



A Mini-Multiresidue Method for the Analysis of Pesticide Residues in Low-Fat Products

1. Aim and Scope

This manuscript describes a method for the analysis of pesticide residues in produce with a low fat content, such as fruits, vegetables, cereals as well as processed products including dried fruit.

2. Short Description

The homogeneous and representative subsample is extracted in frozen condition with the help of acetonitrile. After addition of magnesium sulfate, sodium chloride and buffering citrate salts (pH 5-5.5), the mixture is shaken intensively and centrifuged for phase separation. An aliquot of the organic phase is cleaned-up by dispersive SPE employing bulk sorbents (e.g. PSA, GCB) as well as $MgSO_4$ for the removal of residual water. PSA treated extracts are acidified by adding a small amount of formic acid, to improve the storage stability of certain base-sensitive pesticides. The final extract can be directly employed for GC- and LC-based determinative analysis. Quantification is performed using an internal standard, which is added to the extract after the initial addition of acetonitrile. Samples with a low water content (<80%) require the addition of water before the initial extraction to get a total of ca. 10 mL water. When dealing with samples containing <25% water (e.g. cereals, dried fruit, honey, spices) the size of the analytical sample may have to be reduced (e.g. 1-5 g) depending on the load of matrix-co-extractives expected in the final extracts. **A brief**

overview of the method is shown in the flowchart at the end of this document.

3. Devices and Consumables

- Sample processing equipment: e.g. Stephan UM 5 universal
- Automatic pipettes (e.g. for 10-10 μ L, 200-1000 μ L and 1-10 mL)
- 50 mL Teflon® centrifuge tubes with screw caps (e.g. Oak-ridge from Nalgene 3114-0050) or disposable 50 mL centrifuge tubes (e.g. 114x28 mm, PP, Sarstedt article-no. 62.548.004)
- 10 mL PP-single use centrifuge tubes with screw caps (e.g. greiner bio one article-no. 163270 or Simport/Canada, catalogue no. T550-10AT)
- 10 mL solvent-dispenser for acetonitrile
- Centrifuges for 50 mL and 10 mL centrifuge tubes
- Powder funnels, to fit for the centrifuge tubes
- 1.5 mL vials for GC-autosampler
- plastic cups (stackable) for the storage of the pre-weighed salt mixture (e.g. flame photometer cups 25 mL art. no. 10-00172 from a) GML-Alfaplast (>1000 pieces) or from b) JURO-LABS, D-91239 Henfenfeld (> 100 pieces)
- Sample divider, to automatically portion the salts (e.g. from Retsch/Haan, PT 100 or Fritsch/Idar-Oberstein, Laborette 27). The solids needed for „dispersive SPE“ can be portionated using for example the “Repro” high precision sample divider from “Bürkle” using the 10 mL PP tubes from Simport:

4. Chemicals

- Acetonitrile, pesticide residue grade
- NaCl p.a.
- Disodium hydrogencitrate sesquihydrate (e.g. Aldrich 359084 or Fluka 71635)
- Trisodium citrate dihydrate (e.g. Sigma S4641 or Riedel-de Haen 32320)
- Sodium hydroxide p.a., whereof a 5N-solution (0.2 g/1 mL water) is prepared
- Bondesil-PSA 40 μ m (Varian article no. 12213023/10 g or 12213024/100 g)

- GCB-sorbent, (e.g. Supelco, Supelclean Envi-Carb SPE bulk packing, article no. 57210U). Alternatively isolate material from packed cartridges
- Magnesium sulphate anhydrous coarsely grained (e.g. FLUKA 63135)
- Magnesium sulphate anhydrous fine powder (e.g. MERCK 1.06067)

Note: Phthalates can be removed in a muffle furnace by heating to 550 °C (e.g. overnight)

- Formic acid conc. (>95%ig), prepare a 5 % solution (vol/vol) in acetonitrile
- Pesticide Standards e. g. from Riedel de Haen, Dr. Ehrenstorfer, promochem
- Internal and quality control (QC) standards see Table 1

For the preparation of calibration solutions a dilution of the ISTD solutions is necessary according to the amount of extract used (see 6.3).

Table 1:

Potential internal standards (ISTDs) or quality control (QC) standards.

