Investigations of the feasibility of producing a new "natural" matrix Reference Material for the analysis of pesticide residues in products of plant origin

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I, Helena Saldanha, declare that this dissertation represents my own work, except where due acknowledgement is made.

Helena Saldanha

Dedicated to my Dear Parents, and to all my journeys back Home

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Thus we mortals achieve immortality in the permanent things we create in common."

Albert Einstein in "address to a group of children, 1934"

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Abstract

European Union (EU) legislation sets stringent maximum residue limits (MRLs) for pesticides in products of plant origin. In Council Directives 86/362/EEC3 and 90/642/EEC4 maximum residue levels are fixed for pesticide residues in/on products of plant origin. The maximum pesticide residue level in foodstuffs is 0.01 mg/kg. This general level is applicable 'by default', i.e. in all cases where an MRL has not been specifically set for a product or product type.

Member States are asked to check regularly the compliance of foodstuffs with these levels. Besides national monitoring programmes, the commission services recommended, via Commission Recommendation 2002/1/EC, the participation of each member state in a specific EU coordinated monitoring programme. The monitoring programmes often carried out, serve as an indicator of the level of compliance with these provisions.

The general aim of this thesis is to work towards a system which makes it possible to estimate actual pesticide levels throughout Europe. With all monitoring programmes, analytical data of quality assurance measures have to be massively deployed, otherwise data comparability and thus data based decision making might be compromised. Use of reference materials–where available–for quality control/quality assurance is mandatory under the provisions of ISO 17025, and national accreditation bodies should demand the used of such materials for method validation and other quality assurance/quality control measures.

The specific objective of the work presented here is to study the feasibility of producing a Matrix Reference Material (carrot/potato based) for pesticide analysis. The material is intended as a quality assurance tool in support to european policies regarding pesticide residue legislation. This important component of quality control is not possible in the actual scenario since no natural matrix RM is available in the EU. However this approach, can be modified somewhat to account the unavailability of a natural matrix CRM to control the analytical procedure and validation of results: a validated method, with stated certainty. In this case the method replaces the absence of a CRM to asses the verification of the analytical process and spiking experiments are

used to demonstrate the accuracy of the method. Isotope dilution mass spectrometry (IDMS) is regarded as such a method.

A homogeneity study was carried out for the three candidate reference materials–frozen, freeze-dried and sterilized carrot/potato matrices.Freezing and sterilization were intended to be an alternative to freeze-drying, where a reconstitution step is necessary, to ensure that the matrix format should be as similar as possible to routine laboratory samples. The main reason for the choice of these stabilization techniques is to improve the commutability between real-world samples and CRMs.

Based on the method repeatability and the set-up of the study, in average the uncertainty contribution resulting from the homogeneity assessment is 6.1, 2.6 and 6.2 % respectively for the frozen, freeze-dried and sterilized batches of samples.

In regard to the short stability studies designed for 4 weeks, stability of all 21 target analytes at -20 °C, in the frozen and dried matrices was proven by analytical measurements via GC-MS, along with the stability of the majority of the target pesticides at +4 °C (except phorate, lambda-cyhalotrin, permethrin and cypermethrin) in the dried matrix. This suggests that transport of such candidate reference material would be feasible at +4 °C for all target analytes, if phorate, lambda-cyhalotrin, permethrin and cypermethrin were not of interest, in a freeze-dried matrix.Moreover the determined average content (ng/g dry matter) is in agreement with the values obtained during homogeneity studies. The long-term stability studies enabled to select the best candidate materials.

After conducting homogeneity/stability studies, frozen and freeze-dried materials were elected as the best option for the end-purpose and demonstrated the feasibility of producing a Matrix Reference Material for pesticides in carrots. All studied pesticides remained stable for a period of 5 months in the carrots matrix with an average combined uncertainty contribution of 8.2 % and 10.1 % in the frozen and freeze dried matrix respectively, to the exception of some late elucting compounds in the freeze dried-matrix.

Thus, even if a laboratory would not be interested in (international) comparability of its measurements it would have to utilise references to avoid distortion of their measurements results.

Glossary

ACh	**Neurotransmitter acetylcholine**
ADI	**Acceptable daily Intake**
ANOVA	**Analysis of variance**
ASE	**Accelerated solvent extraction**
Che	**Enzyme cholinesterase**
c.l.	**Confidence level**
CRM	**Certified Reference Material**
CE	**Capillary electrophoresis**
DSI	**Direct sample introduction**
DDT	**Dichlorodiphenyltrichloroethane**
EEC	**European Economic Area**
EC	**European Commission**
EU	**European Union**
EtAc	**Ethyl acetate**
EtOH	**Ethanol**
EQC	**External quality control**
FEP	**Fluoroethylenepropylene**
GAP	**Good agricultural practice**
GC	**Gas chromatography**
GPC	**Gel permeation chromatography**
GCB	**Graphitized carbon black**
Hac	**Acetic acid**
HPLC	**High pressure liquid chromatography**
IDMS	**Isotope dilution mass spectometry**
ISO	**International organization for standardization**
ISTD	**Internal standard**
IQC	**Internal quality control**
LC	**Liquid chromatography**
LOQ	**Limit of quantification**
LTS	**Long term stability**
LVI	**Large volume injection**
MCPA	**2-methyl-4-chlorophenoxyacetic acid**
MASE	**Microwave-assisted solvent extraction**

MeOH	**Methanol**
MeCN	**Acetonitrile**
$MgSO_4$	**Magnesium sulfate**
MRL	**Maximum residue limit**
MRM's	**Multiresidue methods**
MS	**Mass-spectometry**
MS_{among}	**Mean square among bottles from an ANOVA**
MS_{within}	**Mean square within a bottle from an ANOVA**
MSPD	**Matrix solid-phase dispersion**
MeOH	**Methanol**
n	**Average number of replicates per bottle**
NaAC	**Sodium acetate**
PAN	**Pesticide action network North America**
PLE	**Pressurized liquid extraction**
PSE	**Pressurized solvent extraction**
PSA	**Primary secondary amine**
PTV	**Programmed temperature vaporizing**
QC	**Quality control**
QuEChERS	**Quick, easy, cheap, effective, rugged, and safe**
RfD	**Acute reference dose**
RM	**Reference Material**
RSD	**Relative standard deviation**
S bb	**Standard deviation within jars**
SFE	**Supercritical fluid extraction**
SPE	**Solid phase extraction**
SBSE	**Stir-bar sorptive extraction**
SOP	**Standard operating procedure**
SPME	**Solid-phase microextraction**
SRM's	**Single residue methods**
STS	**Short-term stability**
s_{wb}	**Standard deviation within jars**
S/N	**Signal to noise ratio**
t	**Time**
T	**Temperature**
TEPP	**Tetraethyl pyrophosphate**
T MRL's	**Temporary national MRLs**

TPP	**Triphenylphosphate**
u_{bb}	**Uncertainty of homogeneity**
u^*_{bb}	**Degree of inhomogeneity that can be hidden by method variation**
USDA	**United states department of agriculture**
WHO	**World health organization**
$\overline{Y}$	**Average of all results of the homogeneity study**
$\gamma_{MSwithin}$	**Degrees of freedom of MS $_{within}$**
2,4,5 -T	**2,4,5-Trichlorophenoxyacetic acid**
2,4- D	**2,4-Dichlorophenoxyacetic acid**

1. INTRODUCTION

The work presented in this thesis explores the feasibility of producing a (certified) Reference Material for a range of pesticides in a food matrix, in response to EU legislation in the food safety sector.

The proper monitoring of this class of compounds requires the use of CRMs to ensure worldwide comparability of pesticide data.

Several aspects had to be dealt with, specifically the selection of the most important pesticides as covered by current as well as anticipated future EU legislation:

- implementation/optimization/validation of a multi-analyte method(s) for the analysis of the targeted pesticides using GC-MS;
- selection/development/optimization of a suitable sample preservative technique (freezing, freeze-drying and/or sterilization);
- stability and homogeneity studies (to find out whether the pesticide remain stable in the preserved samples at a given storage temperature).

This effort aims at the production of more natural Reference Materials, with little as possible added processing, without compromising the handling and storage of the material. Described are the most important details and findings encountered during the processing stage of such a material, thereby identifying potential occurring problems and possible solutions during the production of a certification batch.

The results of the feasibility study are summarized along with their implications. Depending on the target maximum combined uncertainty resulting from homogeneity and stability studies, decisions will be made in relation to the choice of both the type of processed matrix and pesticides of interest to be certified.

1.1. History

Pesticides are compounds or a mixture of compounds of chemical or biological origin used to mitigate or repel pests that affect food production or human health.

According to the internationally adopted definition of the Food and Agriculture Organization (FAO) of the United Nations (UN) [1], pesticide means any substance or mixture of substances intendined for preventing, destroying, attracting, repelling or controlling any pest including unwanted species of plants or animals during the production, storage, transport, distribution, and processing of food, agricultural commodities, or animal feeds or which may be administered to animals for the control of ectoparasits. The term includes substances intended for use as a plant growth regulator, defoliant, fruit thinning agent, or sprouting inhibitor and substances applied to crops either before or after transport. The term normally excludes fertilizers, plant and animals nutrients, food additives and animal drugs.

They usually act by disrupting some component of the pest's life processes to kill or inactivate it. The concept of pesticides is not new. Around 1000 B.C. Homer referred to the use of sulphur to fumigate homes and by 900 B.C. the Chinese were using arsenic to control garden pests. Major pest outbreaks have occurred, such as potato blight (*Phytopthora infestans*), which destroyed most potato crops in Ireland during the mid-nineteenth century [2]. Between this period and World War II, inorganic and biological substances, such as calcium arsenate, selenium compounds, lime–sulfur, pyrethrum, thiram, mercury, and copper sulfate, were used for pest control. However, the amounts and frequency of use were limited and the majority of the pest control measures employed cultural methods such as crop rotation, tillage, and manipulation of sowing dates. After World War II the use of pesticides bloomed, and there are currently more than 1600 pesticides available and about 4.4 million tons used annually, at a cost of more than $20 billion. The United States accounts for more than 25 percent of this market [1].

The use of pesticides is believed to be one of the major factors behind the increase in agricultural productivity in the 20th century. Products of plant

origin are the world's main source of food. Pesticides are widely used to reduce the loss in crop production caused by harmful organisms and weeds. Pesticides have been the center of controversy for a long time and are associated with risks to human health and/or to the environment. The use of pesticides has also allowed growers to produce crops in otherwise unsuitable locations, extend growing seasons, maintain product quality and extend shelf-life**.** On the other hand, society accepts these risks within certain limits as there are also benefits linked to the use of pesticides, in particular in agriculture. Their usage poses potential risks to humans, animals and the environment, especially if used without having been evaluated for safety and without having been authorized.

1.2 Classification and toxicity of pesticides

Nowadays, pesticides are classified based either on their use or the chemical class they belong to. The Compendium of Pesticide Common Names comprises of more than 1500 compounds. Each major group of pesticides (e.g. insecticide, fungicide) is subdivided into chemical or other classes (e.g. organochlorine, pyrethroid, organophosphate). Individual compounds can occur in more than one group. The compendium lists the official pesticide names that have been assigned by ISO, and it also includes approved names from national and international bodies for pesticides that do not have ISO names.

The classification used in the compendium is based mainly on chemical structure and pesticide activity, not on hazard. However, in 2002 the WHO recommended a classification by hazard taking into consideration the toxicity of the compound and its common formulations. WHO is in the process of adjusting the Pesticide Classification to conform to the Globally Harmonized System of Classification and Labelling of Chemicals.

Information about the toxicity of pesticides can be found in the PAN Pesticide Database (Pesticide Action Network North America) [3]

In the framework of this thesis most of the studied pesticides (14 out of 21 target analytes) are insecticides, belonging to different chemical classes from which newer synthetic insecticides, pyrethroids are also included.

Synthetic pyrethroid insecticides, with structures based on the natural compound pyrethrum, were introduced in the 1960s and include permethrin, lambda-cyhalotrin, and cypermethrin, all used extensively in agriculture. They have very low mammalian toxicities and potent insecticidal action, are photostable with low volatilities and persistence. They act as broad-spectrum insecticides and may kill some natural enemies of pests. They do not bioaccumulate and have few effects on mammals, but are very toxic to aquatic invertebrates and fish. With regard to older insecticides, the first synthetic organochlorine insecticide, DDT (dichlorodiphenyltrichloroethane), developped in Switzerland in 1939, was very effective and used extensively to control agricultural pests in the decades leading up to the 1970s. This insecticide acts by blocking an insect's nervous system, causing malfunction, tremors, and death. All organochlorines are relatively insoluble, persist in soils and aquatic sediments, can bioconcentrate in the tissues of invertebrates and vertebrates from their food webs, move up trophic chains, and affect top predators. These properties of persistence and bioaccumulation led eventually to the withdrawal of authorization and use of organochlorine insecticides from 1973 to the late 1990s in industrialized nations, although they continued to be used in developing countries. Organophosphate insecticides, such as tetraethyl pyrophosphate (TEPP) and parathion, have high mammalian toxicities. Other organophosphates include phorate, malathion, trichlorophon and mevinphos. In insects as well as in mammals they act by inhibiting the enzyme cholinesterase (ChE) that breaks down the neurotransmitter acetylcholine (ACh) at the nerve synapse, blocking impulses and causing hyperactivity and tetanic paralysis of the insect, then death. Some are systemic in plants and animals, but most are not persistent and do not bioaccumulate in animals or have significant environmental impacts [2]. Herbicides such as 2,4,5-T; 2,4-D and MCPA were discovered during the 1940s. They do not persist in soil, are selective in their toxicity to plants, are of low mammalian toxicity, cause few direct environmental problems, but are relatively soluble and reach waterways and groundwater. Contact herbicides, which kill weeds through foliage applications, include dintrophenols, cyanophenols, pentachlorophenol, and paraquat. Most are nonpersistent, but triazines can persist in the soil for several years, are slightly toxic to soil organisms and moderately so to aquatic organisms. Herbicides cause few direct environmental problems other than their indirect effects, in leaving bare soil, which is free of plant cover and susceptible to erosion. Also, many different types of fungicides are used of widely

differing chemical structures. Most have relatively low mammalian toxicities and except for carbamates such as benomyl, a relatively narrow spectrum of toxicity to soil-inhabiting and aquatic organisms. Their greatest environmental impact is toxicity to soil microorganisms, but these effects are short term.

1.3 Effects on the Environment

Pesticides can have considerable adverse environmental effects, which may be extremely diverse, sometimes relatively obvious, but often extremely subtle and complex [2]. In general, improved risk assessment is needed for all types of landside hazards, as are advances in methods of cost-effective mitigation. Some pesticides are highly specific and others broad spectrum, both types can affect terrestrial ecosystems. Bees are extremely important in the pollination of crops and wild plants. Although pesticides are screened for toxicity to bees, and their use is permitted only under stringent conditions, many bees are killed by pesticides, resulting in the considerably reduced yield of crops dependent on bee pollination [2]. The literature on pest control lists many examples of new pest species that have developed when their natural enemies were killed by pesticides [2]. Finally, the effects of pesticides on the biodiversity of plants and animals in agricultural landscapes, whether caused directly or indirectly by pesticides, constitute a major adverse environmental impact of pesticides. Many of the organisms that provide food for fish are extremely susceptible to pesticides, so the indirect effects of pesticides on the fish food supply may have an even greater effect on fish populations. Some pesticides, such as pyrethroid insecticides, are extremely toxic to most aquatic organisms. It is evident that these pesticides can cause major losses in global fish production.

1.4 Natural pesticides from plants and the future role of pesticides in agriculture

Plants contain a largely undiscovered reservoir of potential pesticides that can be used directly or as templates for synthetic pesticides. Numerous factors have increased the interest of the pesticide industry and the pesticide market in this source of natural products as pesticides. These include increased environmental and toxicological concerns with synthetic pesticides, and the high level of reliance of modern agriculture on pesticides. Despite the relatively small amount of previous effort in the development of plant-derived compounds as pesticides, they have made a large impact in the area of insecticides. Minor successes are found in the following classes: herbicides, nematicides, rodenticides, fungicides, and molluscicides. The number of options that must be considered in discovery and development of a natural product as a pesticide are larger than for a synthetic pesticide. Furthermore, the molecular complexity, limited environmental stability, and low activity of many biocides from plants, compared to synthetic pesticides are discouraging. However, advances in natural product chemistry and biotechnology are increasing the speed and ease with which man can discover and develop secondary compounds of plants as pesticides. These advances, combined with increasing need and environmental pressure, are greatly increasing the interest in plant products as pesticides [4].

1.5 Physico-chemical characterization/environmental fate of pesticides

When a pesticide is used in the environment, it becomes distributed among four major compartments: water, air, soil and living organisms [5]. The fraction of the chemical that will move into each compartment is governed by its physico-chemical properties.

Pesticides are distributed in the environment by physical processes which include sedimentation, adsorption or volatilization. Pesticides can equally be degraded by chemical-oxidation, reduction, hydrolysis and photolysis - and/ or biological processes. For the latter the agents of the chemical reactions are living

organisms. The process of degradation will largely be dependent on the physico-chemical properties of the pesticide and on the compartment (water, soil, atmosphere, biota) in which it is distributed (Figure 1).

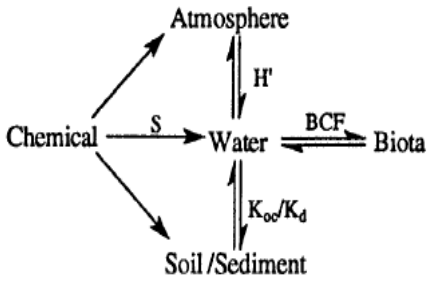


Figure 1: Interaction of chemicals with environmental compartments

S-Solubility
K_{oc}/ K_d - Soil adsorption coefficient
BCF-Bio concentration factor
H'-Henry Law Constant

When a compound's water solubility is known, the distribution of that compound in the environment and possible degradation pathways can be determined. For example, chemicals that have high water solubility will remain in water and tend not to be adsorbed on soil and living organisms. Several factors affect this property: polarity, hydrogen bonding, molecular size, and temperature having the most notable influences.

Hydrolysis is an important reaction that takes place in water. A pesticide reacts with water to form degradation products that can be distributed to the environment.

Adsorption of pesticides on soils or sediments is a major factor in the transportation and eventual degradation of chemicals. Pesticides (Table 1) that are non-polar and hydrophobic tend to be pushed out of water and onto soils which contain non polar carbon material. K_d is called the sorption coefficient and it measures the amount of chemical adsorbed onto soil per amount of water. Values for K_d vary greatly because the organic content of soil is not considered

in the equation. K_{oc} is therefore a preferred value for determining a soil's ability to adsorb chemicals since it considers the organic content of the soil.

$$K_{oc} = \frac{K_d 100}{\% \cdot organic \cdot carbon}$$

The bioconcentration factor (BCF) describes the accumulation of a chemical in living organisms (biota) compared to the concentration in water. It is an indicator of how much a chemical will accumulate in living organisms such as fish.

$$BCF = \frac{Concentration \cdot in \cdot Biota}{Concentration \cdot in \cdot water}$$

Chemicals that have high BCF values are generally no longer used because of possible hazards to living organisms. Once absorbed into an organism, chemicals can move through the food chain as Figure 2 shows with DDT (dichlorodiphenyltrichloroethane), which is one of the best known synthetic pesticides. DDT is an organochlorine insecticide, similar in structure to the pesticides dicofol and methoxychlor. It is a highly hydrophobic, colorless, crystalline solid with a weak, chemical odor. It is nearly insoluble in water but has a good solubility in most organic solvents, fats, and oils. DDT does not occur naturally, but is produced by the reaction of chloral (CCl_3CHO) with chlorobenzene (C_6H_5Cl) in the presence of sulfuric acid, which acts as a catalyst.

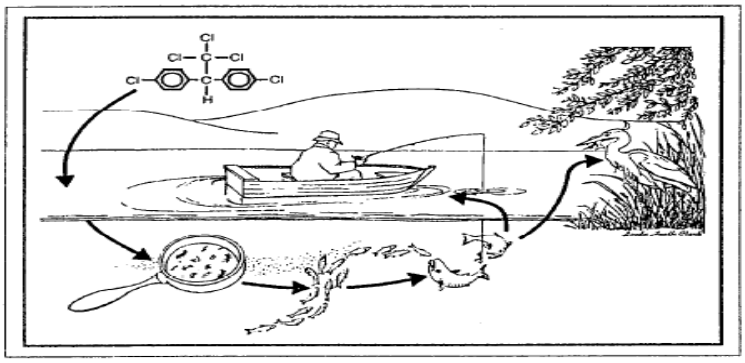


Figure 2: Dichlorodiphenyltrichloroethane (DDT) life cycle.

Henry's law constant, vapour pressure and volatilization are all interrelated and deal with how chemicals are transported from a surface into the atmosphere. Vapour pressure is often used as an indicator of the rate at which a chemical will evaporate. It is defined as the pressure a chemical in the gas phase exerts over a surface. Henry's Law constant (H') is a measure of the concentration of a chemical in air over its concentration in water. A pesticide with a high H' will volatilize from water into air and be distributed over a large area. The H' value is an integral part in calculating the volatility of a chemical. Volatilization is a process where a chemical is transported from a wet or dry surface into the atmosphere. It can be described by the amount of chemical that flows from a unit surface area into the air.

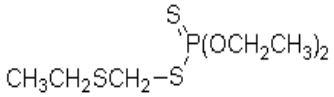
Volatilization is one of the main transport pathways by which pesticides move from water and soil surfaces into the atmosphere. A chemical compound that is extremely volatile is of concern since a pesticide with this characteristic can be quickly spread over a large area by wind. A chemical that is not volatile can accumulate on the soil or water surface and be transported through the soil layer to ground water. Chemicals do not have constant volatilization rates since they greatly depend on climatic conditions (wind, temperature, solubility, polarity, molecular size, vapour pressure). There are mathematical models created that combine these variables which enables researchers to calculate volatilization rates.

Once in the atmosphere, a volatilized pesticide may suffer two major degradation pathways. One is photochemical reaction, caused by sunlight and the second is free radical reactions.

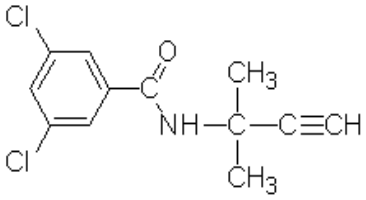
Another pathway includes microbial metabolism in water or soil. The process can take several steps and the end goal is to mineralize the chemical into the basic components - CO_2, H_2O and mineral salts. Higher organisms, such as fish, are able to metabolize but are not able to mineralize them. There are four types of microbes: bacteria, fungi, protozoa and algae. Bacteria and fungi are the most abundant in nature so they are the most important in biological transformation processes.

In Figure 3, the chemical structures of the 21 pesticide analytes under study are shown. Table 1 contains their principal physico-chemical properties.

