Advanced internal standard techniques for quantitation

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Goal

To highlight the capabilities of Chromeleon CDS in performing advanced quantitation using internal standards. These techniques are needed for applications that require the use of multiple ISTDs. This technical note will utilize dioxin analysis as an example to highlight Chromeleon software features such as multiple ISTDs with variable amounts, custom variables, and fixed calibrations.

Introduction

Most chromatographic assays are straightforward when it comes to the calibration of standard solutions and subsequent calculation of sample results. The most commonly used techniques include single or multi-point calibrations from external or internal standards. These can be easily set up by most chromatographers in Thermo Scientific™ Chromeleon™ 7 Chromatography Data System (CDS) software.

For more complex calibration methods, such as those used in the analysis of dioxins, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), polyfluorinated compounds (PFCs), and other halogenated flame retardents, Chromeleon CDS supports multiple internal standard (ISTD) components with variable amount values, eliminating the need to perform the calculations outside the software.

Dioxins (and PCBs) are persistent organic pollutants that bio-accumulate in people and animals and can cause health issues. Due to their persistence in the environment and food chain, dioxins are routinely monitored in human food and animal feed along with air, water, and soil samples. The low limits allowed by regulatory agencies, coupled with the challenging matrices of these types of samples, leads to a difficult analysis due to the extensive sample preparation required. In order to overcome these challenges, dioxin analysis is performed using a technique called isotope dilution mass spectrometry (IDMS). In IDMS an unknown sample is spiked with an internal standard that is an isotopically-altered reference standard of the compound of interest. The spike changes the natural isotopic composition of the compound (giving rise to the name “isotopic dilution”) and when compared to the known internal reference standard composition, gives a highly accurate and reproducible quantitative result.

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An internal standard is a compound that is added to the sample at some point during the sample preparation. Dioxin analysis, as well as many other environmental and food testing methods based on GC-MS, requires the use of multiple types of internal standards. In the case of dioxin analysis, the common internal standards used are known as labelled surrogate standards and syringe (or recovery) standards. Surrogate standards are added to a sample prior to extraction and used to factor in any analyte loss that occurs during the extraction process. In the case of isotope dilution assays, the surrogate internal standard is also used to create the isotope dilution and quantitate the amount of unknowns in the sample. Syringe standards are added to samples directly before analysis to take into account any variability in the injection, from either the syringe or system, by measuring the recovery of the known amount of surrogate standard added. When performing a typical dioxin analysis, there can be as many as seventeen $^{13}C_{12}$-labelled compounds used as surrogates, as well as additional compounds used as syringe standards. To complicate the analysis even more, the concentrations of the surrogates or syringe standards may vary in a single assay.

Adding and identifying the internal standard components

Components are the substances that will be identified and quantitated in a sample being analyzed. In the dioxin example being used, there may be as many as 17 different components present in a single sample. Components also need to be created for each ISTD that will be used in quantitation, so after figuring in the surrogate standards and syringe standards, the number of components can grow very quickly. Each component must be added to the Component Table in the Processing Method prior to unknown sample results being determined.

Adding components to the Processing Method Component Table is a straightforward process. For the purposes of this technical note, the category of the Component Properties box to focus on is Evaluation. In the dioxin example, components have been created for each analyte of interest (i.e., unlabeled 1,2,3,7,8-PeCDD) as well as its surrogate ISTD (i.e., $^{13}C_{12}$-labeled 1,2,3,7,8-PeCDD IS). Figures 1a and 1b show the Standard Method area of the Evaluation category of their Component Properties boxes. The labeled surrogate standard is created as a Variable Internal Standard (Figure 1a), and the unlabeled component designates the corresponding labeled surrogate as its internal standard (Figure 1b).