Name of the compound	Log P (oc-tanol-water)	Chlorine atoms	Exemplary conc. [$\mu\text{g/mL}$] ¹	GC				LC	
				ECD	NPD	MSD EI (+)	MSD CI (-)	MS/MS ESI (+)	MS/MS ESI (-)
Potential Internal Standards									
PCB 8	5.09	2	50	+++	-	++	+++	-	-
PCB 18	5.55	3	50	+++	-	++	+++	-	-
PCB 28	5.62	3	50	+++	-	++	+++	-	-
PCB 52	6.09	4	50	+++	-	++	+++	-	-
Triphenyl phosphate	4.59	-	20	-	+++	+++	-	+++	-
Tris-(1,3-Dichlorisopropyl)-phosphate	3.65	6	50	+++	+++	+++	+++	+++	+
Triphenylmethane	5.37	-	10	-	-	+++	-	-	-
Bis-nitrophenyl urea (Nicarbazin)	3.76	-	10	-	-	-	-	-	+++
Potential Quality Control Standards									
PCB 138	6.83	6	50	+++	-	++	+++	-	-
PCB 153	7.75	6	50	+++	-	++	+++	-	-
Anthracene (or its d10 analogue)	4.45	-	100	-	-	++	-	-	-

¹concentrations exemplary, use acetonitrile as solvent

Annotations 1:

The use of more than one internal and quality control standards is recommended to enable recognition of errors due to mispipetting or discrimination during partitioning or cleanup.

In this method the internal standard (ISTD) is employed at an early stage of the analytical procedure (comparable to a surrogate standard). **To avoid overestimations of results it is important that the compound used as ISTD does not experience any significant losses during the procedure** (e.g. higher than 5%). When analyzing fruit and vegetable samples this criterion is generally met by all compounds listed in the table above.

In the case of **samples with higher fat content**, however, the situation is different. Since the solubility of fat in the acetonitrile layer is very limited, **excessive sample fat will form an additional layer into which analytes may partition and get lost**. The extent of losses depends on the amount of lipids in the sample as well as on the polarity of the analytes with the most non-polar ones showing the highest losses. **In the presence of elevated fat amounts (e.g. > 0.3 g fat/ 10 mL acetonitrile) it is thus recommended to employ the internal standard at the end of the procedure** (to an aliquot of the final extract) assuming the volume of the organic phase as being exactly 10 mL. It should be furthermore noted that the recoveries of pesticides having very low polarity (e.g. hexachlorobenzene and DDT) will drop below 70% at fat contents greater than 0,5 g/ 10 mL acetonitrile.

PCB 138 or 153 may be used as surrogate QC standards to indicate or rule out any significant losses of pesticides. As long as one of those two compounds shows recoveries greater than 70% it is to be expected that this will also be the case even for the most non-polar pesticides. Losses of certain compounds (of low polarity and planar structure) may also occur during „Dispersive SPE“ **when employing GCB sorbent for chlorophyll rich samples** (see 6.3). Some of the potential ISTDs listed above may also be affected. This can be avoided by **employing the ISTD at the end of the procedure**, assuming the volume of the organic phase as being exactly 10 mL. **Anthracene, which shows a very strong affinity towards GCB may be used as surrogate QC standard**. Anthracene recoveries greater than 70% will indicate that no unacceptable losses of pesticides with very high affinity towards GCB (such as hexachlorobenzene, chlorothalonil, thiabendazole) have occurred.

5. Safety annotations

When using dry ice, solvents, solids and standards the corresponding safety direction sheets and the safety information on the vessels have to be taken into account.

6. Procedure

6.1. Sample processing

Subsampling of the laboratory samples is performed following the existing regulations, directives or guidelines. In the case of fruits and vegetables, cryogenic milling (e.g. using dry ice) is highly recommended to increase homogeneity and thus reduce sub-sampling variation and to reduce the size of the sample particles and thus assist the extraction of residues. Cutting the samples coarsely (e.g. 3x3 cm) with a knife and putting them into the freezer (e.g. -18°C overnight) prior to cryogenic milling reduces the amount of dry ice required and facilitates processing.

Annotations 2:

Generally, comminution at room temperature may lead to major losses for several sensitive pesticides but also result in an insufficient degree of comminution thus impeding the extractability of residues enclosed in remaining particles. Furthermore, the degree of homogeneity achieved is generally not as good as in cryogenic processing leading to greater sub-sampling variations. If the necessary degree of comminution cannot be achieved with the means available in the laboratory, the use of larger sample amounts for analysis (scaling up) and/or the use of Ultra-Turrax during the first extraction step may help to overcome these problems (see below).