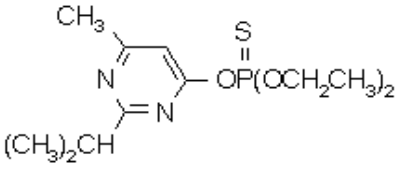
Phorate



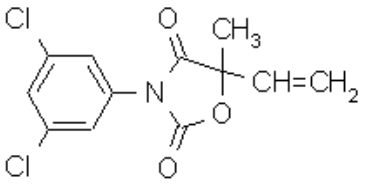
Propyzamide



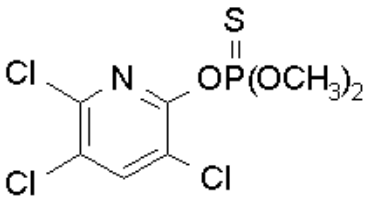
Diazinon



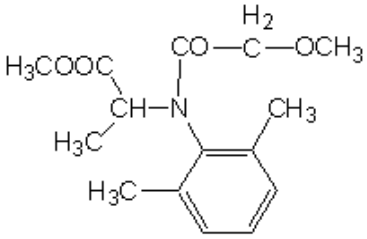
Vinclozolin



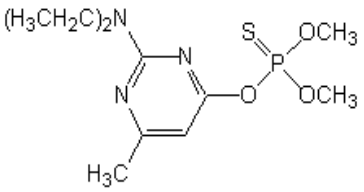
Chlorpyrifos-methyl



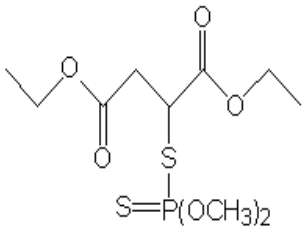
Metalaxyl



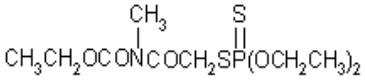
Pirimiphos-methyl



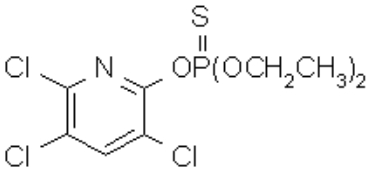
Malathion



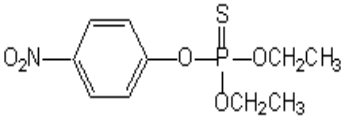
Mecarbam



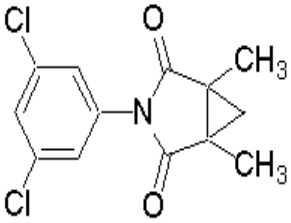
Chlorpyrifos



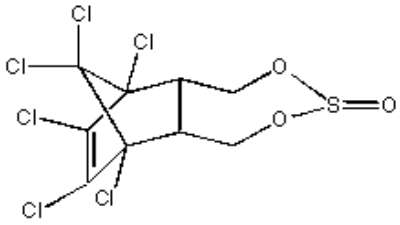
Parathion



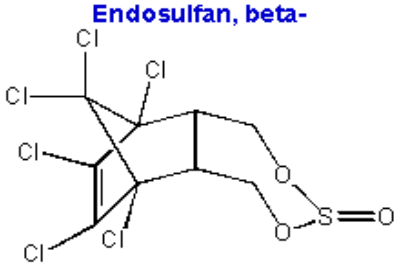
Procymidone



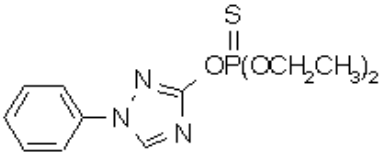
Endosulfan, alpha-



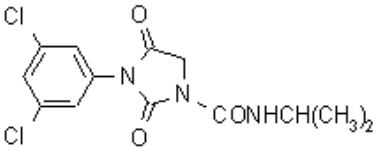
Endosulfan, beta-



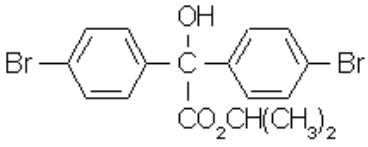
Triazophos



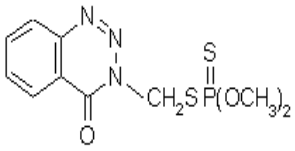
Iprodione



Bromopropylate



Azinphos-Methyl



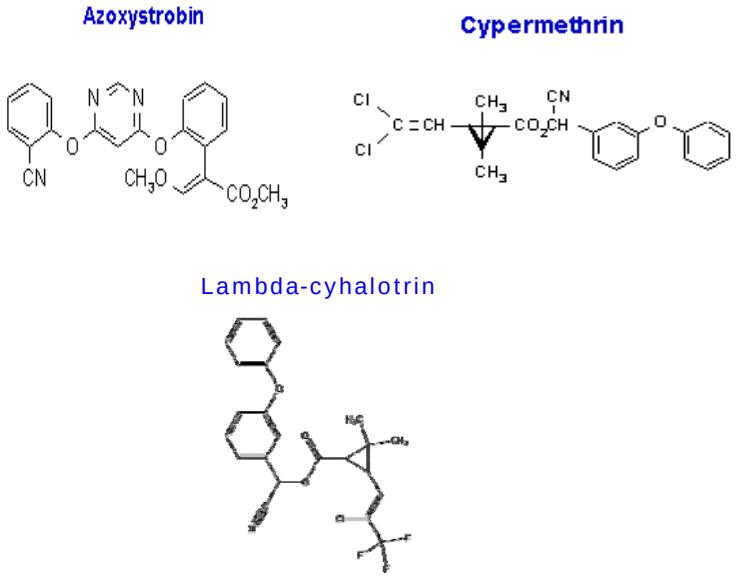


Figure 3: Chemical structures of the 21 pesticide analytes under study.

Endosulfan, permetrhin and cypermethrin have several isomers. Since the MRL's are set for the combined residue of all the isomers the general approach is to sum up the isomers quantified after gas chromatographic (GC) separation. Endosulfan is a chlorinated hydrocarbon insecticide and acaricide in the class of chlorinated cyclodienes, a member of the organochlorine family (Table1). Its distinguishing feature is that it contains only one double bond, whereas most of the cyclodiene class members contain two double bonds. The molecular structures of its two stereochemical isomers, α- and β-endosulfan are depicted in Figure 3.

The α-isomer is asymmetric and exists as two twist chair forms; the β-isomer is symmetric. Isomerization was found to be favored from β- to α-endosulfan [6]. The α-isomer, which is more toxic to mammals, dissipates faster than the less toxic β-isomer.

Technical grade endosulfan is a diastereomeric mixture of roughly 70 % α-isomer and 30 % β-isomer, along with impurities and degradation products. Pure endosulfan is colourless, but technical grade is brown in colour, ranging from light to dark depending on impurities.

Permethrin is a synthetic chemical widely used as an insecticide and acaricide and as an insect repellent. It belongs to the family of pyrethroids and functions as a neurotoxin. It is not known to harm most mammals or birds. It generally has a low mammalian toxicity and is poorly absorbed by skin. Permethrin contains four stereoisomers deriving from the chirality of the cyclopropane ring at the C-1 and C-3 positions. Glenn and Sharpf [7] have shown that the ratio of cis to trans isomers varies with the method of synthesis. Cis-permethrin is more insecticidal than the trans-isomer. The isomers also differ significantly in rates of photolysis and hydrolysis, in biotransformation and in bioaccumulation.Technical grade permethrin contains cis-trans isomers in approximately a 40/60 ratio.

Cypermethrin is a synthetic pyrethroid. The molecule embodies three chiral centres, two in the cyclopropane ring and one on the alpha cyano carbon. These isomers are commonly grouped into four cis- and four trans-isomers, the cis-group being the more powerful insecticide. The ratio of cis-to-trans-isomers varies from 50:50 to 40:60. Cypermethrin is the racemic mixture of all eight isomers (WHO 1989).

Table 1: Information about the selected pesticides for the study (I. insecticide; F. fungicide; A. arcaricide; H. herbicide)

Pesticide	Use	MRL (mg/kg)	Chemical Class	MW (g/mol)	Formula	Vp (mPa)	Water sol. (mg/L) 25 °C	P_{kow}	Analysis	R_t in GC-MS	Characteristic Masses
Azinphos-methyl	I	0.05	Organothiophosphate	317.33	$C_{10}H_{12}N_3O_3PS_2$	0.213	20.9	2.75	GC or LC	18.2	132; 161
Azoxystrobin	F	0.05	Stobilurin	403.4	$C_{22}H_{17}N_3O_5$	1.1x10-7	6	2.5	GC or LC	22.3	344; 345
Bromopropylate	A	0.05	Bridget diphenyl	428.12	$C_{17}H_{16}Br_2O_3$	0.011	0.1	5.4	GC	17.61	341; 343
Chlorpyrifos	I	0.05	OP	350.6	$C_9H_{11}Cl_3NO_3PS$	2.7	1.4	4.7	GC	11.92	197; 258; 314
Chlorpyrifos-methyl	I	0.05	OP	322.5	$C_7H_7Cl_2O_4P$	3	2.6	4.24	GC	10.69	286; 290
Cypermethrin	I	0.05	Pyrethroid	416.31	$C_{22}H_{19}Cl_{12}NO_3$	Negligible	0.004	6.6	GC	19836	163; 181; 209
Diazinon	I	0.01	OP	304.35	$C_{12}H_{21}N_2O_3PS$	11.9	40	3.81	GC	9.54	137; 179; 304
Endosulfan	I	0.05	OC	406.93	$C_9H_6Cl_60_3S$	0.023	0.325	3.83	GC	15.67	339; 341
Iprodione	F	0.02	Imidazole	330.17	$C_{13}H_{13}Cl_2N_3O_3$	Negligible	13.9		GC	15.65	131; 206
Lambda-cyhalothrin	I	0.02	Pyrethroid	449.86	$C_{23}H_{19}ClF_3NO_3$	Negligible	0.000853	7	GC	18.41	181; 197
Malathion	I	0.05	OP	330.36	$C_{10}H_{19}O_6PS_2$	0.0451	143	2.36	GC	11.61	158; 173
Mecarbam	I	0.05	Organothiophosphate	329.38	$C_{10}H_2ONO_5PS_2$	0.431	1000	2.29	GC	13.87	159; 296; 329
Metalaxyl	F	0.05	Anilide	279.34	$C_{15}H_{21}NO_4$	0.749	8400	1.65	GC	11	206; 249
Parathion	I	0.05	OP	291.26	$C_{10}H_{14}NO_5PS$	0.891	11	3.83	GC	11.96	291; 109; 97
Permethrin	I	0.05	Pyrethroid	391.3	$C_{21}H_{20}Cl_2O_3$	0.0015	0.006	6.1	GC	18.98	163; 183
Phorate	I	0.05	OP	260.4	$C_7H_{17}O_2PS_3$	85	50	3.56	GC	8.9	260; 75
Pirimiphos-methyl	I	0.05	OP	305.3	$C_{11}H_{20}N_3O_3PS$	2	8.6	4.2	GC	11.43	290; 305
Procymidone	F	0.02	Dicarboximide	284.1	$C_{13}H_{11}Cl_2NO_2$	18	4.5	3.14	GC	14.13	283; 285
Propyzamide	H	0.02	Amide	256.13	$C_{12}H_{11}Cl_2NO$	0.058	15	3.43	GC	9406	173; 175
Triazophos	I	0.02	Organothiophosphate	313.32	$C_{12}H_{16}N_3O_3PS$	0.387	39	3.34	GC	16.85	161; 162
Vinclozolin	F	0.05	Dicarboximide	286.12	$C_{12}H_9Cl_2NO_3$	0.016	2.6	3.1	GC	10.69	214; 212

Note : the MRLs presented here are the minimum of the EU-MRLs set for each analyte/ matrix combinations (47 analytes in 6 matrixes , resulting from the EU monitoring programme)

1.6 Legal framework regulating the analysis of pesticides in fruits and vegetables within the European Union.

Pesticides contain one or more biologically active substances that have the controlling effect on the unwanted organisms. Unfortunately, these substances are often also harmful to non-target organisms. Therefore, in many countries, pesticides have been subject to strict control for long time already. Specific assessment and approval schemes have been established to prevent unacceptable effects on human health and the environment and to ensure that products are effective and suitable for their purpose.

Pesticide residue levels in foodstuffs are generally regulated in order to:

- minimise the exposure of consumers to the harmful intake of pesticides;
- control the correct use of pesticides in terms of the authorisations or registrations granted (application rates and pre-harvest intervals);
- permit the free circulation within the EU of products treated with pesticides as long as they comply with the Maximum Residue Limits (MRLs) fixed.

A MRL for pesticide residues is the maximum concentration of a pesticide residue (expressed in mg/kg) legally permitted in or on food commodities and animal feed. MRLs are based on Good Agricultural Practice (GAP) data. Foods derived from commodities that comply with the respective MRLs are intended to be toxicologically acceptable. Exceeded MRLs are indicators of violations of GAP. If MRLs are exceeded, comparison of the exposure with Acceptable Daily Intake (ADI) and/or acute reference dose (acute RfD) will then indicate whether or not there are possible chronic or acute health risks, respectively [8].

Legislation for pesticide residues, including the setting of MRLs in food commodities is a shared responsibility of the Commission and the Member States. The Pesticide Authorisations Directive (91/414/EEC) aims to secure greater harmonisation in the pesticide products which are approved in the different Member States. The major initiative under the Directive is a long-term review of all the active substances used in pesticides in one or more of the Member States to ensure that they meet modern safety standards. Some 865 compounds are being considered under the review programme. The main elements of the Directive are as follows:

- to harmonise the overall arrangements for authorisation of plant protection products within the European Union. This is achieved by harmonising the process for considering the safety of active substances at a European Community level by establishing agreed criteria for considering the safety of those products. Product authorisation remains the responsibility of individual Member States;
- the Directive provides for the establishment of a positive list of active substances (Annex 1) that have been shown to be without unacceptable risk to people or the environment ;
- active substances are added to Annex I of the Directive and existing active substances are reviewed (under the EC Review Programme) and new ones authorized;
- Member States can only authorise the marketing and use of plant protection products after an active substance is listed in Annex 1, except where transitional arrangements apply.

Agreed MRLs are published in EC Directives. These Directives can only have force of law if they are transposed to Member States national legislation. MRLs are normally set provisionally for a period of four years. During this period the MRLs can be over-written by temporary national MRLs (tMRLs). At the conclusion of this period the levels are either changed (on the basis of experience/new evidence) or confirmed and set as a definitive MRL, which will apply to all Member States. It is important to note that these MRLs

are not maximum toxicological limits. They are based on GAP and they represent the maximum amount of residue that might be expected on a commodity if GAP was adhered to during the use of a pesticide. Nonetheless, when MRLs are set care is taken to ensure that the maximum levels do not give rise to toxicological concerns. The excedence of a MRL is more an indication of an incorrect use of a pesticide than a risk to the consumer. Excedence is closely monitored, evaluated and communicated to the competent authorities in the Member States through the Rapid Alert System for Food and Feed (RASFF) whenever there is a potential risk to consumers [8].

The EU is committed to establishing a strategy for the sustainable use of pesticides. The aim will be to reduce significantly the risks arising from pesticide use, while not compromising crop protection.

Harmonised MRLs eliminate barriers to trade and increase transparency of trading parameters to ensure equal competition on the EU internal market and a high level of consumer protection. MRLs are set for individual fruits and vegetables in combination with pesticides. Only fruits and vegetables on the internal market and those imported to this market are applicable; this Regulation is not applicable to produce exported to third countries. To facilitate the flow of safe produce from third countries onto the internal market, import tolerances can be set. More than 800 pesticides are currently approved for use in Europe. The procedure for establishing if a new product merits registration is complex. It requires many toxicity and efficacy studies before initial field tests can be carried out. It also includes tests on the degradation of the product and its derivatives in the plant and in the environment. A product should benefit the plant or animal it is intended to help with no negative effect on other species, and should not leave any harmful residues in the plant or animal or in the soil or water.

In EU legislation, pesticides have been divided into two major groups, plant protection products and biocidal products. As many pesticides are deliberately released to the environment, they are also a source of surface and ground water pollution. Therefore they are a subject of water legislation as well.

All in all, the sustainable use of pesticides is an issue recognised to be of major importance in the Sixth Environment Action Program, 2002-2012 [8].

Pesticide legislation at Community level dates back to November 1976 when Council Directive 76/895/EEC fixed MRLs for 43 active substances in selected fruits and vegetables. The MRLs that were set in the Directive were based on the best data available at that time. These older MRLs are gradually being reviewed and, where appropriate, being replaced with newer MRLs based on the newer information and the higher standards of today.

Pesticide residues in food are regulated by four Council Directives: 76/895/EEC, 86/362/EEC, 86/363/EEC and 90/642/EC. A Commission proposal to consolidate and amend these is currently being discussed in the Parliament and the Council.

The legislation puts a regime in place for setting and controlling pesticides residues in crops, food and feeding stuffs. It:

- sets MRLs in food and feeding stuffs
- defines the parts of products to which MRLs apply (e.g. nuts only after removal of the shell)
- specifies how MRLs apply to dried or processed products and composite foods
- defines the residues for all listed active substances (listing all relevant metabolites)
- specifies the methodology to be adopted when sampling and analysing products for residues
- confers powers to seize and dispose of products where MRLs are exceeded

A general MRL level of 0.01 mg/kg is applicable 'by default', i.e. in all cases where an MRL has not been specifically set for a product or product type.

1.6.1 EU Coordinated monitoring programme

Provisions found in Council Directive 86/362/EEC and Council 90/642/EEC oblige Member States to report to the Commission the results of the monitoring programme for pesticide residues carried out both under their national programme and under the EU Coordinated Monitoring Programme. The Commission Services recommended via Commission Recommendation 2002/1/EC the participation of each Member State in a specific European coordinated monitoring programme [8]. These programmes began in 1996 complementing the national monitoring programmes of the Member States. The objectives of the programmes are (I) to ensure compliance with residues legislation and (II) to better estimate the actual exposure of consumers to pesticides residues in food across the EU. The monitoring programme was designed as a rolling programme covering major pesticide-commodity combinations in a series of 5-year cycles and the first cycle was completed in 2000. After that, the time span was reduced to 3 years in order to have a picture of the dietary intake situation after a shorter period of time.

The choice of commodities includes the major components of the Standard European Diet of the World Health Organization (WHO).

1.6.2 Monitored products/active substances

As stated in Annex 1 of Council Directive 90/642/EEC, the legislation covers fresh, dried or uncooked fruit, preserved by freezing and not containing added sugar, whilst the vegetables covered are fresh or uncooked, frozen or dry.

At present no processed fruit or vegetables are included as processing factors (*i.e.* the proportion of pesticide residue from the fresh product which is present in processed fruit) are unknown. It is envisaged that these will be determined and included in an Annex to the Commission's future pesticide residue legislation.

Member States are only able to monitor pesticide residues on a limited number of products per year. As such, 20-30 products which form the bulk of

EU consumers' diets are monitored on a three-yearly basis. It is also well recognised that pesticides show changes over a three-year period, hence each pesticide should be monitored on the 20-30 key products on a three-year cycle. Table 2 shows the agricultural products and Table 3 the relevant MRLs for the pesticides included in the 2005-2007 monitoring programme.

Table 2: Products to be analysed.

2005	2006	2007
Pears	Cauliflower	Apples
Beans	Peppers	Tomatoes
Potatoes	Wheat	Lettuce
Carrots	Aubergins	Starwberries
Oranges or Mandarins	Grapes	Leeks
Spinach	Peas (without pod)	Head cabbage
Rice	Bananas	Rye or Oats
Cucumber	Orange juice	Peaches/Nectarines

Once the data is collected from all Member States, these products are analysed for:

- infringement of MRLs
- the average actual levels of pesticide consumed and relative values based on established MRLs

After analysis, the data is sent to Member States who review the data. Member States may adopt necessary measures such as any action to be taken on a Community level where MRLs are exceeded or whether it is desirable to publish the collected information.

Table 3: Maximum residue levels (mg/kg) in fruits & vegetables of the monitoring programme 2002-2005.

Pesticide	Pears	Bananas	Beans (fresh or frozen)	Potatoes	Carrots	oranges/mandarins	peaches/nectarines	spinach (fresh or frozen)	lowest MRL (mg/kg)
Azinphos-methyl	0.50	0.50	0.50	0.05	0.50	1.00	0.50	0.50	0.05
Azoxystrobin	0.05	2.00	0.20		0.20	1.00	0.05	0.05	0.05
Bromopropylate	2.00	0.05	0.05		0.05	2.00	0.05		0.05
Chlorpyriphos	0.50	3.00	0.05	0.20	0.10	0.3/2	0.20	0.05	0.05
Chlorpyriphos-methyl	0.50	0.05	0.05		0.05	0.5/1	0.50	0.05	0.05
Cypermethrin	1.00	0.05	0.05		0.05	2.00	2.00	0.50	0.05
Diazinon	0.30	0.02	0.02	0.01	0.20	1/0.02	0.02	0.02	0.01
Endosulfan a+b	0.30	0.05	0.05	0.20	0.05	0.50	0.50	0.05	0.05
Iprodione	10.00	3.00	0.02		0.30	0.02/2	5.00	0.02	0.02
Lambda-cyhalotrin	0.10	0.02	0.02		0.02	0.1/0.2	0.20	0.50	0.02
Malathion	0.50	0.50	3.00		0.50	2.00	0.50	3.00	0.50
Mecarbam	0.05	0.05	0.05		0.05	0.05	0.05	0.05	0.05
Metalaxyl	1.00	0.05	0.05	0.05	0.10	0.5/0.05	0.05	0.05	0.05
Parathion	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Permethrin	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Phorate	0.05	0.05	0.05	0.20	0.05	0.05	0.05	0.05	0.05
Pirimiphos-methyl	0.05	0.05	0.05	0.05	1.00	0.50	0.05	0.05	0.05
Procymidone	1.00	0.02	0.02		0.02	0.50	2.00	0.02	0.02
Propyzamide	0.02	0.02	0.02		0.02	1.0/2	0.02	0.02	0.02
Triazophos	0.02	0.02	0.02	0.05	0.02	0.02	0.02	0.02	0.02
Vinclozolin	1.00	0.05	0.50	0.10	0.05	0.05	0.05	0.05	0.05

2 DETERMINATION OF PESTICIDE RESIDUES IN FOOD MATRICES – STATE OF THE ART

The determination of pesticide residues is a challenging topic for analytical chemists. This is a consequence of (I) new compounds, based on new chemical structures, continually being introduced into the market, (II) new regulations, which are becoming ever more restricted concerning the MRLs legally permitted in food, and (III) an increasing social, economic and academic interest in food safety, which has important trade implications.

As a consequence of the specific characteristics of pesticides (i.e. high number of compounds and extremely diverse physical and chemical characteristics) chromatography based techniques are clearly the main choice for their analysis due to their high level of automation, system robustness and analytical performance.

During the last few years chromatography based techniques (GC and LC) coupled with mass spectrometry have become the core of pesticide analysis in food. This has been a result of important developments in and improvements of these techniques, making the great majority of pesticides/levels/commodities amenable to mass spectrometric detection with adequate analytical performance and robustness.