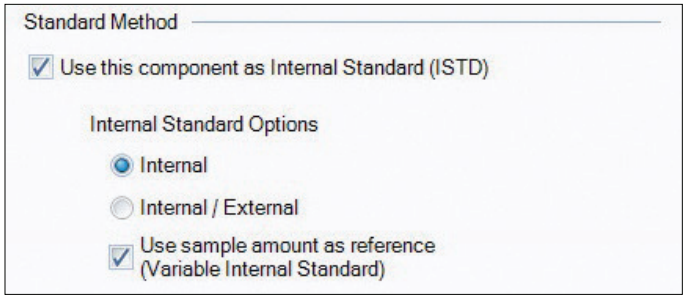

Figure 1a. Component properties of $^{13}C_{12}$-labeled 1,2,3,7,8-PeCDD IS

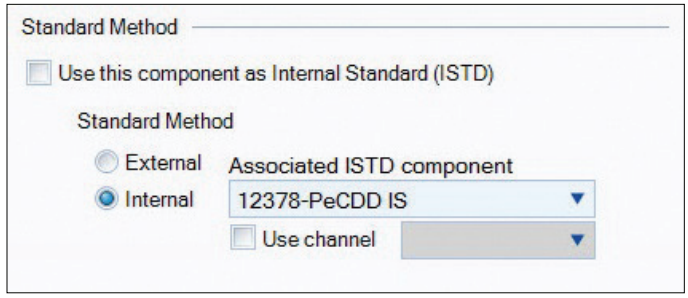

Figure 1b. Component properties of unlabeled 1,2,3,7,8-PeCDD

Quantitation utilizing ISTD typically uses response ratios of the analyte peak and ISTD. Many of these assays require a calibration curve to be generated to compare the unknown response. In the dioxin example, calibration solutions using unlabeled standard solutions of various concentration are spiked with the surrogate standard ISTD, their responses measured, and response factors are determined from resulting calibration curves. The concentration levels of the unlabeled calibration standards are added to the injection list under the **Level** column and the corresponding concentrations are added to each component in the Processing Method's Component Table (Figure 2.)

Component Table

Group Area Drag a column header here to group by that column. Run Component Table Wizard... Show Properties...

#	Name	Ret.Time	Level "Non-ortho PCB & PCDD/F Samples"	Level "PCB CS1/10"	Level "PCDD/F CS1"	Level "PCDD/F CS2"	Level "PCDD/F CS3"	Level "PCDD/F CS4"	Level "PCDD/F LOQ"	Level "PCDD/F LOQ/2"	Level "PCDD/F LOQ/4"	Level "PCDD/F LOQx2"
1	1234678-HpCDD	33.782	32.000000		0.800000	3.200000	16.000000	64.000000	0.040000	0.020000	0.010000	0.080000
2	1234678-HpCDF	32.354	32.000000		0.800000	3.200000	16.000000	64.000000	0.040000	0.020000	0.010000	0.080000
3	123478-HxCDD	29.940	16.000000		0.400000	1.600000	8.000000	32.000000	0.040000	0.020000	0.010000	0.080000
4	123478-HxCDF	29.059	16.000000		0.400000	1.600000	8.000000	32.000000	0.020000	0.010000	0.005000	0.040000
5	1234789-HpCDF	34.521	32.000000		0.800000	3.200000	16.000000	64.000000	0.040000	0.020000	0.010000	0.080000
6	123678-HxCDD	30.029	16.000000		0.400000	1.600000	8.000000	32.000000	0.040000	0.020000	0.010000	0.080000
7	123678-HxCDF	29.170	16.000000		0.400000	1.600000	8.000000	32.000000	0.020000	0.010000	0.005000	0.040000
8	12378-PeCDD	25.960	16.000000		0.400000	1.600000	8.000000	32.000000	0.020000	0.010000	0.005000	0.040000
9	12378-PeCDF	24.337	16.000000		0.400000	1.600000	8.000000	32.000000	0.020000	0.010000	0.005000	0.040000
10	123789-HxCDD	30.351	16.000000		0.400000	1.600000	8.000000	32.000000	0.040000	0.020000	0.010000	0.080000
11	123789-HxCDF	30.715	16.000000		0.400000	1.600000	8.000000	32.000000	0.020000	0.010000	0.005000	0.040000
12	234678-HxCDF	29.848	16.000000		0.400000	1.600000	8.000000	32.000000	0.020000	0.010000	0.005000	0.040000
13	23478-PeCDF	25.710	16.000000		0.400000	1.600000	8.000000	32.000000	0.020000	0.010000	0.005000	0.040000
14	2378-TCDD	20.861	16.000000		0.200000	0.800000	4.000000	16.000000	0.010000	0.005000	0.002500	0.020000
15	2378-TCDF	20.299	16.000000		0.200000	0.800000	4.000000	16.000000	0.010000	0.005000	0.002500	0.020000
16	OCDD	38.386	32.000000		0.800000	3.200000	16.000000	64.000000	0.160000	0.080000	0.040000	0.320000
17	OCDF	38.636	32.000000		0.800000	3.200000	16.000000	64.000000	0.160000	0.080000	0.040000	0.320000