Samples with a **water content between 25 und 80 %** (e.g. bananas) require the addition of water to achieve a total of 10 g water (when 10 g sample is employed). Products with a **water content < 25 %** (e.g. flour, dried fruits, honey, spices), the sample amount may have to be reduced and water has to be added as shown in the table below. The added water should be at a low temperature (e.g. <4°C) to compensate the heat development caused by the addition of the salts.

Homogenous samples (e.g. flour) can be weighed directly into the extraction tube followed by the addition of the necessary amount of water. To avoid a degradation of sensitive pesticides, the temperature during the extraction should be kept as low as possible. When dealing with **inhomogeneous samples which are difficult to comminute** (e.g. dried fruits) **water can be added before processing to assist comminution**. In this case a larger amount of the produce (e.g. 500 g) is weighed and the appropriate amount of water is added (for dried fruits for example 750 g). The mixture is then comminuted (preferably with the help of dry ice). Cold water should be used here as well, to reduce the required amount of dry ice. An aliquot of the resulting homogenate is used for further sample preparation as described below.

Table 2: Water addition for several sample types

Sample type	Weigh	Water	Annotation
<i>Cereals</i>	<i>5 g</i>	<i>10 g</i>	
<i>Dried fruits</i>	<i>5 g</i>	<i>7.5 g</i>	<i>Water can be added during comminution step. 12.5 g homogenate is used for analysis</i>
<i>Fruits and vegetables > 80 % water content</i>	<i>10 g</i>	<i>-</i>	
<i>Fruits and vegetables 25-80 % water content</i>	<i>10 g</i>	<i>X g</i>	<i>X = 10 g – water amount in 10 g sample</i>
<i>Honey</i>	<i>5 g</i>	<i>10 g</i>	
<i>Spices</i>	<i>2 g</i>	<i>10 g</i>	

6.2. Extraction/Partitioning

10.0 g ± 0.1 g of the comminuted homogenous and frozen sample are weighed into a 50 mL centrifuge tube, **10 mL acetonitrile** and the ISTD solution (e.g. **100 µL of an ISTD-mixture**, containing one or several of the compounds listed in table 1 in the concentrations given) are added and the tube is closed and **shaken vigorously by hand for 1 minute**.

Annotations 3:

- 1. If the sample's degree of comminution is insufficient, the extraction can be assisted by a dispenser (e.g. **Ultra-Turrax**). The dispersing element is immersed into the sample/acetonitrile mixture and comminution is performed for about 2 min. at high speed. If the ISTD solution has been already added, no rinsing of the dispersing element is necessary. Nevertheless, the blender has still to be cleaned thoroughly before being used for the next sample to avoid cross-contamination. When using the disposable 50 mL centrifuge tubes (see devices and consumables) the common 19 mm dispersing elements can be used. The Teflon tubes however have smaller openings requiring dispersing elements of smaller diameters (e.g. 10 mm).*
- 2. The described extraction step is scalable as desired, as long as the amounts of solvent and salts used remain in the same proportion (see below). It should be kept in mind, however, that the smaller the amount of sample employed the higher the sub-sampling variability will be. During validation each laboratory should investigate the typical sub-sampling variability achieved when employing the available comminution devices, using representative samples containing incurred residues.*
- 3. For **recovery studies** e.g. 10 g sample is fortified using 100 μL of a pesticide solution in acetonitrile or acetone. A short vibration using a Vortex mixer may help to disperse solvent and pesticides well throughout the sample. Fortification using larger volumes of standard solution (e.g. > 500 μL) should be avoided. If this is not possible, a volume compensation should be performed in the blank samples used to prepare matrix matched calibration solutions, to avoid differences in the matrix concentration of the final extract.*
- 4. **Blank extracts** for the preparation of calibration solutions: The use of matrix matched calibration solutions is necessary to minimize errors associated with matrix induced enhancement or suppression effects during GC- and LC-determination. The blank matrix should be similar to the matrix of the samples to be analyzed and should not contain any detectable residues of the analytes of interest). The blank sample is treated the same way as any other sample, but no ISTD is added during extraction and cleanup. (The preparation of calibration solutions is described below.*

After that add a mixture of:

- **4 g ± 0.2 g Magnesium sulphate anhydrous,**
- **1 g ± 0.05 g Sodium chloride,**
- **1 g ± 0.05 g Trisodium citrate dihydrate** and
- **0.5 g ± 0.03 g Disodium hydrogencitrate sesquihydrate**

Its easier to prepare the necessary number of portions of salts before starting the extraction procedure. The tube is closed and immediately **shaken vigorously by hand for 1 minute** (see annotations on how to prevent the formation of lumps) and **centrifuged** (e.g. 5 min. 3000 U/min).

