Chapter 10
Analyzing Food Samples—Organic Chemicals

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Introduction

The challenge for the analysis of organic chemicals in an advanced total diet study (TDS) is that the most extreme and varied food matrices in the diet of the population must be analyzed for a large number of residues at very low levels. In the United States Food and Drug Administration (FDA) TDS program, approximately 280 foods are analyzed for more than 500 organic chemicals at levels as low as 0.1 part per billion (ppb). This feat is accomplished by using several means. Samples are analyzed by multiple analytical methods ranging from single residue methods designed for specific types of matrices to general screening procedures capable of determining hundreds of analytes found in the full spectrum of TDS matrices. A variety of instruments are employed for the determinations, including some the newest and most sophisticated technologies available, and a few that are older and simpler, yet still fit-for-purpose. Critical attention is applied to the correct identification of residues, the most important task in residue analysis. In addition, all analyses are conducted within an exhaustive quality management system. These topics are briefly addressed in this chapter.

The procedures described within are general outlines and do not include all techniques and cautions. The full set of operational instructions can be found within the references listed below and are available from the FDA Kansas City District Laboratory.

Note: Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the US Food and Drug Administration.

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Analytical Methodologies

Overview

The analysis of pesticide and industrial chemicals (P&IC) in simple matrices is difficult, at best. As analytical screening levels are lowered, the challenge of accurate analyte quantification and identification increases because the analyte responses decrease compared to interferences from instrumental noise and matrix responses. It is significantly more demanding to determine a chemical contaminant in a simple standard solution at 1 ppb than at the percent level because instrumental noise and matrix responses remain constant, but analyte responses diminish as their concentration decreases. At a concentration normally referred to as the Limit of Detection (LOD), the response of the analyte can no longer confidently be distinguished from the interferences and noise. The introduction of complex food matrices complicates the process geometrically.

A P&IC analytical method is essentially a separation process that removes an analyte from the matrix and isolates it for measurement. Methods for the analysis of P&ICs generally consist of three steps: extraction, cleanup and determination. In the extraction step, the chemical residues are dissolved in a solvent, and then physically separated from the solid sample matrix through filtration or centrifugation. The cleanup step selectively removes matrix coextractants that would interfere with the determination. The analyte is detected, characterized, and quantified in the determinative step.

The extent of each step is determined by the scope of targeted analytes for the procedure. For multiple residue methods (MRMs), the analyte scope may range from a few dozen to a thousand P&ICs; and for selected residue methods (SRMs), the scope will generally consist of a single analyte, e.g. perchlorate, or a class of P&ICs, such as the carbamate insecticides.

MRMs provide the most efficient screening proficiency because they cover more residues per analysis than SRMs; however, they present particular challenges. The extraction solvent must be able penetrate complex and varied food matrices to dissolve analytes that have a wide range of polarities and chemical affinities. Acetone and acetonitrile are the two most commonly used solvents for nonfat food matrices because they are mid-polar organic solvents that are able to dissolve most P&ICs; and they are miscible with water, the primary constituent of nonfat foods. Given their universal ability to solvate chemicals and residues, the extraction of foods with acetone or acetonitrile results in extremely complex mixtures of matrix coextractants that can often interfere with the determination of the targeted analytes. Therefore, MRM extracts usually undergo a cleanup step to selectively remove matrix coextractants prior to determination. Cleanup procedures must be applied judiciously, however, because some residues may also be partially or fully removed from the extract with the coextractants. Even with a reasonably applied cleanup, interpretation of instrumental determinations of the residues in these complex matrix extracts can be problematic.

Table 10.1 US Total Diet Study pesticide and industrial chemical analytical methods

SOP*	Method	Analytes	US TDS food items
51[7]	Analysis for pesticide and industrial chemical residues in fatty items	~350 P&ICs	125
52[8]	Analysis for pesticide and industrial chemical residues in nonfat items	~450 P&ICs	155
53[9]	Determination of chlorophenoxy acid herbicides and pentachlorophenol	15 CPAs	20
54[10]	Determination of phenylurea herbicides	10 Phenylureas	56
55[11]	Determination of carbamate pesticides	12 Carbamates	117
56[12]	Determination of ethylenethiourea	ETU	94
57[13]	Determination of benzimidazoles	2 Benzimidazoles	101
71[14]	Perchlorate analysis in food items	Perchlorate	280

*USFDA TDS Standard Operating Procedure

SRMs, on the other hand, have the opposite advantages and disadvantages. By limiting their scope to a single analyte, or class of analytes, the complexity of the extraction method can be reduced tremendously, but their screening efficiency is drastically reduced. The US TDS procedure for ethylenethiourea (ETU) is an excellent example of a classic SRM. ETU is a suspected carcinogen occurring in foods as a result of the degradation of the ethylenebisdithiocarbamate (EBDC) fungicides used extensively to preserve raw agricultural commodities. ETU is extracted using aqueous methanol, an extremely polar solvent that effectively discriminates against nonpolar coextractants. Once dissolved, the polar coextractants are removed using alumina column chromatography. After cleanup, the extracted ETU is determined using high performance liquid chromatography (HPLC) with amperometric detection at a very low voltage, as ETU is oxidized at a much lower potential than most food matrix coextractants. The procedure is quite specific for ETU. Being an SRM, however, this may be considered an inefficient use of resources unless the analysis of the parent fungicides had indicated a potential problem.

Historically, P&ICs have been analyzed in the US TDS program using a combination of MRMs and SRMs [1–8]. Table 10.1 presents the current list of methods and their analytical scope. These procedures are primarily based upon methods found in the FDA Pesticide Analytical Manual [9] (PAM). FDA pesticide laboratories are currently collaborating in the development of a modified QuEChERS [10–14] method that will be used in the US TDS to consolidate the methods for nonfat TDS items (SOPs 52, 54, 55, 56 and 57 in Table 10.1).

MRM Analysis of Fatty Food Items

In the MRMs for fatty food items (SOP KAN-LAB-PES.51), samples are extracted with lipophylic solvents, such as hexane, petroleum ether (hexanes), ethyl ether, or supercritical fluid carbon dioxide (SOP KAN-LAB-PES.61 [15]); solids are

removed by filtration or centrifugation; dissolved lipids are removed by gel permeation chromatography (GPC) per SOP KAN-LAB-PES.63 [16]; and polar coextractants are removed using Florisil chromatography (SOP KAN-LAB-PES.64 [17]). The extracts are analyzed for about 150 organophosphorus P&ICs (OP-P&ICs) using gas chromatography with a flame photometric detector (GC-FPD) and for approximately 200 organohalogen P&ICs (OH-P&ICs) using gas chromatography with an electrolytic conductivity detector in the halogen mode (GC-ELCD).