In addition, the detection step should not be considered as separate from other stages of the analytical methodology, especially sample treatment and clean-up, which are closely linked and together determine the quality and performance of the analyses as a whole. Amongst the most problematic for the analyst are those pesticides that are labile, or volatile, or have no chemical and physical features that differentiate them from co-extractives, or are insoluble in anything, or are of incompletely defined structure. Such analytes tend to require so-called single residues methods (SRMs) and therefore the cost per result of analysis tends to be very high. In contrast, certain large groups of pesticides share physico-chemical properties that render them amenable to the use of multiresidue methods (MRMs) [9]. The analytical process can be divided in the following steps:

1- Field sampling
2- Transport and storage of the sample;
3- Sample preparation (homogeneization and subsampling; extraction, cleanup, concentration)
4- Analysis (quantitation and confirmation)
5- Data processing and quality review
6- Reporting of the results

Of course, the optimization of each step determines the overall quality of the analytical result.

2.1 Food matrix

Carbohydrates, lipids, proteins and water, are the four major components of a food matrix. Food stuffs are often complex matrices with widely varying composition. The matrix constituents are the major factors involved in determining the capability of an analytical method. However, the huge variety of food stuffs limits the endeavour of validating new analytical methods for all types of food matrices. For this reason certain types of foods could serve as a reference for other food stuffs with similar nature. Knowing the composition of the different foods is very important so that trends in pesticide recoveries and interferences can possibly be correlated with respect to water, sugars, lipids or other factors in sample types (e.g. pH).
The USDA provides a wide-ranging food composition database [10].

2.2 Physico-chemical properties of pesticides

The physico-chemical properties of the analyte (s) determine the type of possible approaches to be followed from the field sampling until the laboratory analytical steps that could lead to a successful measurement strategy.

The physical properties of most utility are polarity and volatility. Polarity governs the solubility and chromatographic behaviour of the analyte. It can be estimated through its solubility in water and/or its octanol/water partitioning

coefficient $K_{o/w}$. Volatility governs the vapour-condensed phase distribution of the analyte in such operations as codistillation, headspace transfer, and gas chromatography [11]. Volatility is estimated by the vapour pressure value.

Polarity and solubility considerations play an important role in the choice of extraction and cleanup conditions for the analysis of pesticides; they are also useful guides in designing sampling strategies [11].

In the analysis of pesticides that are weak acids and bases, pH and ionic strength also become critical aspects. Stability, which may indicate precautions to be made to avoid analyte loss is another key element to take into consideration.

Regarding the solvent-pesticide stability issues, Table 4 summarizes possible sources of reduced stability of combinations pesticide-solvent [12].

Table 4: Some problematic pesticide-solvent combinations.

Pesticide (s)	**Solvent (s)**	**Factor (s)**
N-trihalomethylthio pesticides (dichlofluanid, tolylfluanid, folpet, captan, and captafol)	Acetonitrile	pH
Dicofol	Acetone, acetonitrile	pH, light
Pesticides with a thioether group (fenthion, phorate, disulfoton)	Ethyl acetate, acetone	Light, content of acetaldehyde
A-cyano substituted pyrethroids (deltamethrin, λ-cyhalotrin)	Acetone, acetonitrile	pH, activity of the GC system

2.3 Solvents used as extractants in multi-residue methods for pesticide analysis

When developing a multiresidue analytical method one of the most important decisions to be made is the choice of the employed solvents.

Examples of the aspects that must be dealt with include:

1) ability to cover the desired analytical spectrum, ranging from analytes at the polar end to the pyrethroids and organochlorine pesticides at the nonpolar end
2) selectivity that can be achieved during extraction, partitioning and cleanup
3) achieving separation from water
4) amenability to chromatographic separation techniques
5) cost, safety, and environmental concerns and
6) handling aspects (e.g. ease of evaporation, volume transfers) [11].

An ideal solvent for GC analysis of multiclass pesticide residues should be compatible with: (I) the analytes, (II) sample preparation and (III) GC multiresidue analysis. Basically, these three requirements mean that all analytes of interest should be sufficiently soluble and stable in the solvent, the same solvent should be used in the extraction and/or clean-up step to avoid solvent exchange, and physicochemical properties of the solvent should permit an optimal GC analysis of a diverse range of pesticide residues [12]**.** With respect to the GC analysis, an ideal solvent should allow optimum sample introduction and not adversely affect separation and detection of analytes. Optimum sample introduction means highly sensitive, reproducible and fast, resulting in narrow initial band widths and symmetric peaks. Other important attributes of an ideal solvent include: low toxicity, flammability, environmental hazard, and cost. Acetonitrile, acetone and ethyl acetate are three extraction solvents most commonly used for the determination of pesticide residues in produce. Moreover, they often serve as elution solvents in solid phase extraction (SPE) of pesticides from water samples and during clean up steps. If these solvents are involved in post-extraction sample clean-up (alone or in a mixture with other solvents) or if no clean-up is performed, they also constitute the medium in which the final extract is dissolved. Ideally, no solvent exchange and/or concentration step is necessary and final extracts are injected as they are, preferably using a large volume injection (LVI) technique to compensate for a lower analyte concentration. Due to added expense and complications of LVI, however, many methods employ solvent exchange before GC analysis; toluene, isooctane, and hexane being the most popular exchange solvents.

With respect to pesticide stability in organic solvents, Nemoto et al. [13] investigated the stability of 89 pesticides in methanol (MeOH), ethanol (EtOH),

2-propanol, ethyl acetate (EtAc), hexane and acetone for 6 h at room temperature in dark vials. Dicofol degraded rapidly in acetone. All other tested pesticides were stable in the given solvents with the exception of captan in MeOH.

Other authors [14] observed degradation of certain organophosphorous pesticides stored for a longer period of time (4-8 weeks) in EtAc solutions at elevated temperatures (40 or 60 °C). In practice each solvent has advantages and disadvantages with respect to each other.

In a more recent study Mastovska and Lehotay [12] evaluated 6 organic solvents commonly featured in either sample preparation (MeCN, acetone, and EtAc) or solvent exchange (toluene, isooctane, and hexane) in pesticide multiresidue analysis. Their aim was to answer key questions related to the most suitable solvent for sample introduction in GC analysis of pesticide residues, what solvents(s) should be avoided and why, and whether it is necessary to perform solvent exchange after extraction and, if yes, what is the best exchange solvent. Acetonitrile was found to be the most suitable solvent for extraction of a wide polarity range of pesticides residues from food. After acidification, the stability of problematic pesticides in acetonitrile is acceptable, and it can also serve as a medium for GC injection; therefore solvent exchange is generally not required before GC analysis. If sensitivity is an issue in splitless injection, then toluene was demonstrated to be the best exchange solvent due to its miscibility with acetonitrile and a higher response of polar pesticides (e.g. methamidophos) as compared to hexane and isooctane.

Considering that pesticides are usually less volatile than the discussed solvents, direct interferences in the GC separation and/or detection are less likely to occur (although the sample introduction in MeCN in combination with a nitrogen-phosphorous detector may be problematic), the presence of 20 % MeCN in the injected solution may lead to poor chromatography [12]. Also, the use of bonded, cross-linked stationary phases does not restrict the solvent choice, enabling injections in more polar solvents (even water) without the risk of column damage. Thus, the sample introduction step is considered the main critical point in the analysis of pesticide residues by GC.

In splitless injection (which is used in most laboratories), the liquid–gas expansion volume of the solvent dictates the maximum injection volume at any

given set of conditions (temperature, pressure and liner volume). Therefore, the solvent expansion volume should be as small as possible to allow high injection volumes without a risk of liner overflow, which provides high sensitivity without a potential for inlet contamination, sample discrimination and/or a reduction of reproducibility.

This indicates that the selection of an optimal solvent for the GC introduction depends on several factors, one of them being the employed GC injection technique. Another important factor is the actual analyte response obtained in different solvents. Relatively polar pesticides are notorious for interactions with the active sites in the GC system resulting in their loss and peak tailing [12]; therefore they usually constitute the weakest point in multi pesticide residue GC analysis.

Even though solubility per se may not be the factor, the solvent polarity still plays a significant role, because adsorption of some relatively polar pesticides in the syringe may occur when a less polar solvent is used as an injection medium [12].

2.4 Solvents and pesticide reference standards.

For the preparation of stock and working standards one must consider two aspects: the solvent used for long term storage of stock solutions must be compatible with the solvent used in the analytical method and the chosen solvent(s) must be appropriate to the method of analysis and be compatible with the determination system used. Even small proportions or quantities of inappropriate solvents may be detrimental to peak shape in chromatography or to the response of some GC detectors.

Toluene is judged to be the best choice [12] due to its miscibility with acetonitrile and good responses of troublesome pesticides in GC. In addition to these factors, excellent stability of dissolved pesticides, good solubility of a wide range of pesticides and the low volatility of toluene makes this solvent also highly suitable for preparation and long term storage of pesticide stock solutions. Generally, storage at low temperature (refrigerator (+4 °C) or freezer (-20 °C) in dark containers is satisfactory to avoid degradation of many

pesticides [12].Table 5, lists relevant physical properties of some solvents commonly used in pesticide residue analysis.

Table 5: Physical Properties of Solvents.

Solvent	Dielectric Constant (20 °C)	Boiling Point (°C)	Vapour pressure (mm Hg at 25 °C)
Acetone	20.7	56	229.5
Acetonitile	37.5	82	88.5
Cyclohexane	2.0	81	97.6
Dichloromethane	9.1	40	436.5
Ethyl acetate	6.0	77	94.5
Hexane	1.9	69	151.3
Methanol	32.6	65	127.1
Pentane	1.8	36	512.5
Toluene	2.4	110.6	28.5

2.5 Extraction procedures

In an analytical process, extraction of the pesticides from the sample matrix is the first operation and the way of transferring the analysis from the "field" to the laboratory since up to date no method can adequately detect pesticides in the field from a foodstuff.

The desired traits of this operation are among others to be complete and selectively exclude the matrix. The following list of parameters constitutes the main factors that are involved in the extraction process: sample matrix, extraction solvent(s), sample-to-solvent ratio, comminution, water content, amount of salt(s), pH, temperature, time of extraction, and pressure. Each of these factors can have an effect on pesticide recovery, stability and selectivity in the extraction, and these effects on the method being used should be known.

Recently developed extraction techniques used in pesticide analysis include: microwave-assisted solvent extraction (MASE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) [9], which is also known as accelerated solvent extraction (ASE) or pressurized solvent extraction

(PSE), depending on the manufacturer. SFE has many advantages, like (I) higher degree of selectivity, (II) ability to automate, (III) reduced or eliminated solvent usage, (IV) elimination of solvent evaporation steps, (V) and commonly convenient hyphenation with cleanup and/or detection methods. However, SFE is too selective to extract both polar and nonpolar pesticides simultaneously, takes longer than blending methods, may give recoveries dependent on the matrix, requires bulky, expensive instruments, and often involves complicated method development. PLE and MASE use heated and pressurized liquids to potentially increase speed of extraction, but this also acts to reduce selectivity, and the application of heat increases the chance of analyte degradation.

Matrix solid-phase dispersion (MSPD) is another alternative extraction approach that has been evaluated for pesticide residue analysis [9]. MSPD consists in the incorporation of a small portion of sample with a sorbent, and cleanup is performed at the same time as extraction. It has some advantages of convenience over the conventional approach to separately extract the sample, evaporate solvent, and then conduct cleanup.

The very small sample size (0.1 to 2 g) can be an advantage of MSPD if limited sample is an issue, but in most residue applications, it is a crucial disadvantage due to the difficulty of getting a sufficiently representative homogeneous subsample.

Another type of alternative extraction technique is to use a sorptive extraction device. At present, two forms of sorptive extraction have been commercialized: solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE), which are the subject of several reviews [9]. In another format, the coating is contained in a tube, as in a short piece of a capillary column. All three techniques are actually forms of the same approach in which a material, such as polydimethylsiloxane, is coated over a fiber or stir-bar to semiselectively extract chemicals from an aqueous or gaseous sample. The type and amount of chemicals that partition into the coating depend on the partition coefficient, coating volume, sample volume, time, temperature, matrix effects, pH, ionic strength, solvent composition and mechanical factors. Coatings can be prone to memory effects and can become contaminated with non-volatile matrix components.

Matrix components often affect the equilibration process and lead to variable results. Also water is essentially the only liquid medium with which the coatings can be used because analytes do no partition into the fiber from organic solvents. Similarly, polar compounds do not partition into the coatings from water. Different temperatures, phases, volumes, time, sample treatments (e.g. addition of salt) can increase recoveries or speed up the equilibration process, but in reality, the fundamental nature of the sorptive extraction process limits its usefulness. Thus in residues methods, sorptive extraction methods best meet their potential advantages in the analysis of clean water and air matrices.

Despite all of these alternative extraction options, the most common extraction method by far is to simply mix an organic solvent with a solid sample. This approach is rapid, simple, reproducible, cheap, commonly gives high recoveries, and uses compact and rugged devices. Simply blending or shaking, followed by a short centrifugation step is of practical interest above all.

2.6 Cleanup procedures

Ideally, an extraction method gives 100 % recoveries of the pesticides of interest and contains no interfering coextractives from the matrix. This might be true when relatively uncomplicated food matrices, such as melon or cucumber, are under study.

However, one must consider that the ruggedness of the analytical system must be taken into consideration and even if matrix coextractives do not directly interfere in the detection, they often indirectly cause signal suppression or enhancement effects, that lead to the need of greater instrument maintenance.

Nowadays extract cleanup procedures in pesticide residue analysis include separation processes based on molecular size (gel permeation, membrane filtration, dialysis); volatility (distillation); chromatography; solubility (precipitation) or partitioning (liquid-liquid or solid-liquid).

Gel permeation chromatography (GPC) is ussually used to remove large molecules from extracts, that otherwise would contribute to the buildup of nonvolatiles in the analytical instruments. However, some pesticides, such as

pyrethroids elute near the lipids in GPC, thus it is sometimes difficult to get complete recovery of those and still perform adequate cleanup. Also it cannot remove interfering components which tend to have the same molecular weight as the target pesticides. Thus a partitioning type of cleanup procedure is also frequently conducted in combination with GPC.

Liquid-liquid partitioning is commonly used for cleanup in pesticide analysis of high-moisture foods. A water miscible solvent (e.g. acetone) is used for extraction; subsequently a non-polar solvent (e.g. hexane) is added, which separates from water, leaving the most polar coextractives, along with some polar pesticides, separated. The addition of salt to the system helps to force more of the polar pesticides into the organic solvent. In the case of acetonitrile based extraction, salt alone is enough to induce the phase separation between water and acetonitrile, thus the addition of a nonpolar solvent (and the concomitant dilution of the extract) is not necessary. The QuEChERS method (standing for quick, easy, cheap, effective and safe), for the analysis of pesticide residues in food was recently introduced by Anastassiades et al. [15] to provide a much more efficient way to better meet laboratory needs. The use of buffering during the extraction step of the QuEChERS method maintains a pH of 4-5 independent of the commodity, which minimizes degradation of base-sensitive pesticides and increases recovery of the most basic pesticides in acidic matrices. In extensive experiments to develop the QuEChERS method, anhydrous $MgSO_4$ was found to be a salt with excellent features to induce liquid-liquid partitioning between acetonitrile and water and still achieve high recoveries of relatively polar pesticides [15]. $MgSO_4$ in combination with NaCl modifies the partitioning so that sugars tend to remain in the aqueous layer. Another advantage of acetonitrile is that it is not miscible with alkane solvents, thus liquid-liquid partitioning can be used with solvents such as hexane or iso-octane to help remove coextracted lipids (but nonpolar pesticides will also partition into the nonpolar solvent).

In many applications solid phase extraction (SPE) has become the most common cleanup used in pesticide residue analysis. Conventionally, SPE uses plastic cartridges containing 100 to 1000 mg of a sorbent material. The sorbents most common in pesticide residue analysis include C_{18}, silica, Florisil, Alumina, graphitized carbon black (GCB), aminopropyl ($-NH_2$), primary secondary amine

(PSA), and divinylbenzene/polystyrene. In the past analytical pesticide methods often used Florisil columns with fractionation of the pesticides with different elution solvents, but recently the use of a weak anion exchange sorbent, such as -NH_2 or PSA, in combination with GCB has been shown to provide effective removal of fatty acids, chlorophyll, and sterols from foods [15]. GCB strongly retains planar pesticides, such as hexachlorobenzene, thus its usefulness is reduced in multiclass, multiresidue methods. C_{18} can be helpful in removing a small amount of lipids from extracts, but otherwise PSA alone often provides enough clean up of extracts of nonfatty foods. Supelco (Bornem, Belgium), provides ready-to-use clean up tubes for the QuEChERS method. They are available for food/agricultural samples low in fat, or of high chlorophyll or carotenoid content.

2.7 Analysis

The analytical procedure consists of the analytical separation and detection steps: GC and LC have long been established as exceptional methods to separate chemicals in complex mixtures, and at present, there are no better overall alternatives for pesticide separations than GC or LC coupled with the appropriate detection system. Capillary electrophoresis (CE) has shown some promise for the analysis of ionic pesticides [9], but ultimately, the better ruggedness and larger sample injection volumes in LC give it strong adavantages over CE. In pesticide residue analysis, analytical procedures are often divided into pesticide groups that are most effectively analyzed by GC or LC, usually by reversed–phase chromatography**.** In multiclass, multiresidue methods, GC coupled with capillary columns is generally preferred because it gives better separations, has typically lower detection limits, and has more diverse detectors. Thus, LC is generally reserved for ionic, thermally labile, and less volatile pesticides. Due to the lower number of theoretical plates of separation in LC and the mode of separation based on polarity, LC methods are typically designed for single classes of pesticides rather than the more diverse range of analytes possible in a single method by GC, in which separation is largely a function of volatility. However, due to the recent advancements in

LC/MS-MS instruments, LC will likely become the primary approach for the majority of pesticides, and GC will be used primarily for the thermally stable, nonpolar, and semivolatile pesticides [9].

2.8 Matrix effects

The analysis of pesticide residues in foodstuffs is associated with well described phenomena called matrix effects, which are caused by the unavoidable presence of coextracted matrix components in the final extract [9]**.** In GC, matrix effects may impact all steps in the analysis (injection, separation and detection) leading to inaccurate quantitation, decreased analyte detectability, reduced method ruggedness, and or reporting of false positive/negative results. Serious matrix effects occur during sample introduction in GC where degradation and/or adsorption of certain analytes take place. It was first described by Erney et al. [16] as "matrix-induce response enhancement". When a food extract is injected, the matrix components tend to block active sites in the inlet and column (mainly free silanol groups), thus reducing losses of susceptible analytes due to irreversible adsorption and/or degradation (Figure 4). This phenomenon results in higher signals in matrix compared with matrix-free solutions. If analyte standard solutions prepared only in solvent are used for calibration, the calculated concentrations of the affected analytes in food extracts become overestimated. The extent of the matrix-induced enhancement effect is related to both the chemical structure and concentration of the analyte and type and content of matrix components [9]. Thermally labile pesticides and those capable of hydrogen bonding, such as pesticides with hydroxyl (-OH) and amino (-NH_2) groups, imidazoles (-N=), carbamates (-O-CO-NH-), urea derivatives (-NH-CO-NH-), and certain organophosphates (-P=O), are the analytes most susceptible to this effect [17].

Factors involved in the matrix induced effect can be summarized as follows:

- Number and type of active sites in the inlet and GC column
- Chemical structure of the analytes: (I) hydrogen bonding character, (II) thermolability

- Analyte concentration in the sample (known to be most pronounced at trace level)
- Injection temperature
- Interaction time as a function of: (I) flow rate, (II) pressure, (III) injection volume, (IV) solvent expansion volume, (V) column diameter, (VI) retention time
- Matrix type

This phenomenon results in higher analyte signals in matrix versus matrix free solutions, thus precluding the convenient use of calibration standards in solvent only, which would lead to overestimations of the calculated concentrations in the analysed samples.

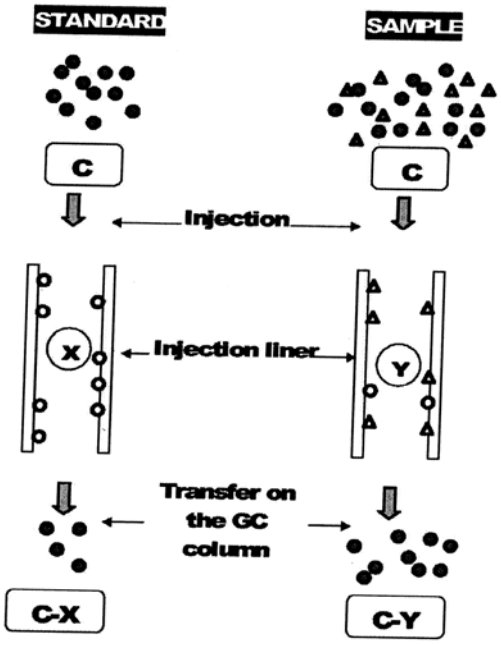


Figure 4: Simplified illustration of the matrix induced chromatographic enhancement effect: **C** - number of injected analyte molecules; **X, Y** - number of free active sites for their adsorption in the injector, ● molecules of analyte in the injected sample; O portion of analyte molecules adsorbed in GC injector; ▲ molecules of matrix components in injected samples; Δ portion of matrix components adsorbed in GC liner; **(C-X) < (C-Y).**

The ratio (C-Y)/(C-X), Figure 4, may dramatically increase when analytes approach trace levels. In some cases the quantification of analytes is no longer feasible since the analyte signal in solvent falls below LOD. In theory, elimination of active sites or matrix components would overcome the matrix induced enhancement effect; however, absolute and permanent GC system deactivation is impossible in practice. Careful optimization of the injection technique, temperature and volume, liner size and design, solvent expansion volume, column flow rate, column dimensions, can lower the number of active sites (due to decreased surface area) or shorten the analyte interactions with them. This results in a reduction but not in complete elimination of the effect.

Alternative injection techniques that decrease analyte thermal degradation and/or residence time in the injection port, such as programmed temperature vaporization (PTV) or pulsed splitless injection, may lead to a significant reduction of the matrix effect, but rarely to its elimination [18,19].

European guidelines recommend the use of matrix matched calibration standards to compensate for matrix effects, which requires the preparation of calibration standards in blank matrix extracts rather then in pure solvent [20]. Nevertheless, this approach has several drawbacks, including a rather time-consuming and laborious preparation of matrix-matched standards, the unavailability of appropriate blanks, the limited stability of certain pesticides in matrix solutions**,** and the increased amount of injected matrix in an overall sequence of samples, which can lead to the increased contamination of the inlet and front part of the analytical column [21].

