher recovery value in the apple sample where it is not shown at all in the other matrices, so it was taken out of this method. The methyl-parathion analyte is low in all matrices. but particularly in carrots, which is a known issue in the laboratory, so these quantitative results are adjusted accordingly. All other compounds are extracted efficiently from their matrices.



Injection Name	Sample Result	Area	ISTD Area	Sample Recovery	RT	Rel. Ret.Time.	RRT Check	RT drift	Test Ratio d'ions	Ratio attendu	Ratio Observé
Selected Peak:	must be < 10 ppbt			ideally within 80-120%	min		must be < 0.5 %	must be < 0.2		Quanti/Quali	Quanti/Quali
	Bromopropylate	Bromopropylate	Bromopropylate	Bromopropylate	Bromopropylate	Bromopropylate		Bromopropylate	Bromopropylate	Bromopropylate	Bromopropylat
SS_APPLE		*12194*	*41427*		*23.36*	*1.728*	*0.0*	*0.0*	*61*	*64*	*61*
PS_APPLE	0.23	286	43593		23.35	1.728	0.0	0.1	68	64	68
SFS_APPLE		22517	44216		23.37	1.727	0.0	0.0	63	64	63
FS_APPLE		10826	37529	113	23.37	1.728	0.0	0.0	58	64	58
SS_BANANA		12335	45874		23.38	1.728	0.0	0.0	59	64	59
PS_BANANA	0.05	56	40480		23.34	1.725	0.1	0.1	IR High	64	123
SFS_BANANA		22512	38915		23.37	1.728	0.2	0.0	63	64	63
FS_BANANA		10299	39690	90	23.38	1.728	0.0	0.0	56	64	56
SS_CAROTS		11898	38840		23.36	1.727	0.1	0.0	56	64	56
PS_CAROTS	0.02	22	35031		23.33	1.725	0.1	0.1	IR low	64	12
SFS_CAROTS		20300	39223		23.37	1.727	0.1	0.0	70	64	70
FS_CAROTS		9606	36399	102	23.36	1.727	0.0	0.0	61	64	61
SS_EGGPLANT		25885	43194		23.37	1.728	0.1	0.0	61	64	61
PS_EGGPLANT	0.00	1	42343		23.39	1.729	0.0	0.0	IR High	64	2997
SFS_EGGPLANT		48963	43889		23.38	1.728	0.1	0.0	60	64	60
FS_EGGPLANT		21078	40097	94	23.37	1.727	0.0	0.0	59	64	59

Figure 4. Reprocessing – Step 2: sequence and results review

Peak Name	Matrix Recovery			
	%			
First Injection	FS_APPLE	FS_BANANA	FS_CAROTS	FS_EGGPLANT
Dichlobenil	104	107	89	75
Chlormephos	98	99	104	87
Propham	140	100	89	101
Pentachlorobenzene	93	94	91	97
Tecnazene	86	94	102	93
Diphenylamine	97	90	93	80
Chlorprophame	94	92	93	99
Ethalfluralin	88	94	90	107
Trifluralin	97	93	89	93
Benfluralin	105	97	87	93
Phorate	93	87	93	87
BHC-alpha	109	88	118	98
Hexachlorobenzene	87	86	78	79
Dichloran	107	88	70	87
Fluorenon	101	106	97	110
BHC-Beta	97	88	91	97
BHC-gamma	96	84	91	101
Profluralin	97	90	96	82
Quintozene	97	83	87	91
Cyanophos	275	n.a.	n.a.	n.a.
Fluchloralin	82	94	90	88
Tefluthrin	99	88	102	96
BHC-delta	108	95	87	92
BHC-epsilon	99	90	92	101
Bromocyclen	91	110	96	92
Monalide	91	94	84	83
Parathion-methyl	*84*	*74*	*52*	*73*
Pentachloroaniline	97	90	103	93
Vinclozolin	98	90	85	102