Figure 2. Component Table with calibration standard levels

Enabling multiple internal standards with variable amounts

The ability to use the functionality of multiple ISTD components with variable amounts in Chromeleon CDS needs to be enabled in the sequence. This is accomplished by first creating a custom injection variable named "**CM7:IntStd_Level**", with the type being **List**, and adding it to the sequence. This custom variable can be imported from another sequence or Data Vault, or can be created as a new custom variable, either of which can be done in the Custom Variable Wizard. If it is created new, it is important that the name is exactly as shown; the custom variables described in this technical note that start with "**CM7:**" have unique features that utilize pre-built code to make them work as described. The list of possible values for the custom variable must be filled with the calibration level names that will correspond to the reference amounts of the ISTD components (Figure 3). Once this custom variable is present in the sequence, all the sample details (weights, dilution factors, processing methods, calibration levels, etc.) can be completed. Reference Figure 4 for an example sequence. Note the * next to CM7:IntStd_Level, indicating that it is a custom variable.

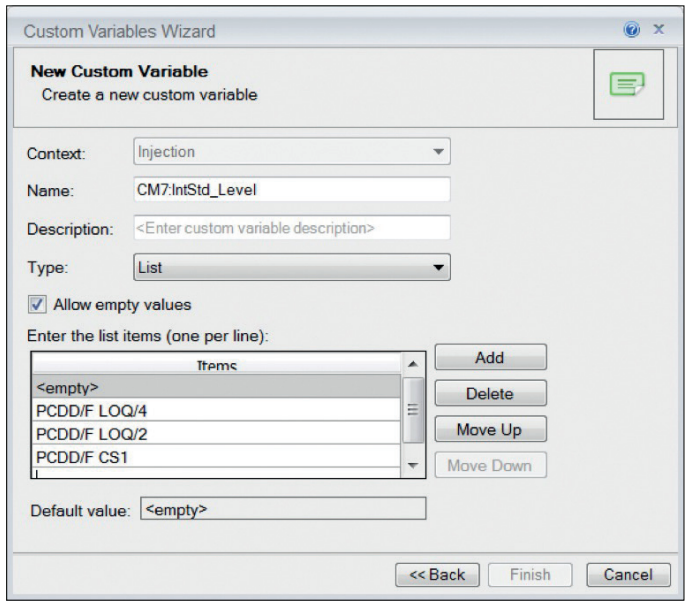

Figure 3. Adding values in the Custom Variables Wizard

#	Name	Type	Level	*CM7:IntStd_Level	Weight	Dilution	Position	Volume [µL]
1	Blank	Unknown			1.0000	1.0000	10	4.00
2	LOQ/4	Check Standard	PCDD/F LOQ/4	PCDD/F LOQ/4	1.0000	1.0000	11	4.00
3	LOQ/4	Check Standard	PCDD/F LOQ/4	PCDD/F LOQ/4	1.0000	1.0000	11	4.00
4	LOQ/2	Check Standard	PCDD/F LOQ/2	PCDD/F LOQ/2	1.0000	1.0000	12	4.00
5	LOQ/2	Check Standard	PCDD/F LOQ/2	PCDD/F LOQ/2	1.0000	1.0000	12	4.00
6	LOQ	Check Standard	PCDD/F LOQ	PCDD/F CS1	1.0000	1.0000	13	4.00
7	LOQ	Check Standard	PCDD/F LOQ	PCDD/F CS1	1.0000	1.0000	13	4.00
8	LOQ*2	Check Standard	PCDD/F LOQx2	PCDD/F CS1	1.0000	1.0000	14	4.00
9	LOQ*2	Check Standard	PCDD/F LOQx2	PCDD/F CS1	1.0000	1.0000	14	4.00
10	CS1	Calibration Standard	PCDD/F CS1	PCDD/F CS1	1.0000	1.0000	15	4.00
11	CS1	Calibration Standard	PCDD/F CS1	PCDD/F CS1	1.0000	1.0000	15	4.00
12	CS2	Calibration Standard	PCDD/F CS2	PCDD/F CS1	1.0000	1.0000	16	4.00
13	CS2	Calibration Standard	PCDD/F CS2	PCDD/F CS1	1.0000	1.0000	16	4.00
14	CS3	Calibration Standard	PCDD/F CS3	PCDD/F CS1	1.0000	1.0000	17	4.00
15	CS3	Calibration Standard	PCDD/F CS3	PCDD/F CS1	1.0000	1.0000	17	4.00
16	CS4	Calibration Standard	PCDD/F CS4	PCDD/F CS1	1.0000	1.0000	18	4.00
17	CS4	Calibration Standard	PCDD/F CS4	PCDD/F CS1	1.0000	1.0000	18	4.00
18	Blank	Unknown			1.0000	1.0000	19	4.00
19	Blank	Unknown			1.0000	1.0000	19	4.00
20	Blank	Unknown			1.0000	1.0000	20	4.00
21	Blank	Unknown			1.0000	1.0000	20	4.00
22	1201 PLA 1	Unknown		Non-ortho PCB & PCDD/F Samples	2.0480	20.0000	21	4.00
23	1201 PLA 1	Unknown		Non-ortho PCB & PCDD/F Samples	2.0480	20.0000	21	4.00
24	9373	Unknown		Non-ortho PCB & PCDD/F Samples	2.1700	20.0000	22	4.00