Pesticides with acidic groups (e.g. phenoxyalcanoic acids) interact with amino-sorbents such as PSA. Thus, if such pesticides are within the scope of analysis, their determinative analysis (preferably via LC-MS/MS neg.) should be performed directly from the raw extract after centrifugation but prior to cleanup. For this, an aliquot of the raw extract is filled into a vial (e.g. 200 µL into a vial with micro-inlay).

Annotations 4:

1. *The preparation of the salt mixtures can be extremely facilitated using a sample divider (see 3. Devices and Consumables). As an alternative the use of portioning spoons is helpful, although not as precise as the divider.*
2. *By adding the citrate buffering salts most samples obtain pH-values between 5 and 5.5. This pH range is a compromise, at which both, the quantitative extraction of sour herbicides and the protection of alkali labile (e.g. captan, folpet, tolylfluanid) and acid labile (e.g. pymetrozine, dioxacarb) compounds is sufficiently achieved. **For acid rich samples (with pH<3)** the pH-value achieved after the addition of buffering salt is normally below 5. To protect acid labile compounds the pH-value can be elevated by **adding 5N NaOH: for lemons, limes and currants 600 µL, for raspberry 200µL NaOH solution** is needed.*
3. *In the presence of water, magnesium sulphate tends to form **lumps**, which can harden rapidly. This can be avoided, if **immediately** after the addition of the salt mixture the centrifuge tube is shaken vigorously for a few seconds. The 1 minute extraction of the entire batch can be performed in parallel after the salts have been added to all the samples.*

6.3. Dispersive SPE:

An aliquot of the **extract is transferred** into a PP-single use centrifugation tube which contains **25 mg PSA and 150 mg magnesium sulphate per mL extract** (e.g.: for 8 mL extract 200 mg PSA and 1.2 g magnesium sulphate are needed). The tube is **shaken vigorously for 30 s** and **centrifuged** (e.g. for 5 min. 3000 U/min).

Annotations 5:

1. **Co-extracted fat and waxes** may negatively affect the ruggedness of the GC analysis. The co-extracted fats or waxes can be separated from the extracts to a large extent by putting them in the freezer (more than 1 hour, e.g. overnight). Both is possible, **freezing out** of the raw extract or the final extract after cleanup and acidification. After a short centrifugation, the required amount of the still cold extract is withdrawn. This procedure is for example applicable for **cereals and citrus fruits treated with waxes**. It has been shown that pesticides and the proposed Internal and QC standards are not affected by this step.

Fats can be also effectively removed using **C18 or C8 silica based reversed-phase sorbents** (25 or 50 mg/mL extract respectively) together with PSA and magnesium sulfate in the dispersive SPE step.
2. For samples, with a **high content of carotinoides** (e.g. red sweet pepper, carrots) or **chlorophyll** (e.g. spinach, lamb's lettuce, rucola, curly kale, wine leaves und Lactuca varieties except iceberg lettuce), dispersive SPE is performed using a combination of PSA and **GCB (Graphitized Carbon Black)**. The cleanup time (shaking) is extended from 30 s to **2 min**. It has to be taken into account, that some planar pesticides have a great affinity to the planar structure of GCB. But recovery studies showed, that no noteworthy losses occur, if the extract after dispersive SPE with GCB still maintains some visible amount of chlorophyll or carotinoides. The following amounts of GCB/mL extract can be used (exemplary): a) 2.5 mg for carrots, romana lettuce, head lettuce and the like, or b) 7,5 mg for red sweet pepper, spinach, lamb's lettuce, rucola and the like. **Please refer to "Annotations 1" for information as regards the use of internal and QC standards.**
3. To simplify the procedure it is helpful to prepare a pre-mixture of pulverized (!) $MgSO_4$ and GCB ($MgSO_4$ to GCB: 60:1 in case a) and 20:1 in case b)). The amount of the magnesium sulphate/GCB mixture and PSA to be employed will depend on the volume of raw extract (e.g. 1 mL spinach extract will require $157.5 \approx 160$ mg of the 20:1 mixture and 25 mg PSA).