MRM Analysis of Nonfatty Food Items

In the MRMs for nonfatty foods (SOP KAN-LAB-PES.52), TDS items are extracted with acetone; solids are removed by filtration or centrifugation; water from the sample is removed by partitioning the acetone/aqueous extract with methylene chloride; the extract is solvent exchanged to acetone and concentrated to approximately 2.8 g sample/ml per SOP KAN-LAB-PES.62 [18]. This extract is analyzed for about 200 OPs on a gas chromatograph with a pulsed flame photometric detector (GC-PFPD) and approximately 120 other P&ICs by gas chromatography with mass spectroscopic detection in the selective ion monitoring mode (GC-MS SIM). Over 150 OH-P&ICs are determined using GC-ELCD after a portion of the acetone extract has been cleaned up using Florisil chromatography to remove polar coextractants.

SRM Analysis for Carbamates

The acetone extract from the nonfatty MRM is also used for the analysis of carbamate pesticides per SOP KAN-LAB-PES.55. The acetone extract is passed thru an aminopropyl solid phase extraction (SPE) column to remove acidic and cationic coextractants before determination by high pressure liquid chromatography with tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) in the multiple reaction monitoring mode.

SRM Analysis for Phenylurea Herbicides

Phenylurea herbicides are analyzed per SOP KAN-LAB-PES.54. TDS items are extracted with methanol/water; solids are removed by filtration or centrifugation; the analytes are partitioned into methylene chloride; polar coextractants are removed by Florisil column chromatography; and the residues are determined by LC-MS/MS.

SRM Analysis for Benzimidazole Fungicides

In the analysis of benzimidazole fungicides (SOP KAN-LAB-PES.57) items are extracted with methanol/water; solids are removed by filtration or centrifugation; the extract is acidified and the fatty acids and nonpolar coextractants are separated by partitioning them into methylene chloride; the extract is basified and the analytes are partitioned into methylene chloride. The residues are determined by LC-MS/ MS.

SRM Analysis for Ethylenethiourea (ETU)

For the analysis of ETU (SOP KAN-LAB-PES.56) the sample is extracted with methanol/water; solids are removed by filtration or centrifugation; the analytes are partitioned into methylene chloride; polar coextractants are removed using alumina chromatography; and ETU determination is by high pressure liquid chromatography with an amperometric electrochemical detector (HPLC-EC) using a mercury and gold amalgamated electrode at a very low potential of 350 mV.

SRM Analysis for Chlorophenoxy Acid Herbicides and Pentachlorophenol

Chlorophenoxy acid herbicides and pentachlorophenol (CPAs) are extracted using acidified methanol to inhibit ionization by the deprotonation of the acid; the CPAs are methylated to volatilize them for determination by GC-MSD; prior to determination the methylated extract is passed though Florisil to remove polar coextractants. This procedure is not posted because it is being replaced by a new method for the analysis of CPAs using an acidified QuEChERS procedure with determination by LC-MS/MS using negative electrospray ionization in the multiple reaction monitoring mode.

SRM Analysis for Perchlorate Ion

The procedure for the analysis of perchlorate ion is provided in SOP KAN-LAB-PES.71. In the method perchlorate ion is extracted with acidified acetonitrile; neutral and lipophylic coextractants are removed by filtering the extract through carbon SPE, and perchlorate is determined using ion chromatography with LC-MS/MS. The use of the $^{18}O_4$ isotope of perchlorate as an internal standard enhances the quality of the analysis because it eliminates extraction volume errors and matrix/analyte interaction biases. Isotope usage for residue work is encouraged due to the aforementioned benefits; however it is impractical for MRMs due to the lack of availability and the cost of isotopes.

Analysis of Nonfat Items by QuEChERS

Since the introduction of the QuEChERS (Quick Easy Cheap Efficient Rugged and Safe) procedure, many residue testing labs around the world have adapted and modified it for inclusion in their surveillance programs. The FDA has recently validated and collaborated the procedure for regulatory analysis of P&IC residues [19]. In the method, residues are extracted with acetonitrile; water is removed by salting out with sodium chloride and magnesium sulfate; dispersive SPE using primary/secondary amines (PSA) is used to remove coextractants from a small portion of the acetonitrile extract and diluted for LC-MS/MS determination of approximately 200 pesticides. The rest of the acetonitrile extract is diluted 1 + 3 with toluene; dispersive SPE using graphitized carbon black is used to remove matrix coextractants; the extract is concentrated for determination of over 300 P&ICs by GC-MS in the SIM mode.

Determination Procedures and Instrumentation

Instrumental determination of TDS samples is largely driven by their selectivity and sensitivity. As previously stated the challenge for TDS analysis of chemical contaminants is that the lowest level of chemical residues are measured in the most extreme and varied food matrices. For the US TDS program the goal is to analyze and detect residues at levels of 1 ppb; however, the nominal reporting limit of 0.1 ppb is routinely achieved and reported. To achieve this, the instruments must be capable of detecting analytes at the 10–100 picogram (pg) levels while discriminating against matrix responses. Additionally, the thermal stability and volatility of the analytes must be considered. In the US TDS, LC-MS/MS is used in the determination of thermolabile and nonvolatile compounds. For thermally stable and volatile compounds, multiple configurations of gas chromatographs (GCs) with selective detection are used: GC-FPD in the phosphorus mode, GC-ELCD in the halogen mode, and GC-MS in the SIM mode.

Determination by GC Using Selective Heteroatom Detection

Amongst chemical residue programs, GC with various detector configurations is the most commonly used determination procedure for the analysis of P&ICs. Separation of analyte and matrix responses is accomplished by temperature-programmed capillary chromatography on multiple GC systems equipped with different stationary phases. Specific instructions and instrument parameters for GC determinations using element selective detectors are provided in SOP KAN-LAB-PES.59 [20]. For the FPD and ELCD element selective detectors capillary column dimensions are 30 m length × 0.53 mm interior diameter (id), and the two most commonly used stationary

phases are 100 % methylpolysiloxane (DB-1) or 50 % phenyl methylpolysiloxane (DB-17). The nonpolar DB-1 stationary phase provides distinctly different chromatographic elution patterns from the mid-polar DB-17 phase. Other stationary phases used to provide additional elution patterns include the cyanopropylphenyl methylpolysiloxane phases with cyanopropylphenyl concentrations of 6 %, 14 %, and 50 %. The 6 % and 14 % phases are mid-polar and the 50 % mixture is considered a polar column. The disadvantage of using the cyanopropylphenyl columns is that they become unstable with prolonged use at temperatures above 200 °C resulting in column bleed.