The concept of "analyte protectants" (compound additives) takes advantage of the response enhancement and optimizes it rather than trying to eliminate it. Analyte protectants are compounds that strongly interact with active sites in the GC system (inlet and column); thus they do not allow access to the analytes most susceptible to the effects [21]. When added to sample extracts and matrix-free standards alike, the analyte protectants can induce the same response enhancement in both instances, resulting in effective equalization of the matrix-induced response enhancement effect. Analyte protectants are defined as compounds that strongly interact with active sites in the GC system, thus decreasing degradation, adsorption, or both of coinjected analytes.

A mixture of ethylglycerol, gulonolactone, and sorbitol (at 10, 1 and 1 mg/mL, respectively, in the injected standards) was found to be most effective in minimizing losses of susceptible analytes [21]. When added to final sample extracts and matrix-free calibration standards alike, these analyte protectants induce a similar response in both instances resulting in effective equalization of the matrix induced response enhancement effect even after a large number of fruit and vegetable extract injections. Ideally, the analyte protectants should provide the same degree of protection (signal enhancement), regardless of whether the solution contains matrix components or not. Figure 5 schematically shows regions of influence of each component of this mixture on signals of susceptible analytes throughout the volatility range of the GC amenable pesticides.

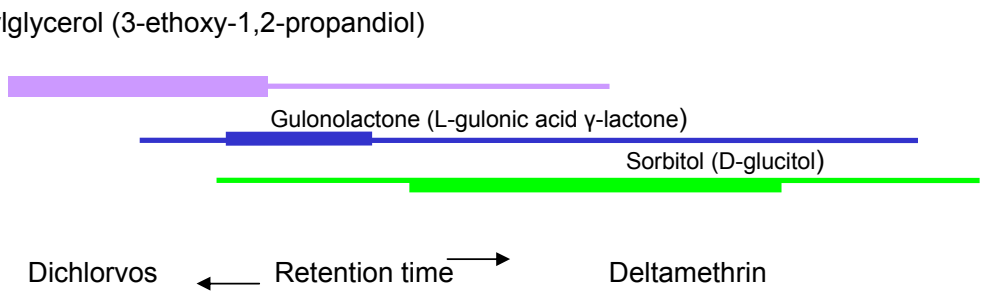


Figure 5: Schematic illustration of the effect of the optimal combination of analyte protectants (3-ethoxy-1,2-propandiol, L-gulonic acid γ-lactone, D-glucitol at 10, 1, and 1mg/mL, respectively, in the injected pesticide solutions in acetonitrile) on the signal enhancement of susceptible analytes throughout the elution range of GC-amenable pesticides. Dichlorvos elutes early, deltamethrin later from appropriate GC columns.

2.9 Injection techniques and its effect on matrix enhancement

For trace analysis, almost only non-splitting injection techniques can be considered. Classical splitless injection is still the most frequently used injection

technique in pesticide residue analysis. It protects the analytical column against the deposition of non-volatile components and is alsorelatively easy to operate. In this type of injection all of the analyte vaporized in the injector enters the column. The constant septum purge helps to (I) keep the septum clean and (II) keep sample components adsorbed on the septum from getting into the gas in the injector, thus preventing the creation of analyte peaks that are carried over from one injection to another.

A significant improvement of this technique can be achieved using a carrier gas pressure pulse during injection. This modification is called pulsed splitless injection. The application of a pressure pulse leads to a higher carrier gas flow rate through the inlet and thus faster transport of sample vapors onto the GC column. Under these conditions, the residence time of the analytes in the injection chamber is much shorter compared to classical splitless injection. It results in a significant suppression of analyte discrimination, adsorption and /or degradation in the injection port [22-23]. In addition, due to the increased pressure larger volumes of sample can be injected without the risk of liner overflow and consequently, lower detection limits can be achieved.

On-column and PTV injection represent other alternatives of sample introduction techniques which may reduce and /or eliminate the matrix-induced response enhancement effect. On-column injection is a superior technique in terms of non-discriminative transfer of sample components [24], however, it provides no protection for the analytical column. In pesticide residue analysis, on-column injection can only be used for simple matrices such as drinking water.

Recently [9], a novel injection technique called direct sample introduction or "dirty sample" injection (DSI) have been introduced. In DSI, up to 30 µl of the sample are added in a disposable micro vial which is then placed (using a holder) into the injector at relatively low temperature. After the solvent evaporation, the injector is rapidly heated and analytes transferred to the GC column. Both these steps must be carefully optimized to avoid losses of more volatile analytes and to quantitatively transfer less volatile ones onto the column. The major advantage versus other large volume injection (LVI) techniques is that non volatile matrix components remain in the micro vial, therefore the DSI approach should eliminate the need for routine maintenance

of the GC system. The potentials of DSI for analysis of pesticide residues in fruits and vegetables without cleanup have been demonstrated by Lehotay [25]. Particularly when no concentration step is conducted in sample preparation, and to achieve low LODs it is desirable to use a LVI technique for sample introduction into GC. In recent years, a number of commercial techniques and inlets have been introduced to permit LVI through the control of pressure and temperature during vaporization [9]. However, the wide volatility and polarity range of pesticides makes LVI a difficult option. The volatile pesticides may be partially or completely lost during the solvent evaporation step, and the analysis of some low-volatile pyrethroids may cause the introduction of some undesirable non volatiles into the column. Certain pesticides interact with active sites and/or degrade on surfaces, and LVI may inherently increases this problem when extended resident times occur in the GC inlet.

2.10 Detection

A traditional approach to multiresidue pesticide analysis is to employ GC with a mass spectrometer (MS) as the detector. It simultaneously serves to quantify and confirm detected analytes, detects a wide range of analytes independent of elemental composition and has the possibility to spectometrically resolve co-elucting peaks. There are many types of mass detectors but the basic principles are the same in all cases: a sample is ionized, ions are separated on the basis of their mass-to-charge ratio (m/z), and accelerated towards a detector where they are counted. The data system compiles a spectrum showing the mass distribution of the ions produced from the sample - a snapshot of the ion intensities plotted against their m/z.

2.11 Mass analysers

The choice of a mass analyser determines the mass range, resolution, sensitivity, scan speed and also the cost of the instrument. Basically they can be divided into two groups: (I) scanning (ion trap, quadrupole, magnetic sector) and (II) non scanning mass analyzers (time of flight).

In pesticide residue analysis, quadrupole instruments are probably the most popular mass analyzers. Any difference in analytical accuracy between these types of MS systems is most likely a function of the injection process and not related to detection [9]

It can be operated in two modes: (I) full scan (of a selected mass range) and (II) selected ion monitoring (SIM). In the SIM mode, sensitivity is enhanced by monitoring only few selected m/z ratios, thus proportionally increasing the acquisition time of the ions of interest, while spectral information is sacrified.

2.12 Ionization techniques

In GC-MS, the most widely used ionization technique is electron ionization (EI), in which sample molecules are bombarded by high-energy (usually 70 eV) electrons, resulting in high-energy, single charged molecular ions that lose excess energy via fragmentation, producing a collection of fragment ions characteristic of the compound. EI can be used for identification of unknowns, determination of the molecular structure and confirmation of target component identity through consistent ion abundance ratios. It all makes it a very suitable ionization technique for pesticide residue analysis, especially for confirmation of results.

2.13 Requirements for confirmation by mass spectometry

Mass spectrometry is capable of providing unequivocal confirmation of residues of most pesticides, but the confirmatory data must comply with certain minimum requirements. This section summarizes the requirements for GC-MS as laid down by the European Guidelines for the monitoring of pesticides in food matrices [26]. Generally, confirmation of the detected analyte should be done by qualitative and quantitative means.

Matrix-matched standards should be used for confirmation but the reference mass spectrum should be derived from a solution of the reference standard in pure solvent. To avoid distortion of the ion ratios, the quantity of

material used for recording the reference spectrum must not overload the detector. Chromatograms of relevant ions should have peaks with similar retention time, peak shape and response ratios as those obtained from a calibration standard, analyzed in the same batch. Intensity ratios for principal ions should be within 80 -120 % of those obtained from the standard. The most abundant ion that shows no evidence of chromatographic interference should be used to quantify a residue. EI full scan spectra generally provide the most suitable identification but sensitivity may be improved by scanning a limited mass range or by SIM.

2.14 General requirements for quantification

Correct quantification is dependent upon correct identification of the analyte. It is also dependent upon a good knowledge of the calibration function and dynamic range of the detection system (e.g. system saturation and "zero" concentration). It is essential to establish the lowest concentration or mass that can be detected. The concentration or mass response of all detection systems to an analyte tends to be variable, even over shorts periods of time and material batch. In this case, internal standardization, particularly with stable isotope-labelled standards, or standard addition may be required. Standard addition is done by means of adding a known quantity of analyte to the sample extract, containing an unknown quantity of the same analyte. The absolute amount of analye in the sample extract before fortifying is calculated via linear regression.

The term "internal standardization" has different meanings [27] and amongst them are: (I) any suitable chemical added to an extract prior the final determination stage. Following detection, its function is to "correct" for uncontrolled changes in the volume of the extract, which is particularly useful where very small volumes of extracts are involved; (II) an extension of this procedure is to utilise an internal standard that shares most or all of the physico-chemical properties of the target analyte. Isotopically labelled standards and standard addition fall into this category. Finally (III), the internal standard may be added to the test portion at the very beginning of the analysis and the

quantity of analyte is determined by the response ratio. The latter provides both calibration and an automatic correction for recovery.

Single-level calibration may provide more accurate results than multilevel calibration, if the detector response tends to drift. Calibration by interpolation between two levels is acceptable where the response factors, derived from replicate determinations at each level, indicate acceptable linearity of response. The higher response factor should not be more than 120 % of the lower response factor (110 % in cases where the MRL is approached or exceeded).

Where three or more levels are utilized, an appropriate calibration function may be calculated and used between the lowest and highest calibrated levels. The fit of the calibration should be plotted and inspected visually, avoiding unique reliance on correlation coefficients, to ensure that the fit is satisfactory in the region relevant to the residues detected. If individual points deviate by more than ±20 (±10 % in cases where MRL is approached or exceeded) from the calibration curve in the relevant area, the function and/or measurements should be reviewed. So the difference between the concentration of analyte in each calibrating standard and the concentration calculated from the calibration curve must be lower than ±20 (±10 % in cases where MRL is approached or exceeded). On the contrary, a more appropriate fit must be used or the individual points must be repeated.

Mostly for reasons of convenience, analytical chemists try to develop methods in which the signal increases linearly with increased concentration in a range as large as possible. This range is called the linear dynamic range. The evaluation of linearity, i.e. the ability of the method to produce signals proportional to analyte concentrations, is part of the validation of methods applied in pesticide residue analysis. Extracts containing high level residues may be diluted to within the calibration range, but where matrix matched calibration is applied the concentration of the matrix in the extract may have to be adjusted accordingly due to the above described matrix enhancement effect [27].

2.15 Quality assurance/quality control aspects in pesticide residue analysis

There is an increasing need in pesticide residue analysis laboratories to ensure the quality of the analytical results. Internal quality control* (IQC) measures are an essential element to ensure reliable results because they allow both the continuous monitoring of the process and measurements and the elimination of causes of unsatisfactory performance [28]. External quality control (EQC) includes proficiency testing and collaborative studies. Although important, participation in EQC activities does not substitute IQC measures and vice-versa. IQC measures involve the use of blanks, certified reference materials (CRMs), quality control samples, calibration standards, spiked samples, replicated samples, and blind samples. Some types of reference materials are: pure substances characterized for chemical purity and/or trace impurities; standard solutions often prepared gravimetrically from pure substances and used for calibration purposes and matrix reference materials. Matrix reference materials are characterized for the composition of specific major, minor or trace chemical constituents, which are prepared from “natural” matrices containing the components of interest with a known uncertainty [28].

*The following definitions given by the International Organization for Standardization (ISO) [29], are widely accepted:

Quality: the totality of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs.

Quality Assurance (QA): all those planned and systematic actions necessary to provide adequate confidence that a product, process or service will satisfy given quality requirements.

Quality Control (QC): the operational techniques and activities that are used to fulfil requirements for quality.

2.16 Principal definitions and terminology related to reference materials

Detailed scientific literature on various aspects of reference materials, together with internationally recognised definitions, exists [29]. This section summarizes aspects on the selection and use of matrix reference materials.

Reference materials are an important tool for a number of aspects of measurement quality and are used for method validation, calibration, estimation of measurement uncertainty, training, and internal and external quality control measures.

Often a measurement operation includes more than one quality purpose and there can be an overlap of functions as illustrated in Figure 6.

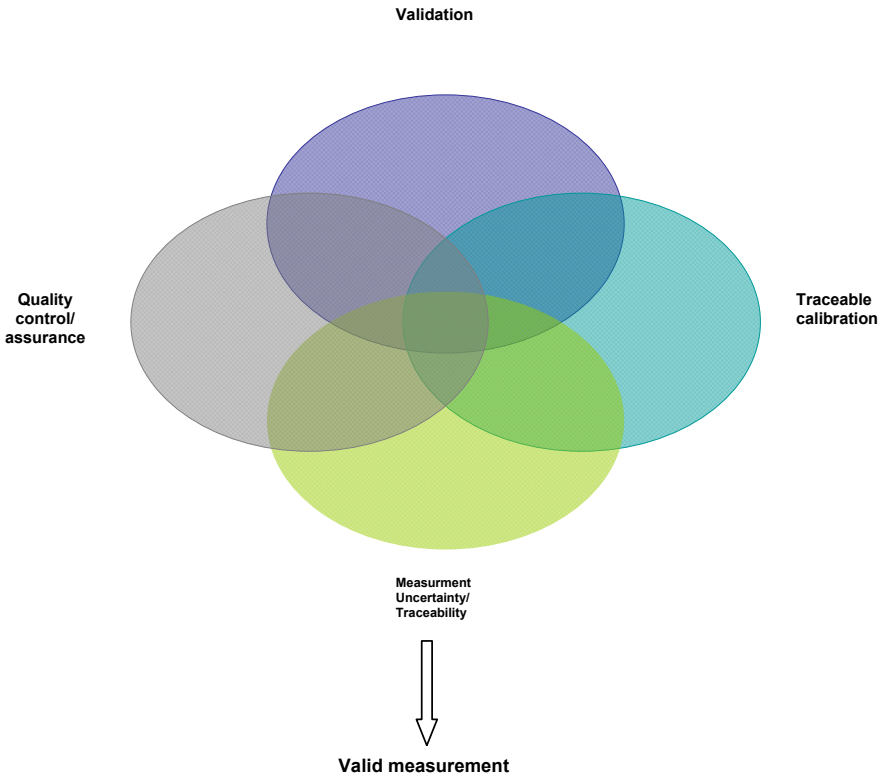


Figure 6: Overlap between functions associated with measurement traceability and analytical quality.

Two classes of materials are recognised by ISO, namely "certified reference materials (CRMs) and "reference materials" (RMs). CRMs must by definition be traceable to an accurate realization of the unit in which the property values are expressed. Different types of reference materials are required for different functions (e.g. a CRM would be used for method validation but a RM would be adequate for IQC).

The suitability of a matrix reference material depends on details of the analytical specification. Matrix effects and other factors such as concentration range can be more important than the uncertainty of the certified value. A protocol for assessing the suitability of matrix RMs is provided in Figure 7. The factors to be considered include:

- Measurand level
- Matrix match and potential interferences
- Sample size
- Homogeneity and stability
- Measurement uncertainty
- Value assignment procedures (measurement and statistics)

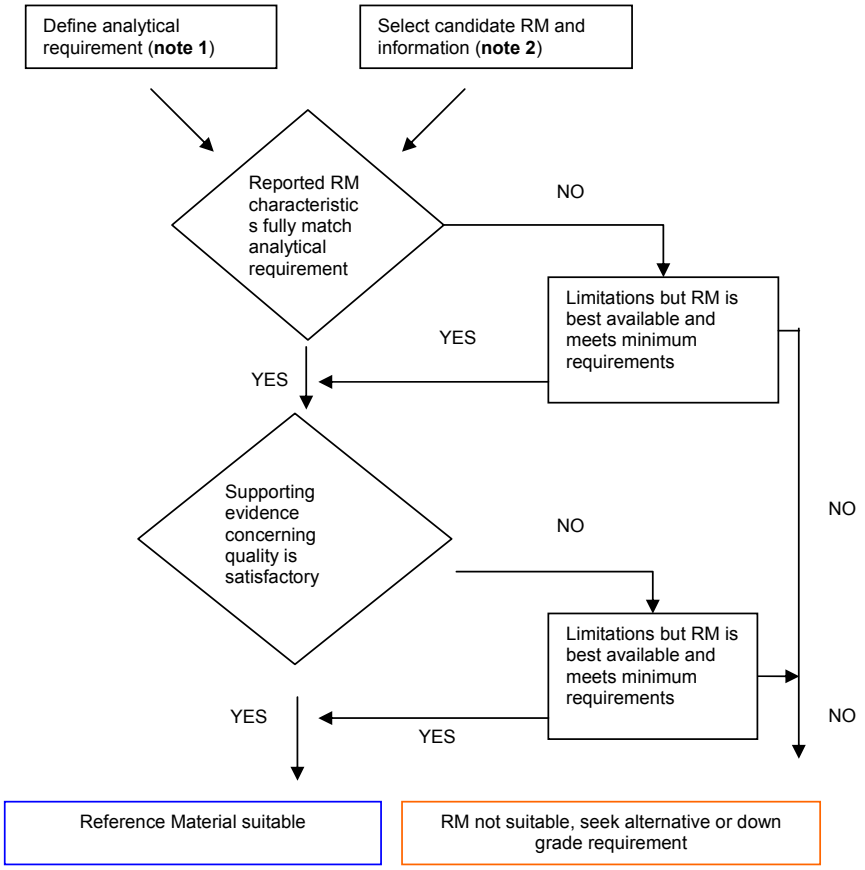


Figure 7: Assesment of the suitability of a Reference Material [30].

note1-The analytical requirements specification should include details concerning the measurand, concentration, traceability, measurement uncertainty, etc.

note 2- Key characteristics should be available in the RM certificate. Additional information, details of the method(s) used for value assignment and the full measurement uncertainty budget should also be available in the certificate or in a supporting report.

In the European Union the most important producers of RM are the European Commission's Joint Research Center-Institute for Reference Materials and Measurements (IRMM), the German Federal Agency for Materials

Research and Testing (BAM) and LGC-Ltd in the United Kingdom, who recently formed the European Reference Materials (ERM®) initiative.

As discussed recently, the issues of accreditation and quality assurance, increasing demands for CRMs, new challenges for RM development and application are appearing each day because of the very broad range and rapidly changing measurement demands [31]. Moreover, the spreading of systems for mutual recognition of measurement competences based on internationally agreed and third-part assessment schemes requires a permanent supply of appropriate reference materials for the proper calibration and quality assurance of measurements [31].

2.16.1 Reference material (RM)

According to a recent definition [32] a RM is a material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process. Notes: RM is a generic term; properties can be quantitative or qualitative, e.g. identity of substances or species; uses may include the calibration of a measurement system, assessment of a measurement process, assigning values to other materials, and quality control; A RM can only be used for a single purpose in a given measurement.

2.16.2 Certified Reference Material (CRM)

A certified reference material (CRM) is a “material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability. Notes: the concept of value includes qualitative attributes such as identity or sequence, uncertainties for such attributes may be expressed as probabilities, metrologically valid procedures for the production and certification of reference materials are given in, among other on ISO Guides 34 and 35, ISO Guide 31 gives guidance on the contents of certificates [33].

2.16.3 Metrological traceability

As layed down recently in an ERM policy for the statement of metrological traceability on certificates of ERM® Certified Reference Materials [34], metrological traceability of measurement results is a key requirement for the comparability of measurement results in time and space with other data, e.g. legal limit values or product specifications. Similarly, traceability of the certified values of a CRM is a prerequisite to be able to compare a measurement result with the certified value.

The certified value is attributed to a quantity representing a property of the CRM (ISO Guide 35). Following the terminology of ISO Guide 99, a "quantity" is a "property of a phenomenon/body/substance, to which a number can be assigned with respect to a reference. This reference can be a measurement unit, a measurement procedure or a reference material".

Consequently, a quantity (e.g., amount-of-substance content of a pesticide in a carrot sample) would be the combination of the identification/description of the property (pesticide) of a body/item (carrot/potato) and the base (or derived) kind of quantity.

The IUPAC Provisional Recommendations on "Metrological Traceability of Measurement Results in Chemistry" [35] describe that combination in form of a sequence.

System *(carrot/potato)* => Component/ analyte *(pesticide*)=> Kind of quantity *(mass fraction)*

A certified value on a CRM certificate belongs to a specified quantity and is the combination of a number (with its uncertainty) and the measurement unit.

Therefore, the key information of an ERM CRM certificate is actually a combination of 5 attributes:

- Identification of the body matrix (e.g. carrot/potato)
- Identification/description of the property/component (e.g. iprodione)
- Description of the certified base or derived quantity/kind of quantity (e.g. mass fraction)
- A number (e.g. 50 - with its corresponding uncertainty (e.g ±0.1))
- The measurement unit (e.g. ng/g)

The combination of these attributes has to be covered by the “traceability statement” of the certificate.
The measurement result has to be related to a stated reference and (as described in ISO Guide 99) such a stated reference can be:

- a value defined by the definition of a measurement unit or
- a value realized by a measurement procedure (including the measurement unit for a non ordinal quantity) or
- a value carried by a measurement standard (i.e. a certified reference material)

For most of the quantities described on CRM certificates, one (or both) of the following cases have to be considered:

- a measurand (quantity) which is defined by its structure alone (e.g. a chemical entity such as a specific ion, atom or molecule)
- a measurand (quantity) which is operationally defined by a described measurement procedure

Obviously a certified value as the mathematical product of a number and the measurement unit has to be described via properly calibrated measurement systems and it is this calibration hierarchy that needs to be described.

2.17 Development of a food based CRM

Analytical chemistry laboratories are continually requested to provide evidence on the quality of their operations. This is mandatory in cases where legislative limits are involved, e.g. in international trade and food analysis. Demonstration of adequate quality is required also in research and development. The general ISO definition of "quality" is given as "totality of characteristics of an entity that bears on its ability to satisfy stated and implied needs"

For a chemical analytical laboratory, the "entity" will in most cases be a measurement result. In a simplified form the quality requirements would then be represented in the form of reliable, comparable (traceable) results, accompanied with stated measurement uncertainty.

Within a laboratory's quality control programme, incorporation of appropriate, compositionally similar RMs is a valuable, cost effective aspect of a good quality control programme, and a way of transferring accuracy from well defined methods of analysis to the laboratory [36-39].

Results obtained with the CRM taken concurrently through the analysis with actual samples are compared with the certified values. Closeness of agreement indicates acceptable performance of the analytical method.

This important component of quality control in pesticide analysis of products of plant origin (fruits and vegetables) is not possible since, at present no natural matrix CRM is available for confirmation of the measurement process in Europe**.** CRMs are available only for persistent organochlorine pesticides in some animal tissues [40] and the National Measurement Institute Australia prepared a natural matrix (pureed tomato) reference material containing pesticide residue relevant to the Australian horticulture industry [41].