After centrifugation the cleaned extract is transferred into a screw cap vial and pH is quickly adjusted to ca. 5 by adding a **5 % formic acid solution in acetonitrile (vol/vol) (pro mL extract ca. 10 µL)**.

The pH-adjusted extract is filled into vials for gas- and liquid chromatography and is used for further analysis.

Annotations 6:

Following contact with PSA the pH of the extracts increases reaching measured values of above 8, thus compromising the stability of base sensitive pesticides (e.g. captan, folpet, dichlofluanid, tolylfluanid, pyridate, methiocarb sulfon, chlorothalonil). If the extracts are acidified quickly to pH 5 the degradation of such compounds is reduced significantly so that storage over several days is possible. At this pH acid-labile pesticides (e.g. pymetrozine, dioxacarb, thiodicarb) are also sufficiently stable over several days. Only some very sensitive **sulfonyl urea herbicides, carbosulfan and benfuracarb** aren't protected sufficiently at pH 5. However, these compounds are stable at the pH of the non-acidified extract (after dispersive SPE) over several days. If these compounds are within the scope of analysis **an aliquot of the non-acidified extract** is used for measurement. If the measurement can be performed quickly, the extract at pH 5 can be used as well. Carbosulfan and benfuracarb (both having individual MRLs) are degraded to carbofuran within the samples as well as in the extracts at pH 5. Thus, merely if carbofuran is present in the acidified extract an additional run of the alkaline aliquot is needed. Normally no residues of sulfonyl ureas are to be expected, because the compounds are very instable and very low doses are used to achieve a sufficient impact in agriculture.

The final extract has a concentration of ca. 1 g/mL. If GC systems with normal split/splitless injectors are used (injection vol. 1 µL) the limits of detection and determination achieved are in many cases not low enough. **The use of GC-inlets that allow injection of larger volumes (≥3 µL) and offer the possibility of solvent venting** (e.g. PTV = Programmed Temperature Vaporizer) are thus highly recommended. The solvent venting protects NPD detectors which can be additionally protected by delaying the hydrogen flow into the NPD during the first minutes of a run.

If large volume injection cannot be performed and the desired detection limits of the compounds of interest cannot be achieved, the **concentration of the extracts** and, if necessary, a solvent exchange may be considered. If GC/MSD is employed a concentration of the extracts by a factor of four should be sufficient. To achieve this e.g. 4 mL of the acidified extract (pH 5) are transferred into a test tube and reduced to ca. 1 mL at 40 °C using a slight nitrogen flow. **Solvent ex-**

change is an option if GC performance using acetonitril is not satisfactory or if NPD is employed (without PTV-injector). For this, an extract aliquot is evaporated to almost dryness at 40 °C using a slight nitrogen flow (some droplets of a keeper e.g. dodecane can help to reduce losses of the most volatile compounds) and resolved in 1 mL of an appropriate solvent. The blank extract (needed for the preparation of calibration solutions) should be treated the same way.

6.4. Preparation of calibration solutions

To prepare calibration solutions a blank matrix containing no detectable residues of the analytes of interest is necessary. The blank is treated as any other sample, but no ISTD is added. To compensate matrix induced effects during chromatography to a large extent, it is best to choose a matrix of the same sample type (e.g. apple for apple samples, carrots for carrot samples and so on).

An aliquot of the blank extract is fortified with the desired amount of a pesticide or a pesticide mixture and a known amount of ISTD solution is added at approximately the same concentration as in the sample extracts. Pipetting ISTD solution in the very same way as in the sample preparation (same pipette, same volume) will help to minimize systematic errors. This means that a dilution of the ISTD is necessary. For example 1 mL of the blank extract is fortified with 1/10 of the amount of ISTD added to the samples. To reduce matrix induced effects during GC, sample and calibration solutions should have the same concentration of co-extracted matrix components. To ensure this a **volume compensation** may be necessary. In the case of MRL violations the quantifications is performed as described in 6.5.

6.5. Calibration following the procedure of standard additions

In case of suspected violative residues, or for compounds which are known to cause severe problems during GC (e.g. strong matrix induced effects), the procedure of standard addition is performed for quantification, where several aliquots of the extract are fortified with increasing amounts of the analyte of interest. This procedure requires a knowledge of the approximate concentration of the analyte in question in the sample.