Temperature programs are designed to elute the full scope of compounds listed in the Pestdata tables in Appendix 1 of PAM I. For example, temperature programs for halogenated P&IC's would chromatograph early eluting compounds, such as dichlobenil, monuron, hexachlorocyclopentadiene, etc., after the solvent front, and late eluters, such as deltamethrin, tralomethrin, fluvalinate, etc., prior to the end of the program. Likewise, for the initial determination of general organophosphates, the temperature program is designed to elute methamidophos, dichlorvos, trichlorfon, etc., after the solvent front and coumaphos, pyrazophos, bensulide, etc., prior to the end of the program. A typical program used in the US TDS for the GC-ELCDs and GC-FPDs is 120–280 °C @ 5 °C/minute, hold 5 min.

OP-P&ICs are determined using GC-FPDs and GC-PFPDs in the phosphorus mode. These detectors are essentially the same with slight variations in their mode of releasing elemental phosphorus from their molecular setting and raising the excitation level of the phosphorus electrons. They are extremely sensitive and selective for residues containing phosphorus. However the PFPD is approximately 5–10 times more sensitive than the FPD. One difficulty with both detectors arises with samples containing high levels of organosulfur (OS) coextractants, such as those found in onions and brassica vegetables, which can overwhelm the detector and obscure OP-P&IC analyte responses. Fortunately, very few products have high levels of OS and/or OP-P&IC coextractants. Some of the more polar OP-P&ICs, e.g. acephate, dimethoate, methamidophos, and omethoate, do not chromatograph well on the relatively nonpolar DB-1 and DB-17 stationary phases, but they perform much more consistently and exhibit greater sensitivity when analyzed by LC-MS/MS; therefore they have been added to the LC-MS/MS screening procedure and will be removed from GC-FPD and GC-PFPD determinations in the future.

OH-P&ICs are determined by GC-ELCD in the halogen mode. Like the GC-FPDs and GC-PFPDs the GC-ELCD responds to high levels of OS coextractants that can overwhelm the detector. It also responds to high levels of hydrocarbon coextractants if it is not maintained properly. GC-ELCDs are temperamental, requiring constant maintenance; however, they are still the most sensitive and selective instruments for the determination of OH-P&ICs.

Recent advances in instrument and computer processing technologies and efficiency indicate that the triple-quadripole GC-MS/MS operated in multiple reaction monitoring mode is approaching the sensitivity needed for the detection of sub ppb chemical residue levels. It is likely the US TDS will replace the use of selective GC detectors, like the GC-FPDs, GC-PFPDS, and the GC-ELCD, with GC-MS/MS in the near future.

Determination by GC-MS in the SIM Mode

Around 2003, GC-MS in the SIM mode was incorporated into the US TDS to detect analytes without halogen and phosphorous heteroatoms. Approximately 135 P&ICs are currently determined using the GC-MS SIM method per SOP KAN-LAB-PES.67 [21]. Analytes are separated on a 30 m length × 0.32 mm id with 5 % phenyl methylpolysiloxane capillary column using a segmented temperature program to optimize resolution of over 130 compounds: 50–130 °C @ 10 °C/minute, 130–230 °C @ 4 °C/minute, 230–290 °C @ 10 °C/minute, hold 7 min. A single quadripole mass spectrometer is programmed to capture the response of 3–4 selected ions characteristic of each analyte. Specificity relies on a combination of selective ion monitoring for brief elution windows, retention time, and agreement of ion ratios.

The SIM method does not generally meet the sensitivity requirements for the US TDS as many analytes cannot be detected below the 100 pg level resulting in LODs of 1–50 ppb. However, the P&ICs targeted by the procedure had not been previously included in the TDS screening regimen. As a result, 26 of the 135 compounds targeted by the procedure have now been reported in the US TDS since its implementation. As with the GC selective detectors, the GC-MS SIM method will likely be replaced by GC-MS/MS because of its increased sensitivity and selectivity.

LC-MS/MS Determination

Until recently, only determination by GC with element selective detection provided the selectivity and sensitivity required for sub ppb level TDS determinations; however, new advances in MS technology have enabled their implementation in the US TDS. In 2009, an LC-MS/MS procedure that replaces the HPLC detection of benzimidazoles, phenylureas, and carbamates was validated and collaborated in the USFDA pesticide laboratories [22], and implemented in the US TDS. The method detects an additional 160 selected P&ICs for a total of over 190 compounds (SOP KAN-LAB-PES.72 [23]). Analytes are separated on a 2.1 mm id x 10 cm long octyldecylsilane column with 3 µm particles. Mobile phase is 0.1 % formic acid/4 mM ammonium formate in water (aqueous) and methanol (organic). The mobile phase composition is programmed from 0 % to 90 % organic modifier in 12 min at a flow of 400 µl/min. Detection is by multiple reaction monitoring of molecular ions: two transition ions are monitored per analyte. A 10–20 µl of a 50 ng/ml standard is used to calibrate the system; Fig. 10.1 is a chromatogram of the standard containing 190 compounds. Samples of 10–20 µl are diluted to 0.5 g/ml before injection. Average LOD for all compounds is about 2–3 ppb, with a range of 0.1–20 ppb.