In general, a number of factors should be considered in the development of food - based CRM. Details of these steps are summarized here:

Definition of analytical methods and overall measurement system: for analytical values to be meaningful, the measurement process must produce numerical values of the property intended to be measured that are free of, or corrected for, all known systematic errors within the practical limits required for the end use of the material. There are internationally agreed protocols in order to establish method performance and validity [42, 43, 44].

Selection of measurands for characterization: the measurands have to be specified as part of the planning of the study (from sampling, sample preparation, calculation and recording of the the results).

Selection of statistical protocols: Statistical protocols for in-house characterization, homogeneity and stability testing, calculation of assigned values and associated uncertainties must be selected. Analysis of variance (ANOVA), t-tests to compare averages as well as F-tests to compare variances, performed on the replicate analysis are of typical use. As mentioned above, metrologically valid procedures for the production and certification of reference materials are given in, among other ISO Guides 34 and 35.

In-house characterization: This step relates to the analytical characterization of the candidate RM, like preliminary analysis to select suitable starting materials.

Material preparation: As a first choice the analyte level should be similar to the level actually present in routine samples, or of monitoring interest (e.g. regarding pesticides the MRLs specific of each analyte/matrix in the current EU legislation).

Material homogeneity and stability: Homogeneity testing of a candidate reference material is of primordial importance in the production of any RM. The risk of inhomogeneity is, with few exceptions (e.g. a metal in drinking water), inherent in any material**.** Therefore care must be taken to ensure that all sub-samples originating from the bulk material have the same properties as the bulk sample. These properties are chemical composition, or physical

and biological parameters. The homogeneity study is designed in a way that detects the differences in the quantity values between the subsamples. Stability testing of a RM is designed to assess chemical, biological or physical processes and reactions that might alter one or more properties of the RM over time (e.g. during transport (short-term stability) and storage (long-term stability). In order to assess and anticipate possible instability problems, reference materials are tested under extreme transport/storage conditions. Stability of a reference material can be seen to a certain extent as its homogeneity over time.

Value assignemnt: This is the final step in certification. The individual steps are summarized in Figure 8.

Generally, the associated standard uncertainty (μ_{CRM}) can be calculatedfromthe four variance components representing the material characterization step (μ_{char}), homogeneity testing (between bottle variation) (μ_{bb}), short term stability testing (μ_{sts}), and long term stability testing (μ_{lts}), according to the following equation (1). The μ_{CRM} is the basis for calculation of a 95 % confident interval or uncertainty interval for a future single observation (1).

$$\mu_{CRM} = \sqrt[2]{\mu_{char}^2 + \mu_{bb}^2 + \mu_{sts}^2 + \mu_{lts}^2} \quad (1)$$

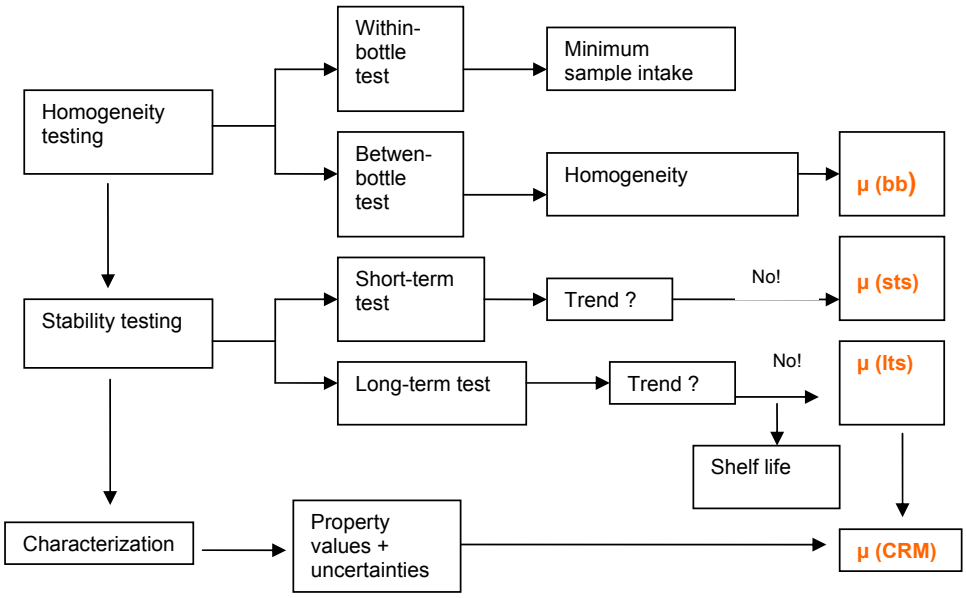


Figure 8: Evaluation of measurement uncertainty in the certification process [45].

Characterization: Characterization is the process of obtaining quantity values that approach as closely as possible the "true" value, together with uncertainty limits. Chemical characterization for quantification or certification purposes encompasses availability of suitable methodologies for the measurement of the analytes in question and expert analysts that could apply conceptually different approaches to selection, development, validation of methodologies and adaptation of statistical protocols for data analysis. Major approaches to characterization/certification may be classified as:

(1) Definitive: a single definitive method used by a single organization of high reputational quality preferably applied in replicate by two or more skilled analysts, in more than one separate laboratories, working totally independently, preferably using different experimental facilities, with equipment and expertise to ensure traceability to the SI system or equivalent. An accurately characterized, different backup method, independently applied is employed to provide additional assurance that the data are correct.

(2) **Independent reference methods:** one organization carries out the material characterization using various reference methods. Two or more independent reference methods, each based on an entirely conceptually different principle of measurement, independent in theory and experimental procedure, applied in replicate, within a single organization, of high reputational quality by two or more expert analysts, working independently. The methods used can naturally include definitive methods. The results should be corroborated by a third or additional, different, accurately characterized, well established, thoroughly validated, definitive, reference or other methods.

(3) Independent reference and validated methods by selected expert analysts: multiple organizations and laboratories carry out the material characterization using independent reference and/or validated analytical methods. Two or more independent reference and/or validated methods, each based on an entirely conceptually different principle of measurement, independent in theory and experimental procedure, applied in replicate, by selected expert analysts of high reputational quality and recognized competence working independently in an ad hoc network of laboratories participating in the collaborative interlaboratory characterization campaingn under very carefully prescribed and controlled conditions. All analytical methods are well characterized, validated, of acceptable demonstrated accuracy and uncertainty. The study can incorporate widely different methods, based on different physical or chemical properties.

(4) Volunteer analysts, various methods: multiple organizations and laboratories carry out material characterization by selecting various types of measurement methods belonging to a hierarchy of method traceability. Analytical methods used are varied, generally self selected, and include reference, validated, non-validated, routine, as well as definitive methods. The interlaboratory characterization exercise is carried out without imposition of prescribed conditions and controls.

(5) Method specific: characterization using a specific, validated method by selected expert or experienced analysts belonging to multiple organizations and laboratories. One specified analytical method applied in replicate, by selected expert or experienced analysts, of high reputational quality and

recognized competence working independently in a network of laboratories participating under carefully prescribed and controlled conditions, giving a method-specific assessed property value.

Approaches 2 and 3 are the most commonly used processes for assigning values to a material.

(6) Reporting of results and information: a document is prepared for each RM developed. Critically important information should be included to define and describe the material, its preparation and characterization, list numerical values for properties together with the associated uncertainties (as well as their definitions), stipulate minimum weight to be taken for analysis, indicate conditions of storage and include other details necessary for the analyst to correctly and fully utilize the material. As referred above, ISO Guide 31 gives guidance on the contents of certificates [33].

2.18 Commutability

Commutability is a property required to avoid undetected bias in routine measurement results when using a RM. It is defined as the equivalence of the mathematical relationships between the results of different measurement procedures for a RM, and for routine representative samples. Vesper at al. [46] have discussed commutability for clinical samples. This article gives a good account on different ways of checking commutability and what is at stake if CRMs are not commutable with field samples. The concept of commutability is obviously applicable to other types of samples as well.

Accurate results over time and location are achieved by standardizing measurements and by establishing traceability to a reference system. The goal of traceability is to have results obtained by a calibrated routine measurement procedure traceable to the highest available level of the calibration hierarchy [47]. Reference materials are key components of such reference systems and for establishing traceability. Commutability of reference materials is a critical property to ensure that they are fit for use.

The trueness of measurement results, defined as the closeness of agreement between the average value obtained from a large series of

results and a reference value [34], is assessed by comparing the measurement results obtained with the procedure in question with the reference value. The established reference is either a reference measurement procedure or a RM characterized with a reference method. Thus RMs are used to establish trueness of measurement procedures through calibration or to asses the trueness of the calibration of a measurement procedure. Furthermore, since commutability is a method-specific characteristic, RMs can be commutable for some analytical methods but may be non commutable for others. An important consideration when determining acceptance of the commutability assessment process is the intended use of the RM (fitness-for-use). The uncertainty in a commutability decision should be smaller when a RM is intended to be used for calibration of a measurement procedure than when it is intended to be used in an EQC program.

A RM would be considered commutable when a measurement procedure produces the same result for a RM as it does for routine samples at the same concentration. Non-commutability of RMs is frequently attributed to differences between the material's matrix and that of the routine samples. The matrix includes all components of a material except the analyte itself. The matrix effects are therefore defined as the influence of a property of the sample, independent of the presence of the analyte on the measurement and thereby on the measurable quantity. This lack of commutability can also be caused by the lack of specificity of the measurement procedures and this can be difficult to distinguish. Material handling, concentration, freeze–thawing cycles, can affect the matrix of the material. Different approaches to assess the comutability of a RM have been described [46]. All are based on determining the mathematical relationship and distribution of results of routine samples measured with different methods and determining if a reference material is a member of the same distribution, provided the sample contained the same analyte concentration. The existing approaches (for calibration, control of bias and accuracy assessment) use descriptive statistics or regression analysis to compare the numeric relationships among methods. Although the impact of non commutable RMs is well documented and international standards and guidance documents require RMs to be

validated for commutability, the assessment of commutability of RMs is still not performed routinely [46] and there is a need for consensus guidelines to enable consistent assessment of commutability of RMs.

3. AIM OF THE WORK

The aim of the present work was to study the feasibility of producing a (certified) reference material for 21 pesticides (Table 1) in a carrot/potato matrix. It was divided in two main tasks:

- Selection and in–house validation of suitable analytical methodology for measurement of pesticides in fruits/vegetables. The analytical method would be used for the homogeneity and stability studies of the candidate reference materials.
- Study the feasibility of stabilizing a matrix material spiked with pesticides by means of three types of physical processes: freezing (at -20 °C), freeze drying and sterilization (at 121 °C for 15 min).

Initially a suitable testing method had to be selected and its performance characteristics assessed by an in–house validation exercise according to internationally agreed protocols [42, 43, 44]. This effort intended to prove that the method was fit for the purpose, and provided traceable measurement results with a known uncertainty, sufficient for carrying out homogeneity and stability studies of the candidate reference materials.

Similarly, it was necessary to investigate the survival rate of target pesticide compounds during the chosen physical processes, and to find out whether these processes will influence method performance (e.g. extractability, repeatability).

Not only the way to stabilize the pesticides in the matrix of interest and its consequences were important for the study, it was also important to answer the key question how it would be possible to achieve a high degree of homogeneity of a large batch of spiked starting material necessary for carrying out the whole feasibility study. This would permit to evaluate if the uncertainty due to potential inhomogeneity would affect significantly the overall uncertainty.

Freezing and sterilization were intended to be an alternative to freeze-drying, where a reconstitution step is necessary, to ensure that the matrix format should be as similar as possible to routine laboratory samples. The main

reason for the choice of these stabilization techniques is to improve the commutability between real-world samples and CRMs.

A frozen material is similar to a routine sample, but it has the disadvantage of the need of being shipped on dry ice (e.g. high quantities of dry ice are necessary for a shipment of 48 h), and this could possibly be avoided if results of freeze dried sample demonstrate that it can be shipped at higher temperature (e.g. +4 °C). Also the sterilization process does not change the matrix format, it is still a wet material but the survival rate might be compromised for labile pesticides and this aspect needed to be addressed.

Three different matrices were tested, namely carrots, spinach and orange. Commercially available baby food was used to simulate the respective fruit/vegetable.

Homogeneity and stability studies were carried out according to experimental/statistical protocols designed for this purpose (Annex 1). Stability testing is of the highest importance as CRMs may be sensitive to degradation by several factors (pH, T, light, etc.).

Short and long term stability were therefore evaluated. Short term degradation studies had to be carried out to simulate degradation during transport and to decide under which conditions the material, once it is certified, has to be dispatched. In addition it enabled the decision whether the material was stable enough to become a reference material. For this purpose storage under extreme conditions (60 °C) was compared to storage at low temperatures (- 20 °C, + 4 °C, +18 °C) during relatively short periods of time (4 weeks). Long term stability test shall ensure the stability of the target analytes during storage of the material and shall allow the definition of shelf life.

The temperature where stability is investigated must include at least one T below the envisaged storage T. This allows the assessment of stability at this lower T (e.g 4 °C) if the results obtained at the higher T (e.g +18 °C) reveals signs of degradation of material.

An isochronous study scheme was employed for the stability study. This method [48] can be used when the total duration of the stability study is known. Consequently it is applicable to the short term study, concerning the possible degradation during transport as well as to the long term study concerning the stability issues during storage conditions. It is based on a storage design of

samples at different temperatures for different intervals of time allowing all measurements to be done at the same time, i.e. at the end of the study.

Using this stability testing method, samples stored at a given temperature, for various times, and either before or afterwards, they are stored at a very low reference temperature (-30 or -70 °C), at which their stability is supposed to be good. At the beginning (t=0) all samples reserved for the stability study will be transferred to a very low storage temperature (-30 °C or even lower) designed as reference temperature. For each of the storage temperatures studied (e.g. +60 °C, + 18 °C ,+ 4 °C, -20 °C) samples will be moved from this very low reference temperature to the corresponding studied storage temperature at different times (t= 0, 2, 4, 5 months, for the long term study and t= 0, 1, 2, 4 weeks, for the short term study). At the defined end time the samples will be immediately analyzed or put back (for a short time) at the reference temperature before analysis. The samples that remained at the reference temperature for the entire study give the starting value of t=0. All samples are then analysed under repeatability conditions in a short period of time. All studies must be carried out using highly repeatable and reproducible methods. This method has the advantage that the evaluation can be made temperature by temperature, starting with the samples stored at highest temperature. If instability is detected after a given time, one may decide not to analyze anymore the samples stored for much longer times and to start analysing samples at the next temperature. If on the other hand, stability demonstrated for the full period at a given T, no further analysis of samples stored at lower temperature are required.

The outcome of the feasibility study will allow IRMM, to initiate the production and certification of more "fresh" certified reference materials to the benefit of measurement laboratories world-wide.

4 EXPERIMENTAL

4.1 Chemicals and consumables

- Ultrapure water (MilliQ-System Millipore, Bedfofd, MA)
- Acetonitrile, of SupraSolv grade (Merck, Darmstadt, Germany)
- Toluene of SupraSolv grade (Merck, Darmstadt, Germany)
- Glacial acetic acid, HPLC grade (Merck, Darmstadt, Germany)
- Formic acid (Merck, Darmstadt, Germany)
- Methanol of SupraSolv grade (Merck, Darmstadt, Germany)
- Magnesium sulphate >98 % pure, anhydrous, fine powder
- (Sigma- Aldrich, Bornem, Belgium), heated overnight in a muffle furnace at 550 °C to remove phthalates
- Fluoroethylenepropylene (FEP) centrifuge tubes (50 mL-Nalgene®, 3114-0050, Supelco, Belgium)
- Adjustable volume solvent dispenser (500 mL, Optifix®, Supelco, Belgium)
- Extraction tube (55234–U, Supelco, Belgium) containing 6 g magnesium sulphate and 1.5 g sodium acetate. Each tube for use with 10 g sample size
- SPE cleanup Tube 1 (55228–U, supelco, Belgium) containing 900 mg magnesium sulphate and 150 mg Supelclean PSA for cleanup of a 6 mL extract of non complicated matrices (e.g. apple/pear based baby food, citrus fruits)
- SPE cleanup tube 2 (55230-U, Supelco, Belgium), containing 900 mg magnesium sulphate, 150 mg Supelclean PSA, 15 mg Supelclean ENVI-carb for samples with moderate levels of carotenoids or chlorophyll (e.g. carrots) and use for 6 mL extract
- SPE Cleanup tube 3 (55233-U, Supelco, Belgium) containing, 900 mg magnesium sulphate, 150 mg Supelclean PSA and 45 mg Supelclean ENVI-Carb for samples with higher level of carotenoids or chlorophyll (e.g. spinach) and use for 6 mL extract

- Dark glass vials (100 mL) with Teflon-lined caps (Supelco, Belgium)
- Glass vials (50 mL,125 mL) with metal screw caps (Supelco, Belgium)
- Reference standards of 48 native compounds (azinphos-methyl, azoxystrobin, bromopropylate, chlorpyriphos, chlorpyriphos-methyl, cypermethrin, diazinon, endosulfan (α+β), iprodione, lambda-cyhalotrin, malathion, mecarbam, metalaxyl, parathion, permethrin, phorate, pirimiphos-methyl, procymidone, propyzamide, triazophos and vinclozolin (validation exercise) and maneb, zineb, metiram, propineb, mancozeb, aldicarb, benomyl, methidathion, carbendazim, methomyl, oxydometon methyl, methiocarb, imazalil, kresoxim-methyl, dimethoate, ometoate, acepahte, methamidophos, folpet, chlorothalonil, captan, dicofol, dichlofluanid, tolyfluanid, deltamethrin thiabendazole, thiophanate-methyl (preliminary studies)
- isotopically labelled pesticides, either deuterated or 13 C labeled (with stated purities >99 %): $^{13}C_4$phorate, D_{10}malathion, D_{10}parathion, $^{13}C_6$cypermethrin, D_6pirimiphos-methyl, D_{10}chlorpyrifos and D_{10}mecarbam, from Cambrige Isotope laboratories (Apeldoorn, The Netherlands)
- GC-autosampler amber vials (amber glass with Teflon-lined caps, 2 mL, Sigma- Aldrich, Bornem, Belgium**)**
- Pasteur pipettes (Sigma Aldrich, Bornem, Belgium)
- Graduated centrifuge tubes (10 mL) for use in evaporator (Sigma Aldrich, Bornem, Belgium)

4.2 Test materials

- Test products (blank samples verified to contain no detectable target analytes): apple/pear, carrots, spinach, and orange based baby food (Olvarit/Nutricia, Belgium)
- Samples to be analysed (spiked test products)

4.3 Analytical equipment

- GC-MS system consisting of a 6890N Network GC and a 5975 Inert Mass Selective Detector (Agilent Technologies, Zaventem, Belgium)
- Analytical balances (ME235-OCE and Genius ME semi-micro balance, Sartorius, Göttingen, Germany)
- Centrifuge, Heraeus Megafuge 1.0R (Thermo Fisher Scientific, Zellik, Belgium)
- Solvent evaporator (nitrogen flow, temperature 50 °C), (Liebisch Labortechnik, Bielefeld, Germany).
- Horizontal mechanical shaker, Model KS501 digital, IKA Labortechnik (Staufen Germany)
- Vortex mixer, Model MS2 minishaker, IKA Labortechnik (Staufen Germany)

4.4 GC/MS operating conditions

The following GC/MS conditions were used for the analysis of the target pesticides (Table 6).

Table 6: GC/ MS optimized method for the analysis of target pesticides

GC-MS settings:
Injection mode: splitless injection, injector temperature 250 °C; pressure: 10.48 psi; total flow 60.0 mL/min; Purge flow: 56 mL/min; purge time: 1.50 min; saver flow 20.0 mL/min; Gas saver: on
Injection volume: 2 µL splitless (autosampler)
Oven temperature program: solvent delay (4 min), initial temperature 80°C for 1.5 min, ramped to 180 °C at 25 °C/min, followed by a 5 °C/min ramp to 230 °C and a 25 °C/min ramp to 290 °C (held for 10 min) –note 1
MS transfer line temperature: 290 °C
Ionization mode: electron ionization mode (EI)
Analytical Column: low bleed 5 % phenylmethylpolysiloxane (DB-5ms). Length: 30.0 m; nominal diameter: 250.0 µm; film tickness: 0.25 µm
Liner: single taper, deactivated, no glass wool, 5181-3316 (Agilent Tecnhologies,USA)
Carrier Gas: He, constant flow 1.0 mL/min, 99.99 % purity
Solvent delay: 4.00 min
MS conditions:
Aquisition mode: SIM (the ions monitored for the target pesticides are provided in Method Validation section)
Dwell time: 20 - 30 ms to get approx. 3 cycles/second for each analyte
MS Quadropole temperature: 150 °C
MS Source temperature: 230 °C
Electron multiplier voltage: 1600-1800 Volts

note 1 - In case of toluene injections, the initial oven temperature was increased to 100 °C and the remaining program kept the same

4.5 Material processing equiment and operation conditions

- Probe blender (Ultra Turrax T 50, IKA, Staufen, Germany)
- Steel mixing vessel which is part of a mixer for paste assembly (IKA, Staufen, Germany)
- Freeze-dryer Epsilon 2-85D (Martin Christ, Osterode, Germany).
- Karl Fischer titrator (IKA, Staufen, Germany)
- Retsch heavy duty cutting mill (Haan, Germany)
- 1.0, 0.5 and a 0.25 mm sieve insert (Haan, Germany)
- PTFE pestle (Supelco, Germany)
- FFP3 breathing mask (Supelco, Germany)
- Dyna-MIX CM200 mixer (WAB, Basel, Switzerland).
- Vibrating feeder and an antistatic blower (IKA, Staufen, Germany)
- Capping machine from Bausch & Ströbel (Ilshofen, Germany).
- Matachana B-4023 Autoclave (Webeco, Ober-Ramstadt, Germany)
- Luminar 4030 Acousto-Optical Tunable Filter Near Infrared Spectrometer (AOTF-NIR, Applitek, Nazareth, Belgium)
- Sympatec Helos laser light scattering instrument (Clausthal-Zellerfeld, Germany).
- Freezer capable of maintaining T at -70 °C, -30 °C and -20 °C. (Liebherr Cinem S.A., Ternat, Belgium)
- Fridge capable of maintaining T at +4 °C (Liebherr Cinem S.A., Ternat, Belgium)
- Glass jars and lids (210 mL) for the frozen samples (Derco, Ittre, Belgium)
- Glass jars and lids (110 mL) for the autoclaved samples (Fränkische Glasgesellschaft Lipfert & Co, Lichtenfels, Germany)
- Amber glass vials with teflon screw cap (100 mL) for the freeze-dried samples, (VWR International, Leuven, Belgium)

4.6 Safety precautions and protection of the environment

Pesticides are known to be toxic and some are carcinogenic. Toluene is toxic and flammable. All applicable safety and waste handling rules were followed, including the proper labelling and disposal of chemical wastes. The following safety precautions were taken when working with the pesticide neat solids and/or solutions in toluene containing these compounds:

- avoid contact with skin and eyes
- wear protective clothing, gloves and eye/face protection
-use the fume-hood for the preparation of the solutions and mixtures if possible, do not inhale the vapours
-do not exceed the safety limits of the centrifuge tubes or rotors used

4.7 Analytical procedure

The QuEChERS. method involves the extraction of the sample with acetic acid in acetonitrile and simultaneous partitioning initiated by adding anhydrous magnesium sulfate ($MgSO_4$) plus sodium acetate (these salts serve to salting out water from the sample) followed by a simple cleanup step known as dispersive-SPE (Figure 9).