25	9373	Unknown		Non-ortho PCB & PCDD/F Samples	2.1700	20.0000	22	4.00
26	9487	Unknown		Non-ortho PCB & PCDD/F Samples	1.9930	20.0000	23	4.00
27	9487	Unknown		Non-ortho PCB & PCDD/F Samples	1.9930	20.0000	23	4.00
28	9370	Unknown		Non-ortho PCB & PCDD/F Samples	2.0730	20.0000	24	4.00
29	9370	Unknown		Non-ortho PCB & PCDD/F Samples	2.0730	20.0000	24	4.00
30	QK1 1	Unknown		Non-ortho PCB & PCDD/F Samples	1.9870	20.0000	25	4.00
31	Blank	Unknown			1.0000	1.0000	26	4.00
32	CS/10	Check Standard	PCB CS1/10	PCB CS1/10	1.0000	1.0000	27	4.00
33	LOQ/4	Check Standard	PCDD/F LOQ/4	PCDD/F LOQ/4	1.0000	1.0000	28	4.00
34	LOQ/2	Check Standard	PCDD/F LOQ/2	PCDD/F LOQ/2	1.0000	1.0000	29	4.00

Figure 4. Example sequence with custom variable included

Setting up the variable amounts

Internal standards are typically added at the same concentration levels for standards and samples throughout an analysis. However, there are times, for example, when analyzing extracted samples acquired from a different laboratory, that different concentrations may be added to certain samples. After the "**CM7:IntStd_Level**" custom variable is added to a sequence, the values of the corresponding concentration levels in the Component Table are created and will be used (Figure 5). Concentration values may be different for each component as well as each level, allowing for complete control of the calibration required. Adjusting the values may be performed directly in the Component Table or in the properties box for the individual component (Figure 6).

Component Table

Group Area Drag a column header here to group by that column. Run Component Table Wizard... Show Properties...

#	Name	*TEF	Level "Non-ortho PCB & PCDD/F Samples"	Level "PCB CS1/10"	Level "PCDD/F CS1"	Level "PCDD/F CS2"	Level "PCDD/F CS3"	Level "PCDD/F CS4"	Level "PCDD/F LOQ"	Level "PCDD/F LOQ/2"	Level "PCDD/F LOQ/4"	Level "PCDD/F LOQx2"
22	1234-TCDD IS		16.000000		16.000000					8.000000	4.000000	16.000000
23	1234678-HpCDD IS		32.000000		32.000000					16.000000	8.000000	32.000000
24	1234678-HpCDF IS		32.000000		32.000000					16.000000	8.000000	32.000000
25	123478-HxCDD IS		16.000000		16.000000					8.000000	4.000000	16.000000
26	123478-HxCDF IS		16.000000		16.000000					8.000000	4.000000	16.000000
27	1234789-HpCDF IS		32.000000		32.000000					16.000000	8.000000	32.000000
28	123678-HxCDD IS		16.000000		16.000000					8.000000	4.000000	16.000000
29	123678-HxCDF IS		16.000000		16.000000					8.000000	4.000000	16.000000
30	12378-PeCDD IS		16.000000		16.000000					8.000000	4.000000	16.000000
31	12378-PeCDF IS		16.000000		16.000000					8.000000	4.000000	16.000000
32	123789-HxCDD IS		16.000000		16.000000					8.000000	4.000000	16.000000
33	123789-HxCDF IS		16.000000		16.000000					8.000000	4.000000	16.000000
34	234678-HxCDF IS		16.000000		16.000000					8.000000	4.000000	16.000000
35	23478-PeCDF IS		16.000000		16.000000					8.000000	4.000000	16.000000
36	2378-TCDD IS		16.000000		16.000000					8.000000	4.000000	16.000000
37	2378-TCDF IS		16.000000		16.000000					8.000000	4.000000	16.000000