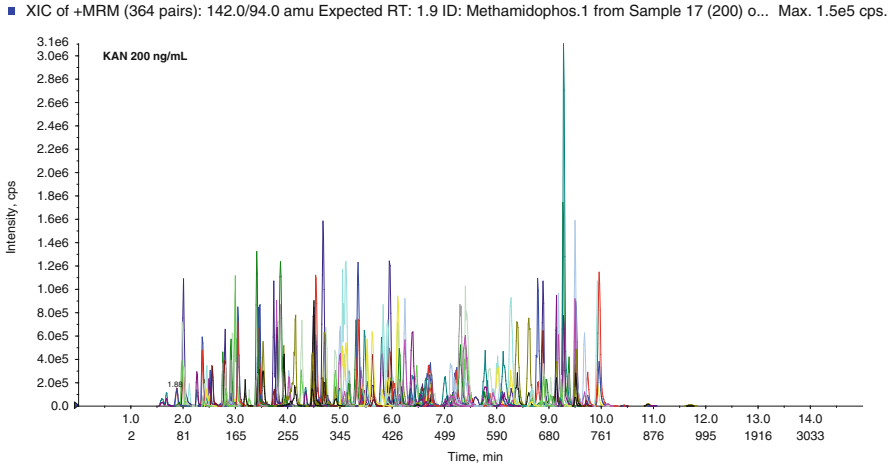

Fig. 10.1 LC-MS/MS chromatograph of 190 P&ICs at 200 ng/ml

Identification of Chemical Residues

The most critical aspect of chemical residue analysis is the correct identification of the residue. Analysis of chemical residues at 1 ppb means exactly that, i.e. the analyte is one billion times less concentrated than the sum of the matrix components. The probability of incorrectly identifying a matrix interference response as the analyte of interest increases exponentially as the concentration of the analyte in the matrix decreases. For mass spectral determination this problem is compounded by the fact most chemical contaminants are small (100–500 Da), therefore they have less distinctive unit-resolved masses and ion fragments than larger molecules found in typical food matrices, such as proteins, that have molecular weights of several thousand daltons.

Identification Point System

The strategy for correctly identifying chemical contaminants is to reduce the probability of misidentification to acceptable levels by comparing empirical evidence of the sample to standard responses. To that end an identification point (IP) system was implemented for the analysis of P&IC residues. It was first developed and adopted in the Europe [24] to standardize the process of identifying residues in light of the explosion of available analytical technologies and has been modified and implemented in various forms in the US [25–27]. In the system IPs are assigned to each

Table 10.2 Assignment of identification points

	Criteria	Point assignment
a.	Low resolution MS ion	1 point per ion
b.	Low resolution MS/MS precursor ion	1 point per precursor ion
c.	Low resolution MS/MS product ion (transition)	1.5 points per ion
d.	High resolution MS (HRMS) ion	2.0 points per ion
e.	High resolution MS precursor ion	2.0 points per ion
f.	High resolution MS product ion (transition)	2.5 points per ion
g.	Matching chromatographic retention time (RT)	1 point per alternative systems
h.	Selective detection with matching RT	1 point per detector
i.	Quantitative agreement between alternate column/detectors	1 point per sample
j.	Isomers with matching RT	1 point

analytical technique, rather than adopting specific identification protocols. Identification of residues is accomplished when enough points have been obtained. While a minimum of 4 IPs are usually required, as few as 3 IPs might be sufficient when other nonempirical evidence is available.

The IP system is extremely flexible, allowing for the use of multiple analytical techniques, such as GC-MS, GC-MS/MS, LC-MS, LC-MS/MS, selective detectors, etc., for the identification of a residue. IPs are assigned by comparing the responses of samples to traceable reference standards analyzed concurrently on the same instrument. Spectral libraries and historical reference determinations may be used to investigate the identity of analytical residues, but IPs are only assigned for matching co-determined samples and standards. Typical analytical techniques used for P&IC residues are listed in Table 10.2 with their assigned IP values.

MS ions found in samples that match ions in standards are not automatically assigned IPs; the probability of encountering an MS ion in complex food matrices that matches a standard is too high. This probability is reduced by using the ion selection and ratio criteria listed below.

Ion Selection Criteria

(a) All selected ions must have a minimum signal to noise ratio of 3:1.
(b) Not more than two diagnostic ions may be selected from an isotopic cluster.
(c) If the molecular ion abundance is at least 10 % of the most abundant ion, it should be selected.
(d) Ions must have unique mass differences, e.g. avoid differences of 18 amu due to water loss, SRMs generated due to loss of adducts, such as ammonium ion (17 amu), etc.
(e) For LC-MS only one molecular ion species may be selected. For example, avoid the use of SRMs resulting from the loss of a adduct ion, such as ammonium adducts ($M\text{-}NH_4^+$) and the corresponding molecular ion (M^+) due to the loss of 17 amu (NH_3).

Table 10.3 Comparison of tolerance windows and percent of base peaks for GCMS and LCMS

Relative intensity	Tolerance window	
(% of base peak)	GCMS	LCMS
> 40 %	± 10 % absolute units	± 20 % relative units
≤ 40 %	± 25 % relative units	± 25 % relative units

Ion Ratio Criteria

Ion ratios are determined using the most abundant ion. In some cases, such as ultra trace residue levels or ion ratios less that 10 %, additional effort might be necessary to meet the criteria. For example, matrix interferences might be removed using background subtraction or standard addition. Ion ratio criteria are segregated between chromatographic technologies (HPLC vs. GC) and the relative intensities to the base peak response. Table 10.3 compares the tolerance windows for GCMS and LCMS as a percentage of their base peaks.

One point is assigned for each alternative chromatographic system provided the column chemistries are sufficiently different and the retention times of the sample and standard are within ± 0.05 min for GC and ± 5 % for HPLC. Matrices may shift analyte retention times in which case matrix matched standards or standard additions might be necessary. Large concentration differences between sample and standard might also cause a shift in retention times requiring the matching of analyte concentration in the sample and standard. Alternative chromatographic column chemistries are defined separately for GC and HPLC.

Alternative GC columns are based upon differences in their polarity ranging from nonpolar to mid-polar to polar chemistries as defined by their Kovats Retention Indices and McReynold's numbers available thru most column vendors. Examples of column chemistries demonstrating sufficiently different polarities include:

- Nonpolar: 100 % methyl, 95:5 methyl/phenyl
- Mid-Polar: 65:35 methyl/phenyl, 50:50 methyl/phenyl, 14:86 cyanopropylphenyl/methyl
- Polar: 50:50 cyanopropyl/phenyl, polyethyleneglycol (PEG)

Alternative HPLC columns are defined by more complex chemical interactions, including polarity, hydro- and lipophilicities, pi-bond interactions, to name a few. Examples of alternative reverse-phase columns include C8 or C18 versus cyano versus phenyl moieties. Alterative reverse phases using hydrophilic interaction chemistries would require empirical demonstration of chromatographic discrimination between analytes and matrices. Additionally, normal phase chromatography systems may always be used to confirm reverse phase systems.