The method is designed for samples with >75 % moisture. Different options in the protocol are possible depending on the analytical instrumentation available, desired limit of quantification (LOQ), scope of target pesticides, and matrices under study.

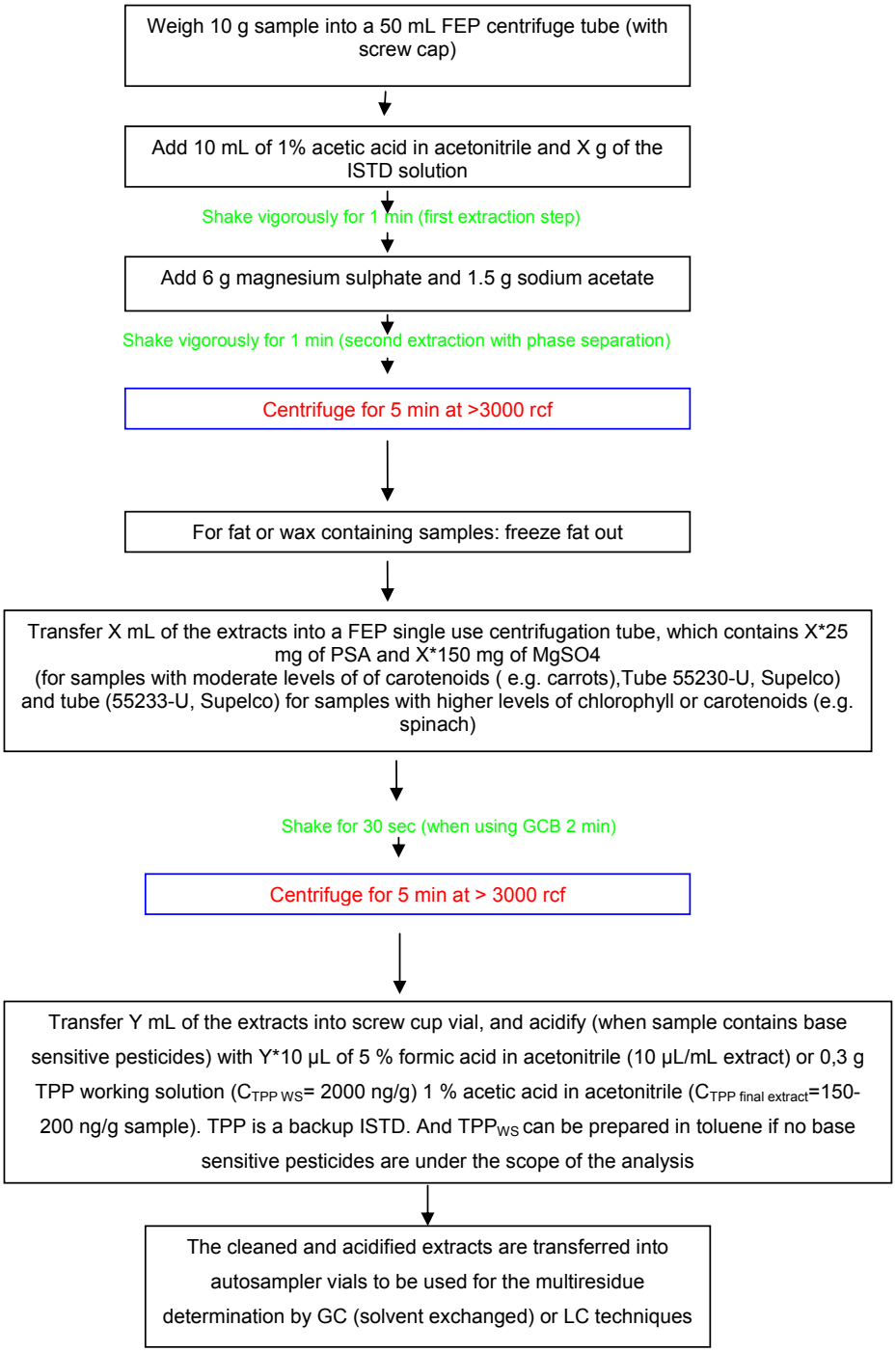


Figure 9: The generic QuEChERS protocol (for 10 g of sample)

4.7.1 First extraction step

4.7.1.1 Weighing

Weigh 10 g ± 0.1 g (m_a) of the wet homogeneous sample into a 50 mL FEP centrifuge tube. For freeze-dried material, weigh equivalent amount on a dry mass basis (provided the water content of the wet (e.g. 90 %) and freeze dried samples (e.g 3 %)) and add sufficient cold water leading to a total water content in the tube of approximately 10 g (e.g., for a water content of 3 % weigh 1 g ± 0.1 g (m_a) of freeze dried sample and add 10 g cold water. In the case of freeze-dried samples vortexing was applied to allow water entering in the freeze dried sample pores before proceeding with the analysis.

4.7.1.2 Solvent and ISTD addition

Add 10 mL of acetonitrile containing 1 % acetic acid and 1 g of ISTD mixture (m_{ISTD}) prepared in toluene and containing each labelled pesticide at a content of 500 ng/g ($C_{\text{singleISTD in the mixture}}$ =500 ng/g). This yields a concentration of the ISTD of 50 ng/g in the samples (spiked) and reagent blank. Wait 10-15 min for equilibration of the working standard solutions stored in the freezer and to be used at room temperature. Detailed example of calculations can be consulted in Annex 2.

For the blank matrix-matched standards ISTD $^{cal\ mix}$ is only added after the evaporation step described in 4.7.2.4 to the matrix blank extracts prepared in toluene. It means that ISTD is added at the same time as the pesticide standards for calibration purposes, without undergoing method losses. TPP working solution, 1 % Hac in acetonitrile ($C_{TPP\ WS}$= 2000 ng/g) was added (approx. 0.3 g) in calibration standards and sample extracts alike (to yield a concentration in final extract of ($C_{TPP\ final\ extract}$=150 ng/g sample) only before the analytical step and it served as a backup ISTD to isolate the analytical step variability. TPP_{WS} can be prepared in toluene when no base sensitive pesticides are under the scope of the analysis [1].

[1]**Note**: the acidification of the QuEChERS extracts before and after PSA cleanup was only performed during the method optimization stage (described in the next section) as a measure to prevent degradation of base sensitive pesticides, because at that stage base sensitive pesticides were under the scope of the test analysis.

4.7.1.3 Extraction

The tube was closed and shaken vigorously for 1 min.

Spiked samples were extracted at room temperature and frozen samples were extracted in the process of thawing, to ensure that no significant degradation or volatilization losses of temperature labile pesticides (e.g. phorate, procymidone, diazinon) occurred during prolonged exposure at room temperature.

4.7.1.4 Second extraction step and partitioning

The prepared-salt mixture (Tube 55234–U, Supelco, Belgium) was added to the suspension from 4.7.3. The tube was closed, immediately shaken vigorously for 1 min and centrifuged for 5 min at 3000 rpm. 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 min extraction of the entire batch was performed in parallel after the salts have been added to all the samples.

4.7.2 Cleanup

4.7.2.1 Cleanup with amino–sorbent ("Dispersive SPE" with PSA)

An aliquot of 6 mL of the acetonitrile phase from 4.7.4 was transferred into a centrifuge tube already containing 150 mg PSA and 900 mg of magnesium sulphate (Tube 55228-U, Supelco, Belgium). The tube was closed, shaken vigorously for 30 s and centrifuge for 5 min at 3000 rcf.

4.7.2.2 Cleanup with a mixture of amino–sorbent+GCB ("Dispersive SPE" with PSA + GCB) for samples with high content of carotenoids or chlorophyll.

For samples, with a moderate content of carotenoids (e.g. carrots) or a high content of chlorophyll (e.g. spinach), dispersive SPE is performed using a combination of PSA and Graphitized Carbon Black (GCB).

An aliquot of 6 mL of the acetonitrile phase from 4.7.1.4 was transferred into a single use centrifuge tube (55230-U, Supelco, Belgium) which already contained 900 mg magnesium sulphate, 150 mg Supelclean PSA and 15 mg Supelclean ENVI-Carb for samples with moderate levels of carotenoids or chlorophyll (e.g. carrots). SPE Cleanup tube (55233-U, Supelco, Belgium) containing 900 mg magnesium sulphate, 150 mg Supelclean PSA and 45 mg Supelclean ENVI-Carb was used for samples with higher level of carotenoids or chlorophyll (e.g. spinach).

The tube was closed, shaken vigorously for 2 min and centrifuged for 5 min at 3000 rcf.

4.7.2.3 Extract storage

The extracts were stored at -20 °C if analysis could not be conducted immediately.

4.7.2.4 Concentration of the end extracts and solvent exchange

The final concentration of the extract corresponded to 1 g sample/mL extract.

A single evaporative concentration of the extracts by a factor of four was performed to increase the amount of equivalent sample injected in splitless mode. To achieve this, 4 mL of the extract were transferred into a test tube and reduced approximately to 1 mL at 50 °C using a slight nitrogen flow, and solvent exchanged to toluene by performing evaporation of the extract to 0,5 mL and then filled up to 1 mL toluene, which acts as solvent keeper for pesticides and has benefits in GC analysis (e.g. smaller vaporization expansion volume than acetonitrile). Anydrous $MgSO_4$ was added to remove residual water, shaken and centrifuged at 1500 rcf for 1 min and approx. 0,6 mL of the final extract transferred to appropriate autosampler vials for analysis via GC-MS. This way the injection of a 2 µL splitless injection of 4 g/mL final extract in toluene (equivalent to 8 mg sample) onto the column was sufficient to achieve LOQ <10 ng/g for some pesticides The blank extract was treated in the same way. In this case the calibration standard spiking solution necessary for the preparation of matrix matched calibration standards was also done in toluene. The $ISTD^{calmix}$ was added to the blank extracts just after the evaporative step, along with the pesticide calibration mixture covering the whole calibration range. TPP^{WS} (approx. 0.3 g, C_{TPPWS}= 2000 ng/g) was added to matrix–matched calibration standards, sample extracts and reagent blank spikes alike [1].

4.7.3 Test for interference and recovery

Reagent blanks (sample was substituted by water), matrix blanks, and recovery tests with the matrix of interest were carried out at levels appropriate to the maximum residue level (MRL) of the pesticide/sample matrix combination. The chromatogram of the reagent blank and matrix blank should not show any significantly interfering peak at the retention time of the analytes (see Method Validation section for detailed information). No evidence of carry over should be present in the reagent blank or toluene reagent, which was injected after the most highly concentrated standard in the sequence, and in the beginning of the same.

4.7.4 Evaluation of results

4.7.4.1 Identification and quantification

The parameters employed to determine the identity of an analyte present in the sample extract included: i) The retention time of the target analyte (Rt_{pest}) or the retention time ratio against the ISTD (Rt_{pest} / Rt_{ISTD}) obtained from the same run; ii) the peak shape of the analyte (left or right tailing indicated poor functioning state of the analytical column) and iii) the relative abundance of the recorded m/z ratios, in general 3 ions for each target analyte. These parameters of the analyte to be indentified were compared with those obtained for the pesticides in the matrix matched calibration solutions.

Pesticides were identified if the following criteria were fulfilled:

- Matching retention time and spectrometric data obtained in SIM mode to those obtained by injecting individual stock solutions in solvent. The retention time (R_t) of the compound in the sample should match the Rt in the standard: the relative retention time should be not less that 0.98 for the same analytical conditions.
- The ions for quantitation and identification in SIM mode (Method Validation section) were selected to maximize S/N ratios of the analyte

while avoiding matrix interferences and had to match the relative intensity of ions listed in literature available for many pesticides [9].

- The molecular ion or in some cases the most abundant ion of each compound was monitored in the SIM mode and was used as quantification ion (Tgt). Additional ions were monitored, as confirmatory ions (Q1, Q2). For the isotopically labelled standards, the molecular ion was monitored for quantitative purposes.
- In each SIM window the dwell time was adjusted to obtain approximately 3 cycles/second for each analyte in order to be able to separate possible co-eluctions of compounds with very close Rt. This optimization parameter is presented in the Method Validation section.
- Results were not reported if they were outside the concentration range covered by the calibration standards.

4.7.5 Calibration

4.7.5.1 Preparation of individual stock and working standard solutions

Weigh about 70 mg of each pesticide standard, using an Analytical balance (Genius ME Semi-Micro balance, Sartorius, Göttingen, Germany) fill up with toluene to a total weight of 30 g (concentration of stock solution around 2000 µg/g).

Dilute the stock solution to obtain a working solution of about 40 µg/g by gravimetry. The final working mixed standard solution was build up in a way to respect the MRLs of the different pesticides (if MRL of pesticide X is 10 ng/g and MRL of pesticide Y is 20 ng/g, the ratio between the concentration of the two pesticides in the final working mixture solution should be 1:2). This solution will be referred later as pest ^{WSmixMRL}.

The ISTD (internal standard) stock solution and working solution were prepared using isotopically labelled pesticides commercially available and TPP. The final mixture was prepared exactly as above described for the native compounds. The desired mass fraction of each labelled

pesticide in toluene in the final mixture ($w_{ISTD}^{cal\ mix}$) was 500 ng/g and the mass fraction of each labelled ISTD compound in the sample extract corresponded to approx. 50 ng/g sample The mass fraction of the TPP working solution in toluene was w_{TPPWS}= 2000 ng/g.

(Annex 2 provides an example of the calculations)

All solutions were stored at -20 °C. Before using them they were left at room temperature at least 30 min to equilibrate.

Currently available data [52] show that stock standards of the large majority of pesticides in toluene are stable for at least 5 years in the freezer when stored in tightly closed glass containers.

4.7.5.2 Solvent–based calibration standards

Five calibration standards (0.25 MRL, 0.5 MRL, MRL, 1.5 MRL and 2 MRL) were prepared by mixing known masses of pesticide working solution ($m_{pest}^{WSmixMRL}$) and a known mass of ISTD solution (m_{ISTD}^{WS}) and filling up to desired mass with toluene (Annex 2 provides examples of the calculations).

The concentration of internal standard was approximately the same in all calibration standards and matched the median of the calibration range (MRL level). The standards were stored in the freezer at -20 °C.

The concentration of an individual pesticide in the calibration standard was as follows (1):

$$w_{pest}^{calmix} = \frac{w_{pest}^{WSmix} * m_{pest}^{WSmix}}{m^{calmix}} [ng/g] \qquad (1)$$

m_{pest}^{WSmix}…mass of mixture of pesticide working solution [g]

$m^{cal\ mix}$…….mass of calibration mixture standard solution [g]

$w_{pest}^{WS\ mix}$…mass fraction of pesticide in mixture working solution [ng/g]

$w_{pest}^{cal\ mix}$…mass fraction of pesticide in calibration mix [ng/g]

4.7.5.3 Calibration in matrix

Matrix matched standards were prepared in the same way as solvent-based standards, however instead of pure toluene, extracts of blank samples were used. The extracts were stored at -20 °C if the analysis could not be conducted immediately after sample preparation.

This was occasionally done by adjusting their volumes with toluene (so that the same dilution of matrix occurred in sample extracts and matrix matched extracts). The stability of pesticides in matrix-matched standards may be lower then that of standards in pure toluene (see Method Validation section for stability of matrix-matched standards). For matrix blanks to be used for the calibration standards first the multiple blank extracts were combined and then the needed amount was transferred into separate dispersive SPE tubes. Annex 2 provides an example of the concentration range of the calibration in solvent and in matrix for each target pesticide (from 0.25 MRL until 2 MRL level of each target pesticide).

4.7.5.4 Calculations of the result

Quantification of the target pesticides was done using the internal standard (ISTD) method. The internal standard consisted of a mixture of isotopically labelled pesticides (3 ISTDs were used for homogeneity and stability studies of the candidate reference materials and 4 ISTDs were used during the validation exercise). For each compound, integration was performed using the corresponding labelled congener. For those pesticides with no corresponding labelled standard, the labelled compound in the same chromatographic window and the closest retention time was used. Calibration was done by internal standardization at five concentration levels.

Calibration functions for each analyte were obtained by plotting the peak area ratio $PR^{cal\ mix}$ ($A_{pest}^{cal\ mix}$/ $A_{ISTD}^{cal\ mix}$) of each calibration level against the ratio of the mass fraction ($w_{pest}^{cal\ mix}$/ $w_{ISTD}^{cal\ mix}$) of the standard solutions.

From the corresponding calibration graph, described by the following formula:

$$PR^{\text{cal mix}} = a_{cal} \times w\,pest^{\text{cal mix}} / w_{ISTD}^{\text{cal mix}} + b_{cal} \quad (2)$$

each expected mass fraction ratio ($w_{pest}^{\text{cal mix}} / w_{ISTD}^{\text{cal mix}}$) can be calculated as follows:

$$w_{pest}^{\text{cal mix}} / w_{ISTD}^{\text{cal mix}} = (PR^{\text{cal mix}} - b_{cal}) / a_{cal} \quad (3)$$

The mass fraction ratio $w_{pest}^{\text{sample}} / w_{ISTD}^{\text{sample}}$ in the final extract depends on the mass fraction W_r of the pesticide in the test portion m_a, the mass fraction of the ISTD and its mass m_{ISTD}^{sample} added to the test portion.

$$w_{pest}^{\text{sample}} / w_{ISTD}^{\text{sample}} = (W_r * m_a) / w_{ISTD} * m_{ISTD}^{\text{sample}} \quad (4)$$

The mass fraction W_r is calculated as follows:

$$W_r\,[mg/kg] = ((PR^{\text{sample}} - b_{cal}) * (w_{ISTD}^{\text{sample}} * m_{ISTD}^{\text{sample}})) / a_{cal} * m_a \quad (5)$$

Variables used:

mass fraction of internal standard (ISTD) in the ISTD solution	w_{ISTD}^{sample} [µg/g]
mass of test portion	m_a [g]
mass of ISTD added to test portion	m_{ISTD}^{sample} [g]
Peak area of pesticide obtained from calibration mixture	$A_{pest}^{\text{cal mix}}$ (counts)
Peak area of ISTD obtained from calibration mixture	A_{ISTD}^{calmix} (counts)
Peak area of pesticide obtained from final extract	A_{pest}^{sample} (counts)
Peak area of ISTD obtained from final extract	A_{ISTD}^{sample} (counts)
Peak area ratio obtained from calibration mixture	PR^{calmix} (dimensionless)
Peak area ratio obtained from final extract	PR^{sample} (dimensionless)
Slope of calibration graph	a_{cal}
Y-intercept of calibration curve	b_{cal}
Mass fraction of pesticide in the sample	W_r [µg/g]

4.7.5.5 Measurement uncertainty

The expanded uncertainty was calculated using the following mathemathical expression (6):

$$U = k * \sqrt{u_{(Cst)}{}^2 + u_{(cali)}{}^2 + \frac{u_r{}^2}{n_1} + \frac{u_{ip}{}^2}{n_2} + \frac{u_{rec}^2}{n_3}} \qquad (6)$$

Where:

U	expanded uncertainty;
k	coverage factor (k=2)
u (c_{st})	uncertainty of standards used
u (cali)	uncertainty of calibration
u_r	uncertainty of repeatability
n_1	total number of measurements
u_{ip}	uncertainty of intermediate precision
n_2	total number of days
u_{rec}	uncertainty of recovery (coefficient of variation for the results of recovery)
n_3	total number of independent samples used in the recovery experiments

4.7.6 Measuring sequence and performance qualification

The sequence given bellow was followed when performing the analysis:

- Measurement of the 5 calibration solutions covering the working range prepared as described above (0.25 MRL to 2 MRL), 3 replicates each (in randomized order, e.g. 1^{st} replicate – 1^{st}, 2^{nd}, 3^{rd} , 4^{th}, 5^{th}, calib. point, 2^{nd} replicate – 5^{th},4^{th}, 3^{rd}, 2^{nd},1^{st} calibration point. etc) and two injections per each standard. The calibration standards were injected at the beginning and in the end of each analytical run for QC purposes
- The first sample in a sequence was a solvent blank (*i.e.,* toluene) followed by reagent blank and matrix blank (zero standard). No interfering peaks (relate to validation report) must be detected at the retention time of the target compounds in the matrix blank. No evidence of carryover should be present in the reagent blank or toluene (injected in the beginning of the sequence and after the most highly concentrated standard in the sequence). If a potential carry over was detected corrective action was then taken, such as checking the toluene used for possible contamination
- TPP was used as a QC measure to isolate the variability of the analytical step from the sample preparation method and it was spiked just before the analytical step in calibration standards and sample extracts alike. Although pipets and balances were periodically calibrated to ensure accuracy, random and systematic errors[2] in volumetric transfers are inherent in analytical methods, and the ISTD should improve the accuracy of the results. The recoveries of the ISTD were assessed by comparing the peak areas of the ISTD in the samples with those from the calibration standards. The TPP/ISTD peak area ratio should remain consistent (<10 % RSD) in the method. In case any extract gave a substantially different ratio from the others, the results of this extract were questioned

- Furthermore, if the QC spike yielded recoveries <70 % or >120 %, then the results from all samples were questioned. If all pesticide recoveries were outside the acceptable range, then most probably a systematic bias occurred
- Samples: at least two independent replicates per sample were prepared. Samples were injected at least 2 times each
- Peak shapes were Gaussian, and peak widths at half-heights were less than 5 s.

Table 7: Example of injection sequence for calibration purpose.

	Sample description
1	toluene
2	reagent blank 1
3	reagent blank 2
4	matrix blank 1
5	matrix blank 2
6	1st cal. point in matrix 1st replic. (2 inject.)
7	2nd cal. point in matrix 1st replic. (2 inject.)
8	3rd cal.point matrix, 1streplicate (2 inject.)
9	Etc. All 5 calibration levels injected randomized, 3 replicates each, (2 inject.)
10	Sample 1st replicate (2 inject.)
11	Sample 2nd replicate (2 inject.),etc.

[2] **Note**: *Random error*, is a component of the error which, in the course of a number of test results for the q same characteristics, varies in an unpredictable way. It is not possible to correct for random error.

Systematic error is a component of the error which, in the course of a number of test results for the same c haracteristics, remains constant or varies in a predictable way.

Gross errors, such as accidental loss of sample, do not fit into the usual pattern of errors associated with a particular situation. They should normally be absent and avoided by strict observance of a given SOP.