Figure 5. Completed Component Table displaying calibration levels for various components

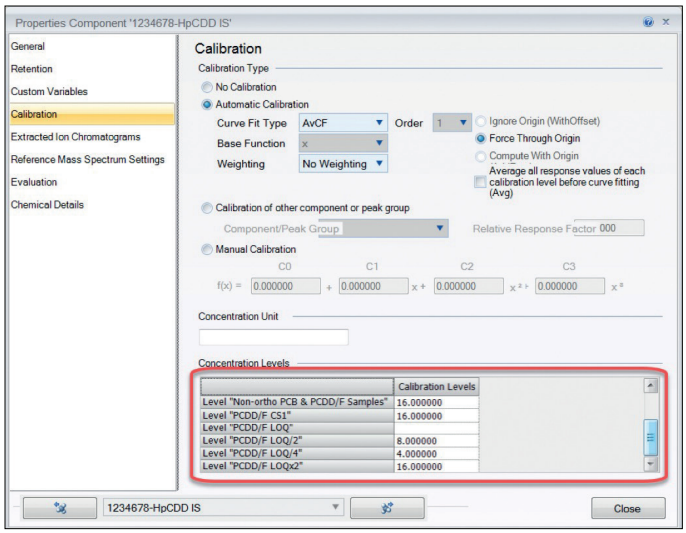

Figure 6. Component Properties box

Adding syringe standards – using an ISTD to reference another ISTD

The inclusion of syringe standards presents a unique situation where an ISTD (the syringe standard) must be used to perform a calculation on another ISTD (the surrogate standard.) This can be performed by using a custom component variable to identify the correct syringe standards. Figure 7 shows the Properties Component dialog box for a surrogate ISTD. In the Custom Variables category there is an additional custom variable of type **List** present **– CM7:SyringeStandard**. This custom variable (similar to **CM7:IntStd_Level**, in that it must be named exactly) can be used to identify which syringe standard is to be used to determine recovery for each surrogate standard. Figure 8 shows the completed Component Table, which includes the syringe standard for each component. The custom variable **Entry_Type** is not required, but was added to make identification easier and sorting/filtering possible.