One IP is assigned when alternative selective detectors are utilized. Alternative selective detectors must respond to different heteroatoms in the analyte. An example of alternative detectors would be a GC with a flame photometric detector (GC-FPD) in the phosphorus mode that responds primarily to phosphorus in

organophosphate residues, and a GC with electron capture detector (GC-EC) that responds primarily to electrophylic heteroatoms, such as halogens and oxygen. If a sample residue response matches the retention time and relative intensity of the same standard on a GC-FPD and a GC-EC, then one IP may be assigned. Only one IP may be assigned for alternative detectors.

An IP may be assigned for quantitative agreement between alternate columns or detectors. For some analytes that are difficult to quantify and for concentrations near the limit of quantification (LOQ) this requirement might be increased based upon the discretion of experienced analysts. A maximum of one IP may be assigned in this manner.

A single IP is assigned for each low resolution MS ion, including selected ion monitoring (SIM) and full scan acquisitions. Higher IP values are assigned based upon their probability of uniqueness to the analyte. For example, 1.5 points are assigned for low resolution product ion (transition) obtained using MS^n acquisition, including selected reaction monitoring and full scan acquisitions, because product ions are generated from specific parent ions that have been isolated and fragmented in the mass spectrometer. The probability of encountering a product ion in the sample that matches a standard product ion within the same chromatographic retention window is significantly reduced. That probability is further reduced when using high resolution mass spectroscopy. Two points are assigned for each high resolution ion as opposed to one point for a low resolution ion; and 2.5 points are assigned for each high resolution product ion compared to 1.5 points for low resolution product ions.

For residues with multiple isomers, one IP is assigned for the detection of isomers with matching retention times and relative responses. For example, one IP is assigned if all four isomers of cyfluthrin are detected. This IP may only be assigned once per analyte.

Some examples of positive identification of analytical residues using the IP system might include:

(i) Three ions from low resolution GC-MS in the SIM mode that meet the ion selection and ratio criteria and the retention time of sample and standard responses are within 0.05 min – 4 points (1 IP for each ion and 1 IP for the RT match). Note that although this meets the point criteria, identification using at least 4 GC-MS ions in the SIM mode is encouraged, but not always possible.

(ii) Two LC-MS/MS MRM product ions that meet the ion selection and ratio criteria and the retention times of the analyte and standard match within 5 % – 4 points (1.5 IP per product ion plus 1 IP for retention time match).

(iii) Analyte response of sample and standard have matching retention times on two different GC detection systems, e.g. GC-FPD and GC-ECD, that use a nonpolar column, and on an additional GC-FPD that uses an alternative GC midpolar column, and agreement of quantification between all three detection systems is within ± 30 % – 4 points (1 IP for each alternative detector plus 1 IP for matching retention times on alternative chromatographic systems plus 1 IP for the agreement of the quantifications).

Nonempirical Tools for Residue Identification

Heretofore, the process for the identification of P&IC residues in complex matrices has been limited to examination of empirical data, i.e. by comparing sample and standard analyte responses. However, judicious use of nonempirical information can augment the identification process. One extremely powerful tool for residue identification in a continuous US TDS is the table of historical findings. For example, an examination of the list of all the residues found in the TDS item "whole wheat bread" reveals that the pesticide malathion has been found in the item for 100 % of the samples analyzed. This is consistent with the fact that malathion is used extensively on grain products in the US. Given the historical information, one could say malathion is "expected to be detected" in whole wheat bread. Historical findings tables provide the analyst with an invaluable head start when investigating complex trace level instrument responses in samples. They are also useful when negating a suspect residue. If the empirical evidence is questionable and the suspected residue is not listed in an item's historical findings, then the probability of the residue being incurred in the item is unlikely. Additional evidence to support a new residue/item combination is required.

Maximum Residue Limits (MRLs), or tolerances, and regulated uses of P&ICs are another sources of nonempirical evidence of the likelihood that a suspected P&IC is legitimate. Some multicomponent TDS items can limit the effectiveness of this tactic because MRLs and prescriptive uses for chemical contaminants are assigned to specific raw agricultural commodities. All of the P&ICs reported with frequencies of 2 or more findings in the historical findings for whole wheat bread have tolerances and prescribed uses for wheat grain with the exception of a few industrial compounds commonly found in processed foods and the ubiquitous (in the US) perchlorate ion.

An additional tool to assist with the identification of residues is characterization of matrix responses, sometimes called "product peaks". Figure 10.2 contains two chromatograms that exhibit typical matrix responses of the brassica products cauliflower and cabbage, where: Fig. 10.2a is a chromatogram of cauliflower from a GC-ELCD; and Fig. 10.2b is a chromatogram of cabbage extract from a GC-PFPD. Sample responses labeled "Cole product peaks" are characteristic for all Cole products analyzed in the US TDS. The product peaks can be characterized by a retention index, which requires some work to establish a retention database. Another simpler practice is to catalog chromatograms of product peaks for easy visual reference.

Of course, the danger of using historical data, MRLs, and product peak characterization is self-evident, i.e. residues might be falsely reported positive or negative based upon nonempirical data. False reporting of a residue (false positive) can be avoided by requiring all residues to comply with the identification point criteria. The converse problem of not reporting a residue (false negative) can only be overcome by a healthy diligence to uncover and report trace level residues.

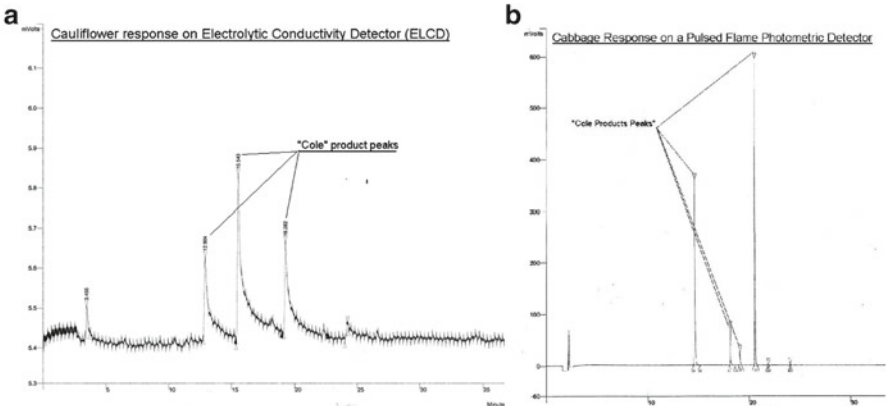

Fig. 10.2 Typical matrix responses. (**a**) cauliflower response on GC-ELCD, and (**b**) cabbage response on GC-PFPD