5 RESULTS AND CONCLUSIONS

5.1 Optimization of the analytical method for the determination of pesticides in food matrices

Before proceeding with the development of the candidate RMs, the analytical procedure described in Materials & Methods section was optimized. In this section the optimization of the analytical method is described. Also the parameters regarding the performance of the analytical procedure were assessed via an in–house validation exercise, using spiking experiments. This optimized procedure was then used in all the experiments and tests carried out.

5.1.1 Method set-up

Apple based baby food was chosen as the initial test material for the study of the performance of the analytical method, because it is easily found on the market, it was considered comparable to the target matrices of fresh fruit and vegetables and has potentially no occurring contaminants (it is pesticide free). Moreover, apple is not considered a very complex matrix so it is possible to give relatively clean extracts.

The choice of an appropriate internal standard was very important because it must not be present in the sample. A relatively inexpensive deuterated pesticide (d_{10}-parathion) was chosen as the ISTD for initial studies of the performance of the analytical procedure.

Individual working pesticide standards prepared in toluene (40 µg/g) of all target analytes (except dithiocarbamates which were prepared in 10 % methanol in toluene) were injected at the GC-MS specified conditions and spectra recorded in full scan mode (50-400 m/z). Table 1 in Annex 3 gives the particular R_t and quantitation ions for the SIM mode analysis. Some pesticides did not show a good chromatographic response in GC-MS (peak shape), e.g. thiabendazole, thiophanate-methyl, aldicarb, and for those R_t are omitted in the table.

This was followed by the injection of a solution (in toluene) containing all GC-amenable analytes at the specific MRL level in SIM mode (11 windows) to verify if equally good chromatographic separation could be achieved. Only after adjusting dwell times in each window to get approximatelly 3 cycles/s, quantification of the analytes was possible and co-eluctions resolved. In the majority of the SIM windows only two ions were chosen to characterize the analyte (for quantitative and qualitative purposes). In exceptional cases one confirmation ion was added. The relative intensities of the detected ions in SIM mode, expressed as a percentage of the intensity of the most intense ion, and R_t measured under the same conditions were used for identification and confirmation purposes in an unknown sample.

The total ion chromatogram in SIM mode of a solution containing all GC amenable pesticides at the MRL level (in toluene) is shown in Fig. 10-11. All GC amenable pesticides were detected at the MRL level of each analyte/matrix combination as set out in the 2002-2005 EU monitoring programme. This list of analytes resulted initially in 48 pesticides to be analysed in the 2002-2005 monitoring scheme for 8 commodities: pears, bananas, beans, potatoes, carrots, oranges/mandarins, peaches/nectarines and spinach (Table 3).

Out of these the dithiocarbamates (maneb, zineb, metiram, propineb and mancozeb) cannot be included in a GC-amenable multiresidue method because dithiocarbamates are heat-sensitive and will degrade during GC. Usually they are measured by liberating carbon disulfide through acid hydrolysis and its determination by head-space GC. Actually, they require a special homogenization because they can easily be lost during sample preparation of acidic matrices using the QuEChERS method. As for thiabendazole and thiophanate-methyl, their solubility in toluene is low. From the remaining 41 pesticides to be analysed with the QuEChERS method, for 13 LC is preferred (aldicarb, benomyl, methidathion, carbendazim, methomyl, oxydometon methyl, methiocarb, imazalil, kresoxim-methyl, dimethoate, ometoate, acepahte, methamidophos), phorate is GC amenable but LC preferred (better peak shape) and 6 analytes (folpet, chlorothalonil, captan, dicofol, dichlofluanid and tolyfluanid) are all base sensitive pesticides and the addition of 1 % acetic acid during sample preparation does not solve their degradation and detection in both GC and LC [15]. However, their degradation products can serve in routine

monitoring when no alternative method for these types of pesticides is available. Therefore, 22 pesticides were chosen to be analysed with the selected methodology in GC-MS.

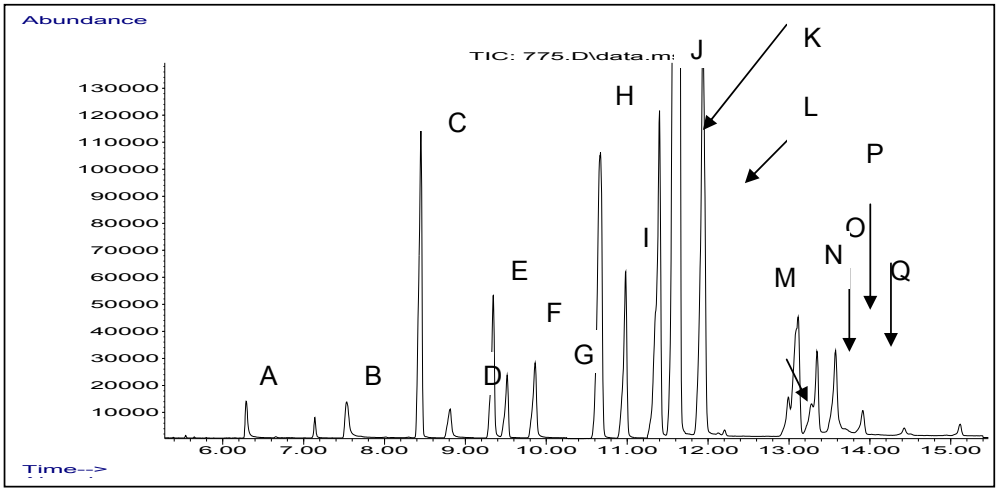


Figure 10: Total ion chromatogram of a mixture of target analytes at the MRL level prepared in toluene and injected in GC-MS, run time from 6.0 to 15.3 min. A–acephate (R_t-6.2 min); B–omethoate (R_t-7.53 min); C–phorate (R_t-8.45 min); D–dimethoate (R_t-8.81 min); E–propyzamide (R_t-9.34 min); F–diazinon (R_t-9.51 min); G–chlorothalonil (R_t-9.86 min); H–chlorpiriphos-methyl and vinclozolin (R_t-10.64, 10.67 min); I–metalaxyl (R_t-10.97 min), J–pirimiphos-methyl, methiocarb (R_t-11.39, 11.41 min), K–dichlofluanid, malathion (R_t-11.59, 11.60 min), L–ISTD, chlorpyriphos, parathion (R_t-11.60, 11.82, 11.83 min); M–tolylfluanid (R_t-12.99 min); N–mecarbam (R_t-13.11 min); N–folpet (R_t-13.28 min); O–procymidone (R_t-13.34 min); P–methidathion (R_t -13.57 min), Q–α-endosulfan (R_t-13.91 min).

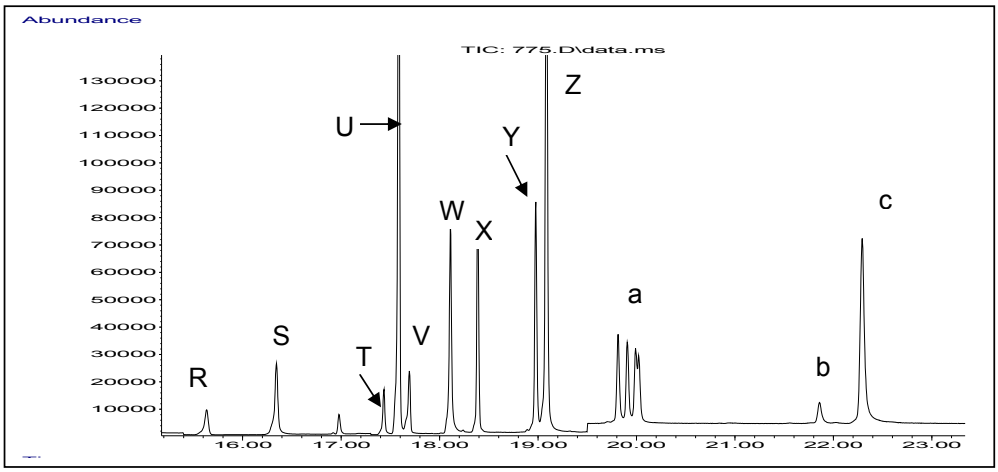


Figure 11: Total ion chromatogram of a mixture of target analytes at the MRL level prepared in toluene and injected in GC-MS, run time from 15.5 to 23 min. R–ß-endosulfan, imazalil (R_t-15.63 min), S–triazophos (R_t-16.34 min), T–iprodione (R_t-17.43 min), U–bromopropylate (R_t-17.58 min), V–dicofol (R_t-17.69 min), W–azinphos-methyl (R_t-18.11 min), X–lambda-cyhalotrin (R_t-18.39 min), Y–permethrin isomer-1 (R_t -18.97 min), Z–permethrin isomer 2 (R_t -19.08 min),a–α,β,γ cypermethrin (R_t-19.81,19.90,19.99 min),b–deltamethrin (R_t-21.86 min),c–azoxystrobin (R_t 22.29 min).

5.1.2 Calibration in solvent

Pesticide standards were prepared in toluene by adding the proper amount of stock solution (50 µg/g) or a dilution of this (1 µg/g) and ISTD solution (1000 ng/g in all standards) to achieve concentrations ranging from 10 ng/g to 6000 ng/g, (10, 100, 250, 600, 1000, 2500, 4500 and 6500 ng/g solvent).The MRLs for the initial target list of pesticides varies from 10 ng/g to 10000 ng/g sample.The average (3 injections for each standard) of the peak area ratio and the mass fraction ratio, obtained by a 2 µL spiltless injection in GC-MS were calculated and plotted (Figures 12-18).

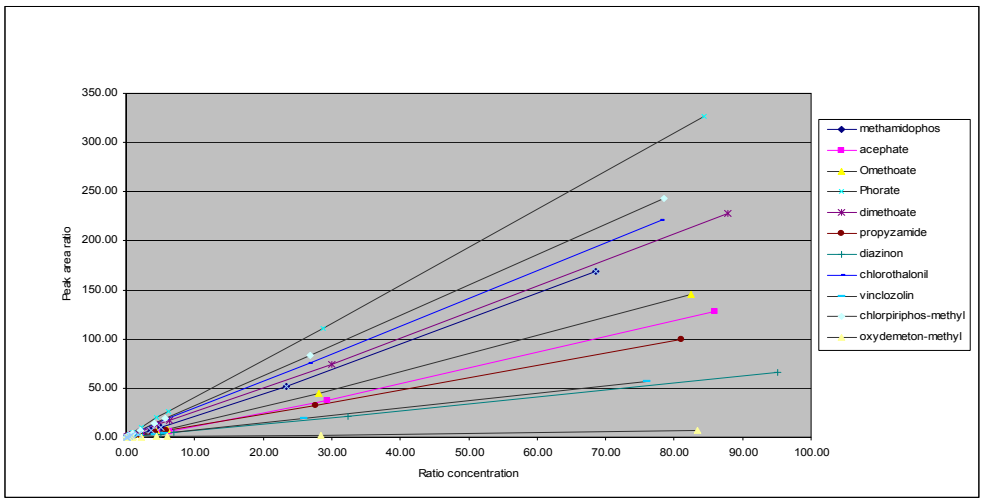


Figure 12: Calibration curves in solvent (toluene) - peak area ratio vs. concentration ratio, for methamidophos, acephate, omethoate, phorate, dimethoate, propyzamide, diazinon, chlorothalonil, vinclozolin, chlorpiriphos -methyl and oxydememton-methyl covering concentration range from standards 1 to 8.

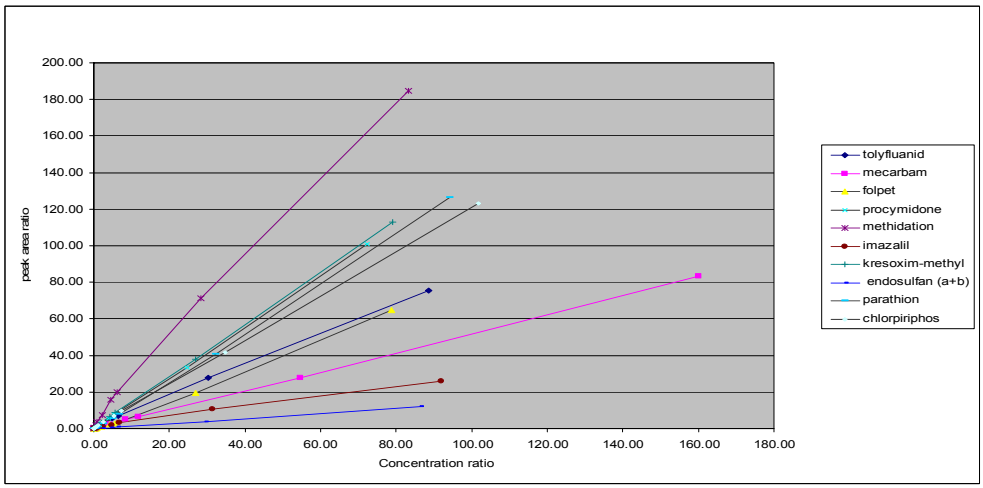


Figure 13: Calibration curves in solvent (toluene) - peak area-ratio vs. concentration ratio, for tolylfluanid, mecarbam, folpet, procymidone, methidathion, imazalil, kresoxim-methyl, endosulfan (a + b), parathion and chlorpiriphos, covering concentration range from standards 1 to 8.

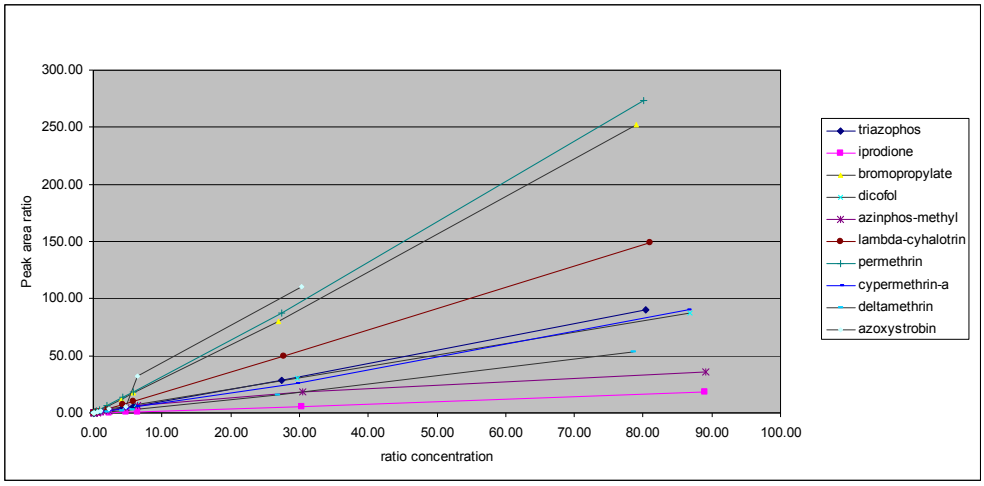


Figure 14: Calibration curves in solvent (toluene) - peak area ratio vs. concentration ratio, for triazophos, iprodione, bromopropylate, dicofol, azinphos -mehyl, lambda-cyhalotrin, permethrin, cypermethrin, deltamethrin and azoxystrobin, covering concentration range from standards 1 to 8.

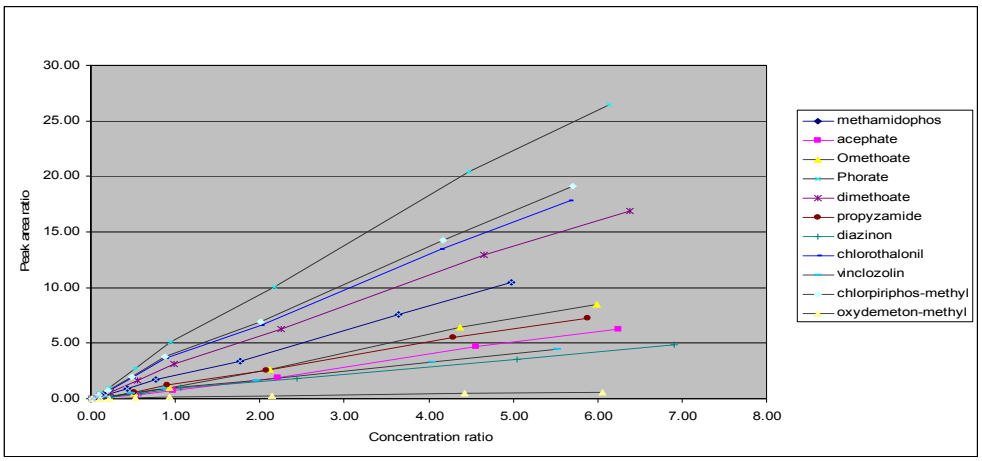


Figure 15: Calibration curves in solvent (toluene) - peak area ratio vs. concentration ratio for methamidophos, acephate, omethoate, phorate, dimethoate, propyzamide, diazinon, chlorothalonil, vinclozolin, chlorpiriphos -methyl and oxydememton-methyl,covering concentration range from standards 1 to 6.

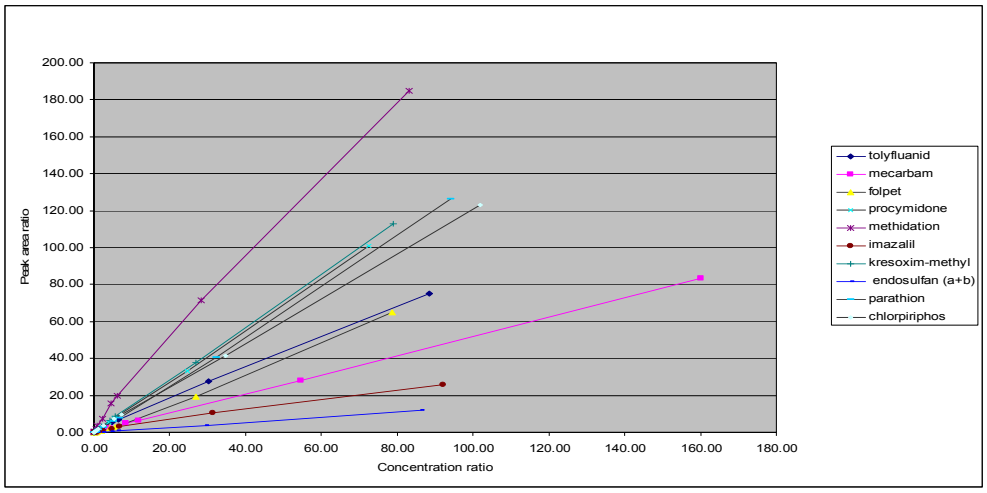


Figure 16: Calibration curves in solvent (toluene) - peak area ratio vs. concentration ratio, for tolylfluanid, mecarbam, folpet, procymidone, methidathion, imazalil, kresoxim-methyl, endosulfan (a + b), parathion and chlorpiriphos, covering concentration range from standards 1 to 6.

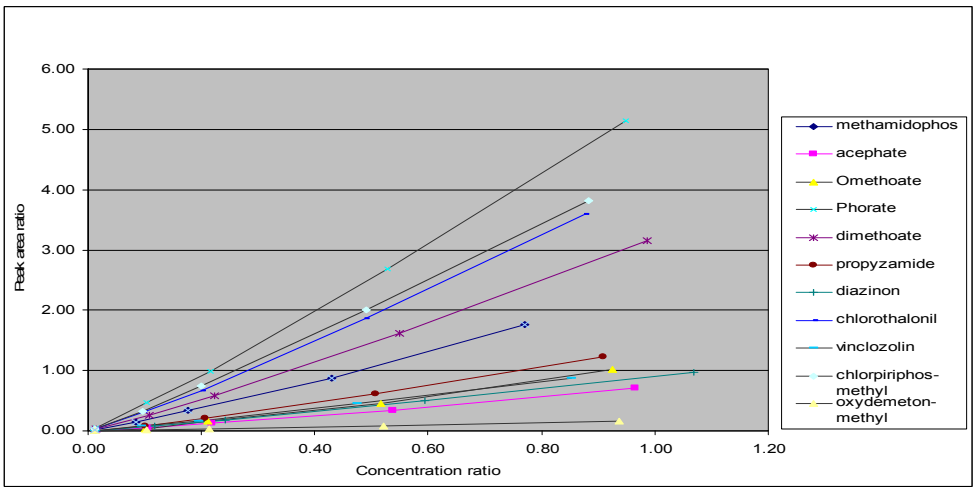


Figure 17: Calibration curves in solvent (toluene) - peak area ratio vs. concentration ratio for methamidophos, acephate, omethoate, phorate, dimethoate, propyzamide, diazinon, chlorothalonil, vinclozolin, chlorpiriphos-methyl and oxydememton-methyl, covering concentration range from standards 1 to 5.

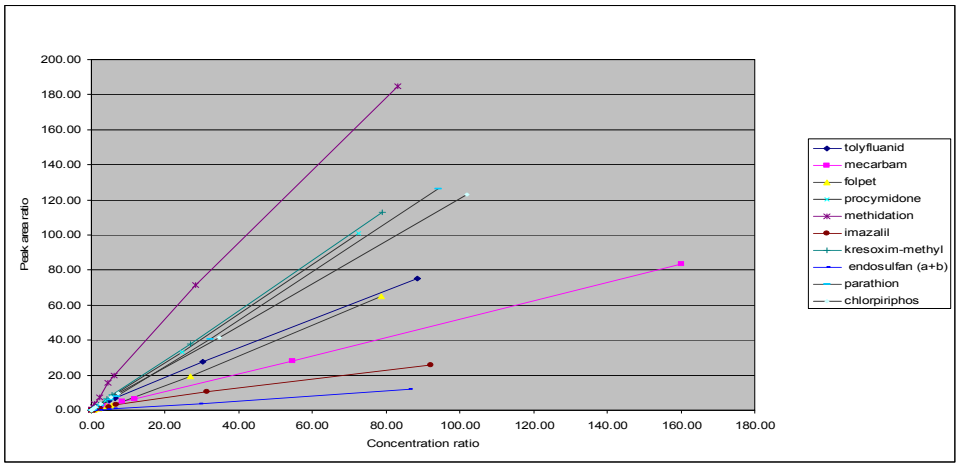


Figure 18: Calibration curves in solvent (toluene) - peak area ratio response vs concentration ratio, for tolylfluanid, mecarbam, folpet, procymidone, methidathion, imazalil, kresoxim-methyl, endosulfan (a + b), parathion and chlorpiriphos, covering concentration range from standards 1 to 5.

Also, for each injected standard response factors (R_f) were calculated by the following equation (1):

$$R_f = \frac{Area_{x \cdot extract}}{Area_{ISTD \cdot extract}} * \frac{w_{ISTD \cdot spk \cdot std}}{w_{x \cdot spk \cdot std}} * \frac{Mass_{ISTD \cdot spk \cdot std}}{Mass_{spk \cdot std \cdot x}} \qquad (1)$$

Where:

Area $_{x.extract}$ - peak area of analyte x in the extract

Area $_{ISTD.extract}$ - peak area of internal standard in the extract

w $_{ISTD.spk.std}$ - mass fraction of internal standard in the spiking standard

w $_{x.\ spk.\ std}$ - mass fraction of analyte x in the spiking standard

Mass $_{ISTD.spk.std}$ - mass of ISTD spiking standard

Mass $_{spk.std.\ x}$ - mass of spiking standard of analyte x

Although good chromatographic separation was achieved for the majority of the pesticides studied, high variability of Response Factors (R_f) for each analyte was observed across the entire concentration range of the standards (stds 1-8) and their values were as follows: 13 % < RSD R_f < 54 %). Response Factors were then evaluated within a smaller range of standard concentrations. It was observed that the R_f RSDs were in the range 10-40 % for the concentration range 10 to 2500 ng/g solvent (std 1-6) and 9-40 % for concentration range 10-1000 ng/g (std 1-5). For the range 2500 to 6500 ng/g solvent (std 6-8) the RSD's decreased substantially to 1-20 %, suggesting that R_f values are concentration dependent.