Figure 5. Target compounds recoveries in all matrices

Peak Name	QUANTI PPB			
First Injection	PS_APPLE	PS_BANANA	PS_CAROTS	PS_EGGPLANT
S421	n.a.	n.a.	0	0
Fenitrothion	n.a.	0	0	0
Anthraquinone	2	0	0	0
Aldrin	n.a.		0	n.a.
Isodrin	16	n.a.	n.a.	n.a.
Internal Standard				
Parathion-ethyl	0	0	1	0
Dichlorobenzophenone	0	0	0	n.a.
Chlorthal-dimethyl	0	0	0	0
Isobenzan	n.a.	0	0	0
Chlorthion	0	n.a.	0	0
Fenson	0	0	0	0
Bromophos-methyl	0	n.a.	n.a.	0
Isopropalin	0	n.a.	n.a.	n.a.
Heptachlor-epox-cis	0	0	0	n.a.
Chlordane-oxy	0	n.a.	n.a.	0
Heptachlor-epox-trans	n.a.	n.a.	80	3
Chlozolinate	0	0	0	0
Procymidone	0	0	0	0
Chlorbenside	0	0	n.a.	0
Chlordane-alpha	0			n.a.
Bromophos-ethyl	0	0	0	n.a.
DDE-op	0	0	0	0
Endosulfan-alpha	0	n.a.	n.a.	0
Chlordane-gamma	0	0		0
Chlorfenson	0	0	0	0
Iodofenfos	0	n.a.	0	
DDE-pp	0	0	0	0
Dieldrin	0		0	n.a.
DDD-op	0	n.a.	n.a.	n.a.
Oxyfluorfen	n.a.	0	1	0
Dibromobenzophenone	0	0	0	0
Nitrofen	0	0	n.a.	0
Endrin	n.a.	n.a.	2	1
Fluazifop-P-butyl	n.a.	0	n.a.	0
Hexaconazole	n.a.	n.a.	n.a.	n.a.
Chloropropylate	0	n.a.	n.a.	0
DDT-op	0	n.a.	0	n.a.
DDD-pp	n.a.	0	n.a.	n.a.
Benodanil	0	0	1	0
TRIS-IS				
Edifenphos	n.a.	0	0	n.a.
Endosulfan-sulfate	0	0	0	0
DDT-pp	n.a.	0	n.a.	n.a.
Propargite	n.a.	4	3	n.a.
Piperonyl-butoxide	n.a.	1	1	n.a.

Figure 6. Overview of the quantitation results for the target compounds in all matrices

Another option could be to review the global quantitation table, showing all targets in all samples at a glance (Figure 6).

The compounds showing a quantitation value above the maximum acceptable value can be red-flagged, as shown by the two examples in this table.

The operator simply selects the flagged value in order to visualize the corresponding chromatogram. The quantitation value, confirmation status, actual ion ratio and expected ion ratio are shown, which can help the operator to determine if the result should be validated. Figure 7 shows two examples: the top peak (Heptachlor Epoxide) is above the maximum acceptable value, but not confirmed; the bottom peak (Piperonyl butoxide) is confirmed, but below the reporting limit.

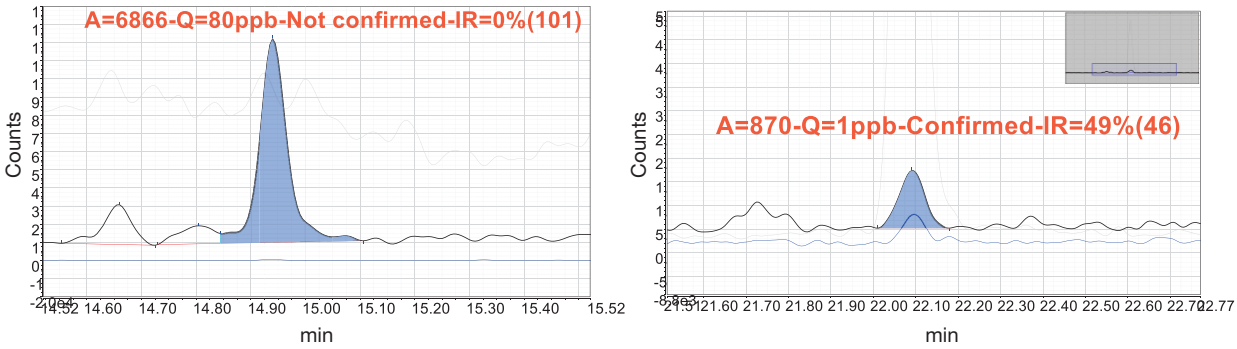


Figure 7. Red-flagged values from the interactive table showing the corresponding peaks