Contraindicating Data

Contraindicating data is any empirical evidence that a suspected residue is not present in an item. Regardless of how much data is generated to support positive identification of a suspected residue, when data is available that contradicts that identification it must be considered and overcome. For example, if a residue analyzed on a GC-MS generates retention and spectral data that meets the minimum IP criteria of 4 points, but data from a different analysis on a different instrument, e.g. an LC-MS/MS, is negative for the same residue, contradicting the GC-MS data, then the residue cannot be positively identified until the contraindicating data has been investigated and negated. In this example the GC-MS data would need to be carefully reviewed to determine its validity. The investigation might include determining whether the residue is present in blank or control sample analyses on the GC-MS, whether both instruments were calibrated correctly, whether the LC-MS/MS could detect the residue in the matrix, whether the sample integrity is violated because of cross-contamination, etc. In this example, the import of the investigation extends beyond the sample itself, because the capability of both the GC-MS and LC-MS/MS determinations is being questioned, so the investigation must be conclusive to resolve the contraindication. Additional examples of contraindicating data include:

- Unexplained or abnormal analytical behavior
- Abnormal chromatographic peak shape
- Lack of response on expected detector
- Unexplained differences between original and check analysis
- Absence of an expected diagnostic MS ion or the ion ratio is not within criteria

Quality Control

Quality assurance (QA) is a management system that assures data generated by a laboratory is of acceptable quality. Critical to the success of a QA program is the incorporation of quality control (QC) into the routine analytical regimen. QC is the empirical real-time measure of method and instrument performance, including analysis of method blanks and fortified samples and verification of instrument calibration initially and throughout the analytical determination. QC in analysis is also discussed in Chap. 13 – Quality Control and Assurance Issues Relating to Sampling and Analysis in a Total Diet Study. Procedures for the implementation of QC in the US TDS program are provided in KAN-LAB-PES.50 [28] and key aspects expanded briefly below

Method and Batch Quality Controls

Typical method performance QCs used in the pesticide laboratory include the analysis of blanks and fortified samples (spikes) with each batch of samples. Sample batches are defined as a group of samples that are analyzed concurrently using the same reagents and laboratory resources. While batch size could be as high as hundreds of samples, practical and logistical considerations of pesticide analysis generally limit batch sizes to less than 50.

Method, or reagent, blanks are analyzed with each batch to document interferences from laboratory contaminants that are occasionally detected during P&IC analysis. Matrix blanks, or control samples, would be optimal because they allow for the additional determination of matrix interferences; however control samples are very seldom available for P&IC analysis. Detection of actual target analytes in the blank is extremely rare and normally indicative of cross-contamination. More commonly detected are cleaning chemicals used in washing of the labware, equipment lubricants, hand lotions, creams, antimicrobial agents, and cleansers used by maintenance personnel. For example, shortly after the introduction of antimicrobial hand cleansers an Unidentified Analytical Response (UAR) was detected on the GC-ELCDs used for the detection of OH-P&ICs. The levels were too low to analyze by GC-MS until one sample had particularly high response of the UAR. Analysis by GC-MS in the full scan mode identified the UAR as triclosan, a common antimicrobial agent used in hand cleaners. Further investigation found the source of the triclosan was from several bottles of hand soap distributed within the lab by a well-intentioned maintenance worker. The bottles of hand cleanser were removed and the triclosan cross-contamination diminished but was not removed altogether; traces are still detected occasionally, probably from food-handling establishments and consumers.

Method accuracy and precision are demonstrated by the analysis of spikes with each batch of samples. The use of standard reference materials containing certified levels of P&IC residues would be ideal, as in the case of elemental analysis;

Table 10.4 Spike recovery limits for US TDS P&IC methods

Analysis	Analyte	Level (ppm)	Limits[a] Recoveries	RPD
GC determination of P&IC residues in fatty items	Dieldrin	20	50–130	40
	Parathion	20	45–115	40
GC determination of P&IC residues in nonfat items	Dieldrin	20	45–125	35
	Parathion	20	55–140	35
LC-FL determination of benzimidazole fungicides	Benomyl	100	60–110	20
LC-MS/MS determination of carbamate pesticides	Carbaryl	80	60–110	20
LC-EC determination of ethylenethiourea (ETU)	ETU	50	50–115	25
LC-MS/MS determination of phenylurea herbicides	Diuron	50	70–120	20
GC determination of chlorophenoxy acids and pentachlorophenol residues	2,4-D	100	40–120	40

[a]Calculated at the 99 % confidence level

however, they are generally not available for P&IC analysis. Method accuracy is verified by the calculation of the spike recovery. For example, in the US TDS duplicate samples are fortified at 20 ppb of dieldrin and parathion and analyzed for P&ICs using the general pesticide MRMs for the analysis of fatty and nonfat items by GC. A spike with a net residue concentration of 16 ppb parathion, i.e. after subtracting the amount of parathion in the sample, the recovery would be 80 % = 16/80*100 %.

Method precision is verified by statistical analysis of multiple spike recoveries. The best statistical indicator of precision is the Relative Standard Deviation (RSD); however, this statistic requires a minimum of 5 iterations to provide valid calculation of the standard deviation. In some P&IC programs each sample is fortified with a nontargeted analyte(s) that is not anticipated to be found by the screening procedure. Ideally, the spiked analyte does not interfere with the targeted analyte(s) and nearly approximates their performance. The RSD of the recoveries of the spiked compound provides an excellent measure of method precision. Alternatively, analytical precision can be estimated by calculating the Relative Percent Difference (RPD) between duplicate spike recoveries. RPD is determined by comparing the difference of the two spike recoveries with the average spike recovery. Typical spike recoveries of 90 % and 110 % would result in an RPD of 20 % = [110–90]/[(110 + 90)/2]*100 %.

Specifications for acceptable accuracy and precision are evaluated annually by statistical analysis of spike recoveries and RPDs. Limits are calculated for each spike analyte corresponding to the 99 % confidence level of the average recovery ± 3 SD. Table 10.4 contains the current US TDS spike recovery and RPD limits for each analytical/procedure combination. Spike recoveries outside the limits indicate the analysis may have failed and must be investigated.