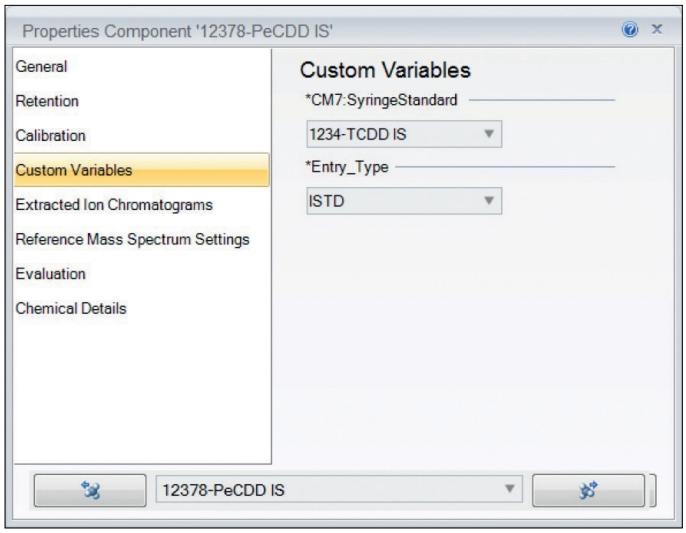

Figure 7. Custom Component Variable identifying syringe standards

Component Table

Group Area — Drag a column header here to group by that column. Run

#	Name	Ret.Time	*CM7:SyringeStandard	*Entry_Type
1	1234678-HpCDD IS	33.770	123789-HxCDD IS	ISTD
2	1234678-HpCDF IS	32.342	123789-HxCDD IS	ISTD
3	123478-HxCDD IS	29.935	123789-HxCDD IS	ISTD
4	123478-HxCDF IS	29.053	123789-HxCDD IS	ISTD
5	1234789-HpCDF IS	34.508	123789-HxCDD IS	ISTD
6	123678-HxCDD IS	29.935	123789-HxCDD IS	ISTD
7	123678-HxCDF IS	29.165	123789-HxCDD IS	ISTD
8	12378-PeCDD IS	25.946	1234-TCDD IS	ISTD
9	12378-PeCDF IS	24.320	1234-TCDD IS	ISTD
10	123789-HxCDF IS	30.707	123789-HxCDD IS	ISTD
11	234678-HxCDF IS	29.840	123789-HxCDD IS	ISTD
12	23478-PeCDF IS	25.696	1234-TCDD IS	ISTD
13	2378-TCDD IS	20.858	1234-TCDD IS	ISTD
14	2378-TCDF IS	20.287	1234-TCDD IS	ISTD
15	OCDD IS	38.369	123789-HxCDD IS	ISTD
16	OCDF IS	38.625	123789-HxCDD IS	ISTD
17	PCB 126 L	20.817	PCB 111 L	ISTD
18	PCB 169 L	25.370	PCB 170 L	ISTD
19	PCB 77 L	16.795	PCB 70 L	ISTD
20	PCB 81 L	16.317	PCB 70 L	ISTD
21	1234-TCDD IS	20.108		SYR_STD
22	123789-HxCDD IS	30.346		SYR_STD

Figure 8. Component Table identifying syringe standards for each component

Using previous calibrations

Chromeleon CDS allows calibrations to be created from standards run in the current sequence or from calibration standards run in a previous sequence. The latter is especially useful in an analysis such as dioxins because the surrogate standards are extremely expensive due to their isotopic makeup. Instead of using fresh standards each time samples are analyzed (which would get very expensive very quickly), the calibration from a previous sequence can be used and QC checks run in order to verify the system is operating as expected. This functionality within Chromeleon software is the fixed calibration and can be enabled in the Calibration tab of the Processing Method (Figure 9.) All of the functions mentioned in the technical note are compatible with fixed calibrations.

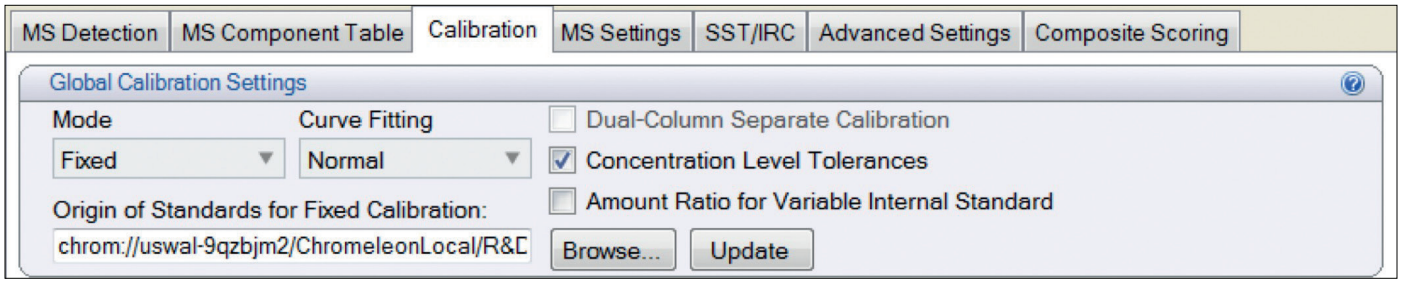

Figure 9. Fixed calibration mode settings

Summary

The complicated matrices found in many environmental, food, and other testing areas gives rise to the need for advanced use of internal standards. The complex sample extractions required for these types of samples (for example, testing for dioxins and/or PCBs in soil, water, air, or food) raises the potential for analyte loss, so the use of surrogate standards and their associated syringe standards is common. Chromeleon CDS has built-in capabilities designed to handle these advanced internal standard methodologies, allowing the CDS to automatically perform the calculations, eliminating the need to perform them outside the software.

Reference

1. EPA Method 1613b, Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS

Find out more at **thermofisher.com/chromeleon**

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