Alternatively, a consolidated table can be created as a report template to view both the recoveries and quantitation (Figure 8).

First Injection	PS_APPLE	FS_APPLE	PS_BANANA	FS_BANANA	PS_CAROTS	FS_CAROTS	PS_EGGPLANT	FS_EGGPLANT
Peak Name	quan & recovery	quan & recovery	quan & recovery	quan & recovery	quan & recovery	quan & recovery	quan & recovery	quan & recovery
	ppb & %	ppb & %	ppb & %	ppb & %	ppb & %	ppb & %	ppb & %	ppb & %
Dichlobenil	0	104	n.a.	107	0	89	0	75
Chlormephos	n.a.	98	n.a.	99	n.a.	104	0	87
Propham	1	140	1	100	0	89	1	101
Pentachlorobenzene	0	93	n.a.	94	n.a.	91	0	97
Tecnazene	0	86	0	94	n.a.	102	0	93
Diphenylamine	0	97	0	90	0	93	0	80
Ethalfluralin	n.a.	88	0	94	0	90	0	107
Chlorprophame	0	94	n.a.	92	0	93	0	99
Benfluralin	0	105	0	97	0	87	0	93
Trifluralin	0	97	n.a.	93	n.a.	89	0	93
Phorate	0	93	0	87	n.a.	93	0	87
BHC-alpha	0	109	0	88	0	118	0	98
Hexachlorobenzene	n.a.	87	n.a.	86	n.a.	78	0	79
Dichloran	0	107	0	88	0	70	0	87

Figure 8. Consolidated table showing the target compounds' quantitation and recovery data in all matrices

Workflow 3: LC-MS with validated recoveries

When a large number of recovery determinations are required, the number can be reduced to a more practical size, per the SANTE guidelines. In many labs, the recovery value is included in the validation method and re-validated every 6-12 months. In this case, the number of required injections is reduced to two, and view settings can be also adjusted in LC-MS experiments.

ISTD review

As noted in the GC-MS workflow, the first step is to assess the internal standard. If it does not pass both the recovery and retention time criteria, the peaks and quantification of the individual targets will not be accurate.

The criteria can be adjusted according to lab requirements; however, most regulations require that the ISTD peak area in the unknown sample must be within 30% of the ISTD peak area in the calibration standard sample.

With the LC method, the ISTD's retention time in the sample must not shift to more than 2.5% of retention time value in the calibrant.

The Chromeleon software will automatically measure and validate those points (Figure 9), showing visual alerts if a failure is detected. In this example, the extraction from the banana sample must be repeated because the variation of area (47%) is red-flagged because it is >30%.

If all the other matrix extractions meet the requirements, the next step of reprocessing can be done to review the sequence and results (Figure 10).

Conclusion

The Chromeleon CDS can be adapted to any workflow process in the testing lab. On-going method performance verification analysis is conducted daily to meet the SANTE/12682/2019 guidelines. Moreover, working at the reporting limit and measuring recoveries in all matrices while using a single level calibrant in the matrix enables the most accurate and precise results, with a minimum number of injections. Identification criteria, such as ion ratio and retention times, are also automatically checked.

This software provides excellent operator versatility, including customized view settings, interactive results tables, and exportable reports. In addition, it offers personal visual settings, alerts and automatic calculations that will enable the user to reprocess data with high efficiency, reducing time and improving productivity.

Learn more at **thermofisher.com/chromeleon**