Ideally, each matrix would be spiked with all the compounds within the scope of the procedure to assure acceptable accuracy of analytes in all matrices. Good examples of this technique are the analyses of perchlorate and dioxins that use isotopes

as internal standards. Because they are chemically identical to their respective analyte, analysis of isotopes provides the best measure of analyte performance; however, they are very costly, not always available, and require MS determination.

Analysis of every matrix fortified with all target analytes is not practical for a typical P&IC screening analysis, except in cases where the scope of analytes and matrices is extremely limited. One solution is the use of marker compound recoveries to represent the performance of all analytes. Marker compounds are chemicals that are known to be fully recovered by the methods employed. The analytes in Table 10.4 are the marker compounds utilized for their respective methods in the US TDS.

Other P&IC survey compounds may also be included in the fortification of the sample. Recoveries of these compounds are used to establish and maintain the scope of chemicals for the procedure; they are not generally used to assess the quality of an analysis.

Fortification standard solutions are prepared so their concentration result in a fortification level approximately 10 times their LOQ. In some cases, incurred residue levels or the presence of interfering sample coextractants may require the use of higher fortification levels. Fortification levels for the US TDS are also listed in Table 10.4. The dilution solvent used in preparation of the spike solution is chosen to minimally interfere with the extraction chemistry and volume of the procedure. Because spike recoveries are not useful to evaluate or monitor extraction efficiency, the spike sample is typically fortified during the initial sample extraction step, rather than fortifying the sample itself.

Instrument Quality Controls

In addition to the method, instrument performance is also monitored. Routine QC to monitor pesticide instrument performance includes the analysis of an initial calibration verification standard (ICV) and subsequent analysis of continuing calibration verification (CCV) and limit of quantification (LOQ) standards. The ICV is a standard solution prepared separately from the calibration standard solution that contains at least one of the calibration standard analytes. The response of the ICV is monitored to verify the calibration standard has been properly prepared, and the instrument has been calibrated correctly. Once the calibration has been shown to be acceptable, the LOQ standard is analyzed. The LOQ standard is one of the calibration standards diluted 5–10 times lower than the calibration level. In some P&IC analysis programs, the response of the LOQ standard is visually examined to ensure it is greater than the 5 times the noise level of the instrument. In the US TDS because so many residues are determined at the trace level, the LOQ standard is quantified and must be ±50 % of its nominal concentration.

After the ICV and LOQ standards have been analyzed and found acceptable, samples are analyzed. The calibration standard is intermittently analyzed at least once every 10–20 injections to verify the instrument calibration is maintained throughout

Table 10.5 ICV and CCV specifications

	ICV limits		CCV limits	
Determination	Low	High	Low	High
GC-FPD	55	135	70	130
GC-ELCD	50	150	55	145
GC-MSD	55	135	80	120
HPLC-FL (phenylureas)	80	120	80	120
HPLC-FL (carbamates)	80	120	80	120
HPLC-EC (ETU)	80	120	80	120
HPLC-FL (benzimidazoles)	80	120	80	120

an analytical run. As in the case of the marker compound spike recoveries, the specifications for the ICVs and CCVs are determined statistically each year based upon a 99 % confidence level. Table 10.5 lists some of the current ICV and CCV limits for the US FDA TDS program.

Quality Assurance

The FDA laboratories have incorporated all the fourteen management and ten laboratory requirements for the ISO 17025 standard into a total national quality management system. A complete discussion of the laboratory quality assurance program is beyond the scope of this chapter; however some aspects of the QA program as applied to P&IC analyses are highlighted, including control charting of QC data, reference standard preparation, review, and standard operating procedures. Chapter 13 – Quality Control and Assurance Issues Relating to Sampling and Analysis in a Total Diet Study also addresses QA in analyses.

Control Charting QC Data

As discussed earlier, method accuracy and precision are monitored in real time by comparing the batch spike recoveries and RPDs with the annually calculated statistical limits for the method/analyte combination. Method accuracy and precision are also evaluated for outliers and trends over time by control charting marker compound recoveries and RPDs on scatter plots. Figure 10.3 is a control chart of the marker compound parathion recoveries for a 12 month period. Examination of the recoveries reveals no outliers or trends, i.e. the recoveries are evenly scattered around the average recovery of 96 %. The three standard deviation values calculated from the graphed data of 62 % and 135 % are within the annually calculated control limits of 55 % and 140 % percent listed in Table 10.4.

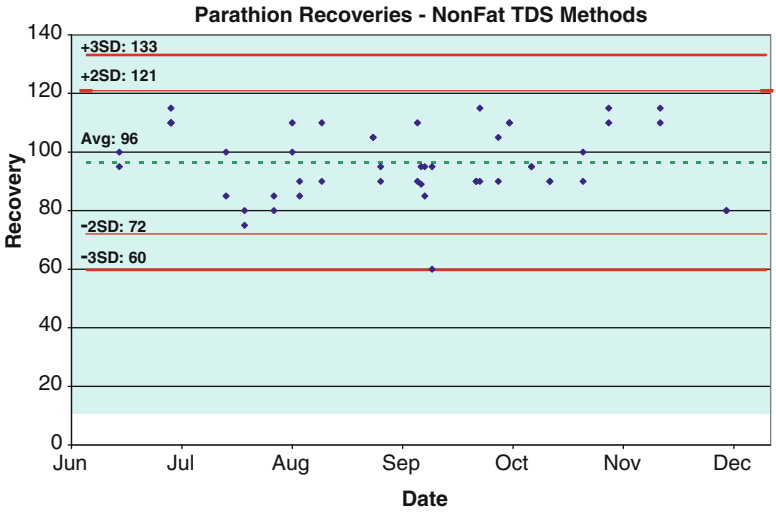

Fig. 10.3 Control chart of marker compound (Parathion) recoveries for nonfat TDS MRMs

Figure 10.4 is a scatter plot of the RPDs of the duplicate batch recoveries plotted in Fig. 10.3 with two and three standard deviation levels calculated from the data. The three standard deviation RPD level of 30 % is slightly better than the annually calculated limit of 35 % listed in Table 10.4 calculated for parathion and nonfat methods. Although no trends are apparent, one RPD of 45 % corresponding to duplicate spike recoveries of 60 % and 95 % is clearly an outlier, both of which are within the current limits of 55–140 % listed in Table 10.4. An investigation of the data uncovered no apparent reason for the disparity of the recoveries, so the data was not rejected.