The standard solutions should be miscible with the sample extract solution. Also, all vials should have the same end volume and the same solvent composition.

Pipette scheme 1:

Additions	Vial 1	Vial 2	Vial 3	Vial 4
Sample extract	1000 μ l (1 g sample)			
ISTD	Already in- cluded	Already in- cluded	Already in- cluded	Already in- cluded
Thiabendazole standard solu- tion, (2 μ g/mL)	-	100 μ L (0.4 μ g)	200 μ L (0.8 μ g)	300 μ L (1.2 μ g)
Solvent	300 μ L	200 μ L	100 μ L	-
Final volume	1300 μ L	1300 μ L	1300 μ L	1300 μ L

(exemplary for an expected thiabendazole concentration of 0.8 mg/kg – or 0.8 μ g thiabendazole/1 g sample)

The analyte concentration in the sample is calculated using the area proportions analyte to ISTD as shown in Fig. 1 by calculating the linear regression. It is important to check that the generated standard addition curve is linear since any curvature can influence the slope and thus the result.

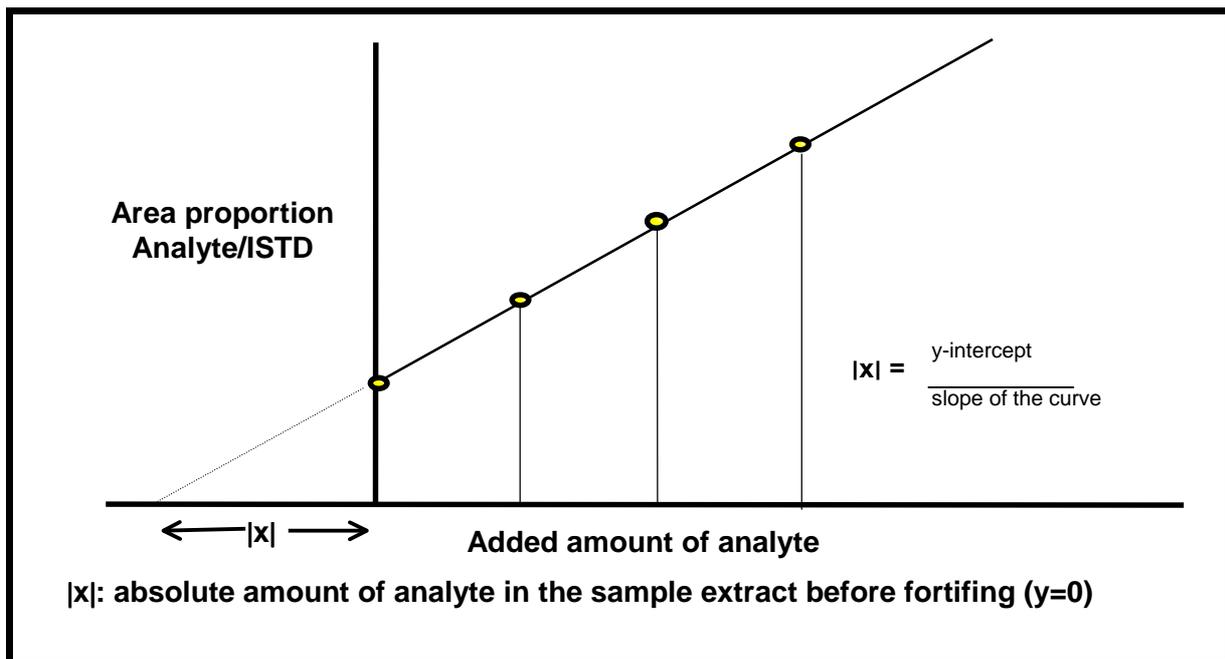


Fig. 1: Internal calibration using the procedure of standard additions, schematically

7. Reference

M. Anastassiades, S. J. Lehotay, D. Stajnbaher, F. J. Schenck
Fast and Easy Multiresidue Method Employing Acetonitrile Extrac-
tion/Partitioning and “Dispersive Solid-Phase Extraction” for the Determi-
nation of Pesticide Residues in Produce, J. AOAC Int., **86** (2003) 412-431

8. Procedure schematically (for 10 g sample)