Standards Preparation and Analysis

P&IC standards are prepared per KAN-LAB-PES.60 [29] and the general guidelines provided in the PAM. Reference standards are traceable to a certifiable source with the exception of a few for which a certifiable source is not available. Reference standard mixes used for routine P&IC analyses are prepared annually. Reference solutions prepared from neat standards are validated prior to use. In most cases the newly prepared standards are compared to the current reference standard mixes; agreement between them must be within 10 %. P&ICs not included in the current reference standard mixes are prepared in duplicate by different analysts, and then compared to assure they are within 10 % agreement.

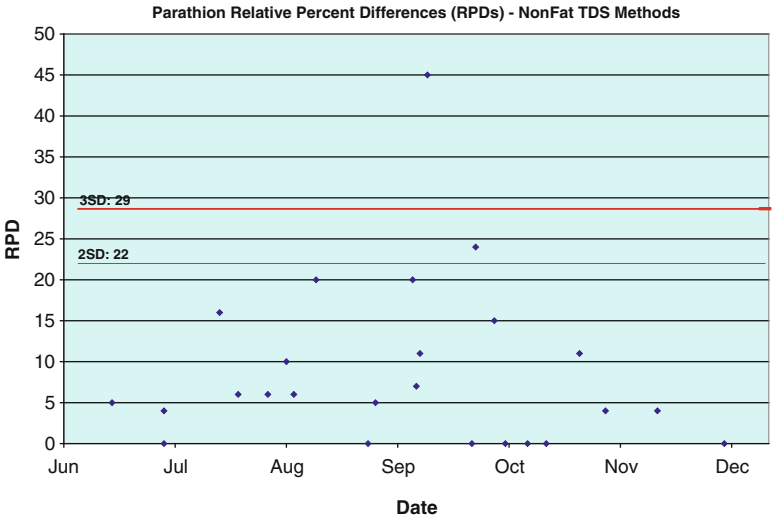

Fig. 10.4 Control chart of relative percent differences of duplicate marker compound (Parathion) recoveries for nonfat TDS methods

The reference standard mixes are designed by pesticide specialists who review historical residue findings, notifications from the US Environmental Protection Agency that establishes the MRLs, and other literature to determine anticipated residue findings in the coming year. This information is used along with known GC elutions and sensitivities to design screening standards composed of compounds with similar modes of detection. For example, several mixtures of organohalogens are prepared for determination by GC-ELCD, thermolabile and water soluble compounds are included in the LC-MS/MS mixtures. Once the screening standards are designed, concentrated mixes are either prepared or purchased from a certified vendor. Final injection standards are diluted from the concentrated mixes.

Review

As noted in the introduction, P&IC analysis is extremely difficult under the best circumstances; hence multiple levels of review are essential for the accurate identification and quantification of chemical residues in complex food matrices. Initially, all analytical work is reviewed by peers to ensure that analytical findings are accurately reported, e.g. identification criteria were met, integration of chromatographic responses are appropriate, instruments were properly operated and calibrated, no transcription errors were made, etc. A secondary review is conducted by a residue specialist to confirm the proper identification of the residue and the scientific plausibility of the finding. A third review is conducted to evaluate the historical and

regulatory significance of the residue and matrix combination. Finally, all P&IC results are recorded in a national database that is reviewed for accuracy.

The US TDS undertakes four regional market baskets (MBs) per year, each MB covering a different region across the US, and three different cities per region (See Chap. 41 – United States Food and Drug Administration's Total Diet Study Program for more details). After the data from each MB has been entered into the national database, several reports are generated to evaluate the data for trends. Spike recovery statistics are calculated to determine if average marker compound recoveries and RPDs are consistent with past MBs. Duplicate incurred residue findings are examined for agreement; and residue frequencies for each compound are compared to previous MBs. All new residue/item combinations are investigated and referenced to current and past US and international MRLs; items with a residue that is not listed in the US MRLs are reanalyzed. After all review is completed, the TDS MB report is prepared summarizing the MB logistics, program changes, residue frequencies, and new/unusual findings.

Standard Operating Procedures

Almost every aspect of the US TDS is addressed in Standard Operating Procedures (SOPs) specifically written for the TDS program; including the pesticide procedures previously mentioned. SOPs are controlled documents from their inception thru their retirement. Management approves and oversees the development of each procedure, ensures they are reviewed and updated annually, and controls user access to them. SOPs for the analysis of P&ICs in the US TDS provide specific instructions and specifications for all methods including an overview to the analysis of pesticides (KAN-LAB-PES.66 [30]), determination of moistures (KAN-LAB-PES.151 [31]), maintenance of instrumentation (KAN-LAB-PES.65 [32]), preparation and maintenance of standards, and quality assurance. The preparation of the TDS samples is addressed in SOPs KAN-LAB-PES.152 [33] and KAN-LAB-PES.161 [34]. The TDS procedures mentioned here are just a small fraction of the many SOPs, protocols, policies, and manuals required to assure quality and good laboratory practices in the laboratory.

Conclusion

The challenge of analyzing ultratrace levels of organic chemicals in an advanced TDS is substantial, but the benefits are invaluable. Residue incidence and levels found in table-ready foods provide overwhelming evidence of the effectiveness of the regulation of pesticide use and application. In regulatory pesticide programs, unprocessed raw agricultural commodities are analyzed for chemical contaminants and the levels found are compared to maximum residue levels to ensure their proper

use and application, however regulatory pesticide analyses do not provide information about the levels of contaminants in the diet of the consumer. The real evidence that the regulatory pesticide program is protecting the consumer from unsafe levels of chemical contaminants is found in the TDS program.

Furthermore, because TDS programs are designed around actual food consumption levels, the residue levels found in the TDS program can be converted to exposures and compared to the Acceptable Daily Intakes (ADIs) and other reference values established by the World Health Organization.

In the US TDS program the exposure levels of the most frequently found pesticides in the highest risk group (infants and toddlers) are more than 200 times below their ADIs. Even for the most extreme case, such as dieldrin, which has an ADI of 0.0001 mg/kg body weight/day that is 10–100 times lower than the typical level, the average exposure levels determined in the US TDS are 50 times below their ADI. These exposure levels provide solid evidence of the effectiveness of the pesticide regulatory program and ultimately the safety of the food supply; the challenge to protect the consumer is achieved.

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