5.1.3 Matrix interferences

Apple based baby food was purchased on the local market (Geel, Belgium) and it was verified to be pesticide free (Fig. 19).

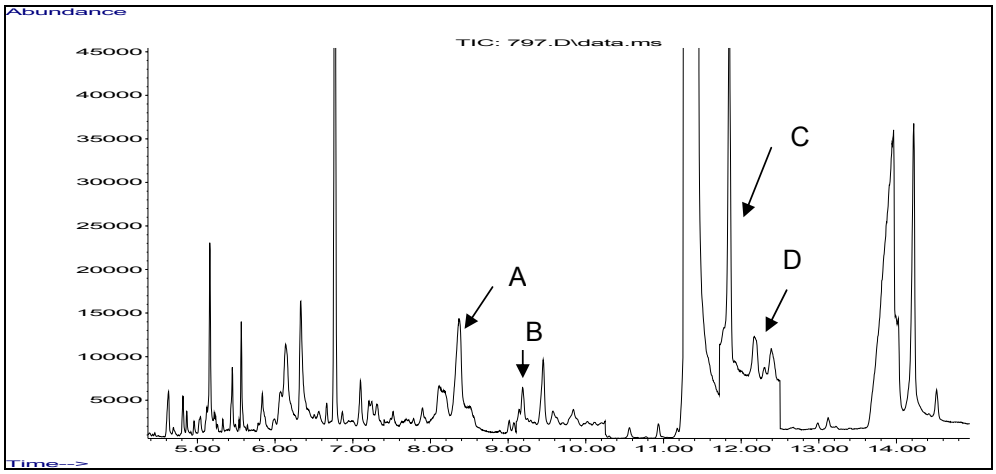


Figure 19: Total ion chromatogram of an extract of apple based baby food from R_t 4.80 to 15.00 min.
ISTD is represented by C (R_t-11.84 min). Interference peaks are A, B, D respectively at the R_t of phorate (8.42 min), propyzamide (9.14 min), chlorpiriphos, (11.93 min).

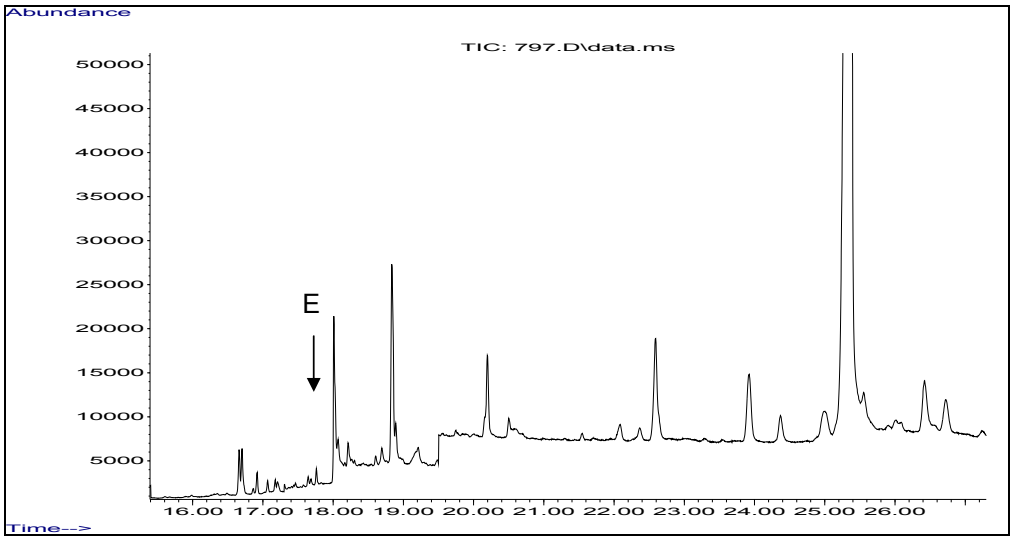


Figure 20: Total ion chromatogram of an extract of apple based baby food from 15.00 to 26.50 min. E represents an interference peak at the R_t of dicofol (Rt - 17.68 min).

In order to verify if equally good chromatographic separation could be obtained in the presence of matrix components, spiking of a blank sample was done at concentration levels of 5 ng/g sample ("spike low") and 600 ng/g sample ("spike high"). This was done by the addition of proper amounts of mixed stock solution (40 µg/g or 1 µg/g) and IS solution (5 µg/g) per each 10 g sample to be extracted by the QuEChERS method.
Good chromatographic separation was achieved (Figures 21-26).

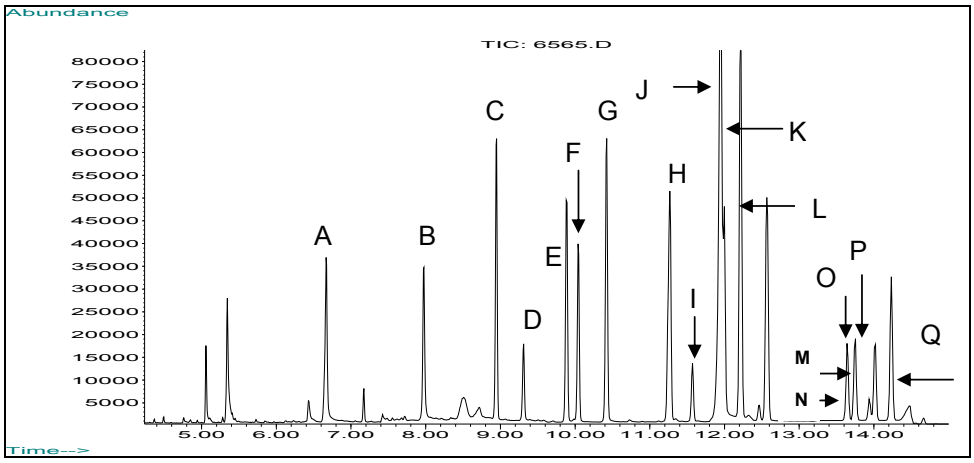


Figure 21: Total ion chromatogram (retention time from 4.50 to 15.00 min) of apple baby food spiked with a mixture of GC amenable pesticides at 600 ng/g. A – acephate (Rt-6.67 min), B – omethoate (Rt-7.98 min), C – phorate (Rt-8.95 min), D – dimethoate (Rt-9.31 min), E – propyzamide (Rt-9.89 min), F – diazinon (Rt-10.05 min), G – Chlorothalonil (Rt-10.42 min), H – chlorpyriphos-methyl and vinclozolin (Rt-11.27 min), I – metalaxyl (Rt-11.57 min), J – methiocarb and pirimiphos-methyl (11.95 and 12.00 min), K – malathion and dichlofluanid (Rt-12.22 min), L – chlorpyrifos and parathion (Rt 12.57 min) and ISTD (Rt-12.47 min), M – tolylfluanid (Rt -13.64 min), N – mecarbam (Rt- 13.75 min), O – folpet (Rt-13.94 min), P – procymidone (Rt-14.02 min), Q – methidathion (Rt -14.25 min).

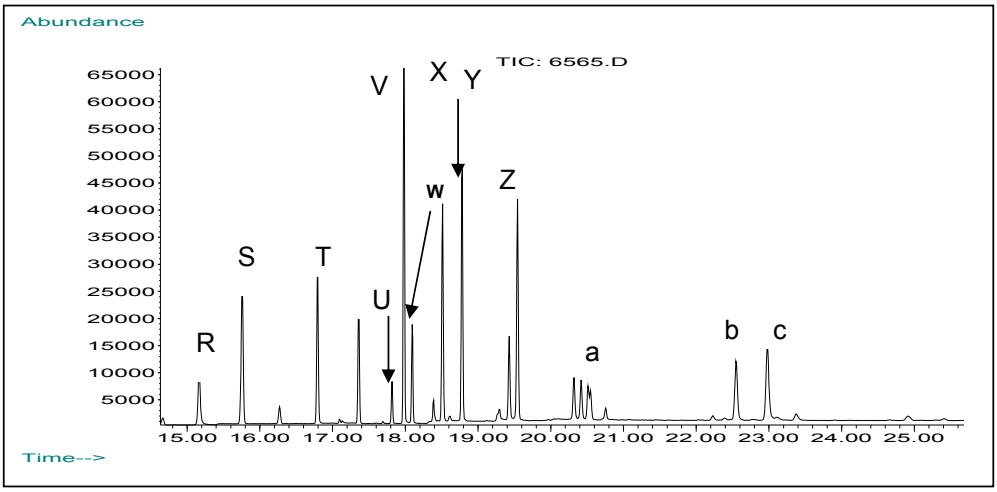


Figure 22: Total ion chromatogram (retention time from 15.00 to 25.00 min) of apple baby food spiked with a mixture of GC amenable pesticides at 600 ng/g of a mixture of GC amenable pesticides.

R – imazalil (R_t-15.17 min), S – kesoxim-methyl (R_t-15.76 min), T – triazophos (R_t- 16.80 min), U – iprodione (R_t-17.82 min), V – bromopropylate (R_t-17.98 min), W – dicofol (R_t-18.10 min), X – dicofol (R_t-18.10 min),Y – lambda-cyhalothrin (R_t-18.78 min), Z – permethrin isomer 1and 2 (R_t-9.43 and 19.55 min), a – α,β,γ cypermethrin (R_t- 20.32;20.42 and 20.52 min), b – deltamethrin (R_t-22.55 min), c – azoxystrobin (R_t-22.98 min).

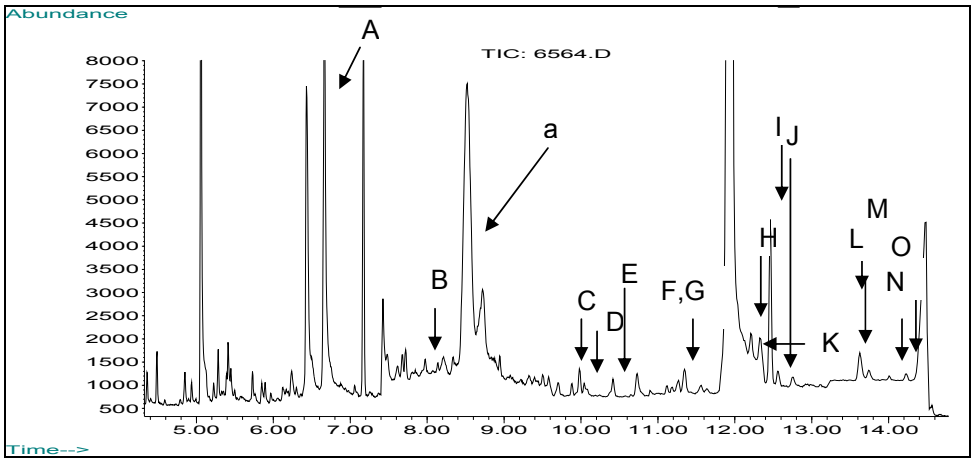


Figure 23: Total ion chromatogram (retention time from 4.50 to 15.00 min) of apple baby food spiked with a mixture of GC amenable pesticides at 5 ng/g.
A – acephate (R_t-6.67 min), B – omethoate (R_t-7.98 min), a – phorate (R_t-8.95 min), C – propyzamide (R_t-9.89 min), D – diazinon (R_t-10.05 min),
E – chlorothalonil (R_t-10.52 min), F – chlorpiriphos-methyl, vinclozolin(R_t-11.24, 111.27) G – metalaxyl (R_t-11.57 min), H – methiocarb (R_t-12.21 min),
K – dichlofluanid, malathion, pirimiphos-methyl (R_t-12.33min),I – ISTD (R_t-12.46 min), J – chlorpiriphos and parathion (R_t-12.57 min), L – tolylfluanid (R_t-13.64 min), M – mecarbam (R_t-13.73 min), N – procymidone (R_t-14.00 min), O – methidation (R_t-4.25 min).

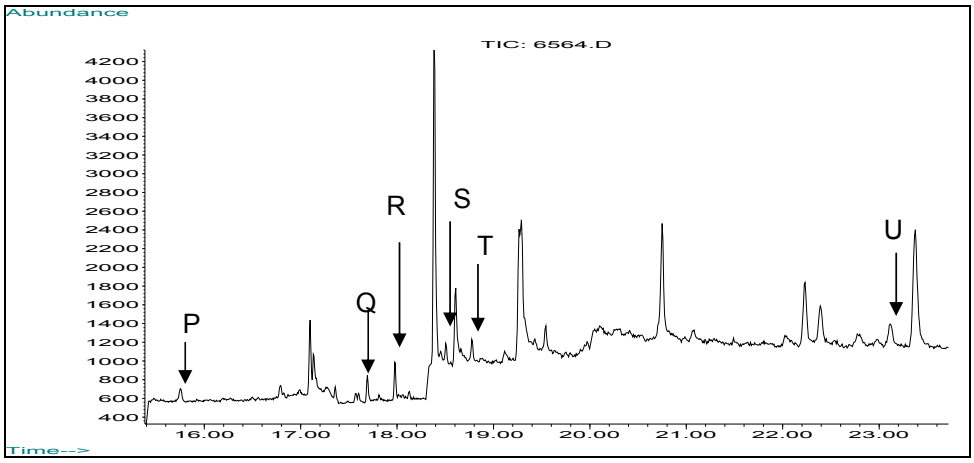


Figure 24: Total ion chromatogram of apple baby food spiked with 5 ng/g with a mixture of GC amenable pesticides 5 ng/g sample, of a mixture of GC-amenable pesticide analytes injected in GC-MS, run time from 14.50 to 23.50 min.

P – kresoxim-methyl (R_t-15.76 min), Q – iprodione (R_t-17.80 min),
R – Bromopropylate (R_t-17.98 min), S – azinphos-methyl (R_t-18.51 min),
T – lambda-cyhalotrin (R_t-18.78 min), U – azoxyxtrobin (R_t-22.98 min).

R_f in "spike low" were on average 10 * R_f in "spike high, and this observation confirms the known fact that the matrix enhancement effect is more pronounced at trace levels [17]. Standards injected in solvent resulted in lower and less reproducible responses. This is mentioned in the literature as a typical case of matrix induced response enhancement [17]. During injection of analytes in pure solvent, they block the active sites (mainly free silanol groups) in the inlet and consequently there is a lower transfer of these analytes to the GC column resulting in lower signal intensities and peak tailing. Instead, when a real sample is injected, co-extractives block the active sites in the inlet, increasing the transfer of target analytes to the GC column resulting in higher signals and better focused peaks. Compounds prone to matrix effects are either thermolabile or rather polar and they are typically capable of hydrogen bonding [9, 49].

According to literature findings [23-24], it was sought if this effect could be overcome to a certain extent by on-colum injection, which shortens the

interaction of analytes with active sites and minimises the contact surface area.

Two pesticides were tested, malathion (1) and chlorothalonil (2). Both of them are known to be prone to matrix effects.

(1) $y = 0.0067x-0.0325$; $R^2 = 0.9973$

(2) $y = 0.0232x-0.0861$; $R^2 = 0.9978$

Although it was possible to obtain calibration curves using 5 calibration points with good correlation coefficients, the R_f variability (RSD_{Rf}) over this calibration range was still high (14 and 12 % respectively), but lower when compared with the same standards injected in splitless mode (29 and 21 % for chlorothalonil and malathion respectively). Repeatibility of the injections was 3 % for on column injection and 5 % for splitless injection.

5.1.4 Extent of matrix effects

A study was conducted to evaluate whether all of the targeted pesticides were prone to the matrix effects described above and to what extent. Accurate measurements at the LOQ largely depend on this matrix effect. Additionally, it was important to determine if the presence of matrix affected the response functions. Generally, the matrix-induced response enhancement should be investigated when the response in matrix versus matrix-free (solvent) exceeds the upper limit of the mean recovery requirement for quantitative methods. The EU criteria for pesticide residue analysis require mean recovery within the range of 70-120 % for a pesticide concentration range of 10-100 ng/g and 70-110 % mean recoveries for concentrations of > 100 ng/g [43].

Calibration standards in solvent (5,10,20,50,100,250 and 500 ng/g) were prepared by adding a proper amount of calibration mixture working solution containing all target analytes dissolved in 0.1 % acetic acid in acetonitrile to the ISTD solution prepared in acetonitrile. Base sensitive pesticides were under the scope of the analysis and therefore acetic acid was added to prevent their degradation. Calibration solutions in matrix where prepared in the same way but blank extract of apple based baby food obtained with the QuEChERS methodology was used instead of pure acetonitrile. In all matrix-matched

standards the content of blank extract was 50 % of the total mass of the solution and the analyte content ranged from 5 ng/g to 500 ng/g, (5,10,20,50,100,250 and 500 ng/total g solution). Peak areas and concentrations were normalized to the ISTD. The ISTD content in all standards was 100 ng/g. TPP $_{\text{ISTD solution}}$, prepared in 1 % acetic acid in acetonitrile, was added to all final extracts and standards alike, in solvent to isolate the GC step variability.

This study demonstrated that the addition of 50 % blank extract in calibration standards was sufficient to induce higher responses in these standards compared to the standards prepared in solvent only (matrix free), for the same concentration range. Some examples to illustrate the effect of matrix on the calibration functions are given in Figures 25-30. The calibration curves are based on peak areas and mass fraction values normalized to the ISTD (d_{10}-parathion). Each data point corresponds to the average value of 3 injections in the GC-MS system in splitless mode.

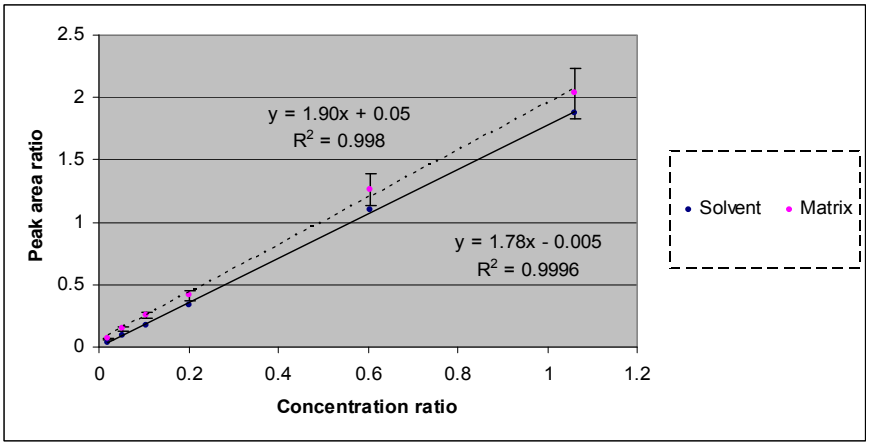


Figure 25: Calibration curve of permethrin in solvent (0.1 % Hac in acetonitrile) versus calibration curve of permethrin in matrix (QuEChERS extract in acetonitrile).

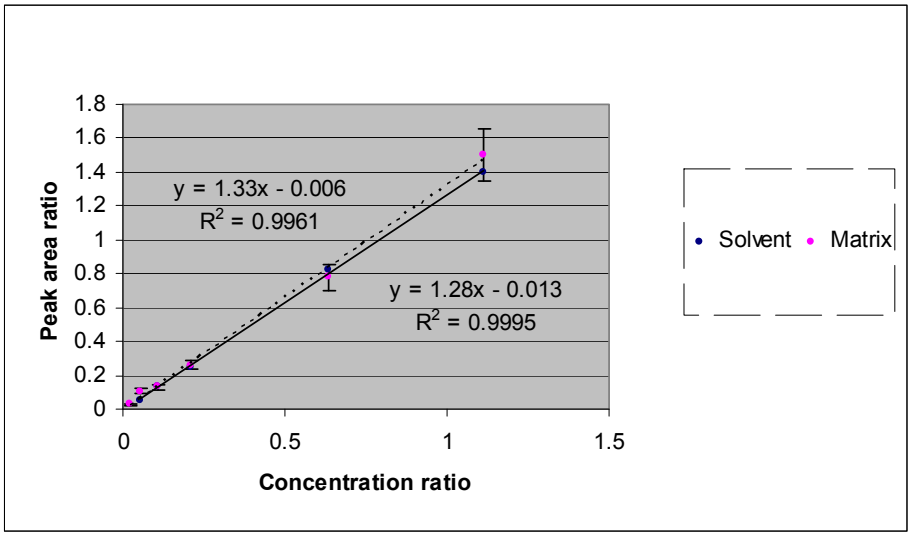


Figure 26: Calibration curve of parathion in solvent (0.1 % Hac in acetonitrile) versus calibration curve of permethrin in matrix (QuEChERS extract in acetonitrile).

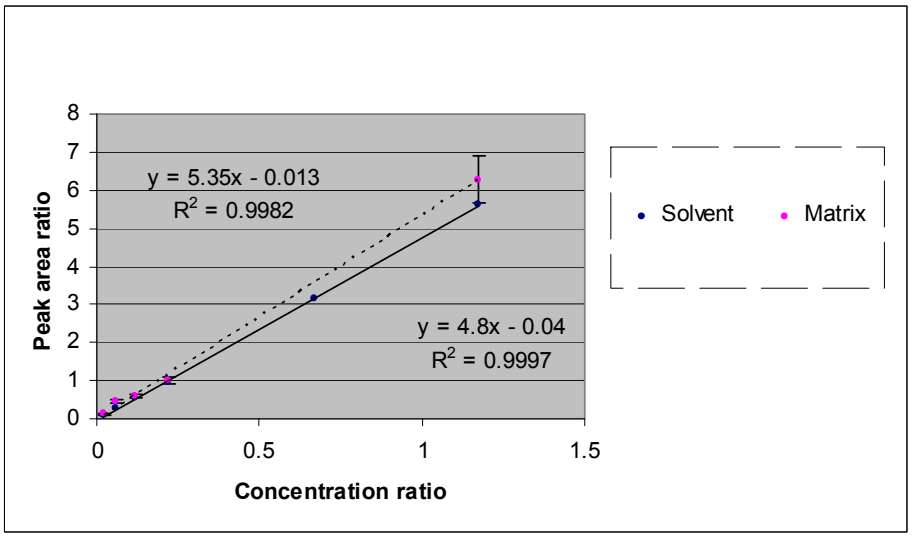


Figure 27: Calibration curve of bromopropylate in solvent (0.1 % Hac in acetonitrile) versus calibration curve of bromopropylate in matrix (QuEChERS extract in acetonitrile).

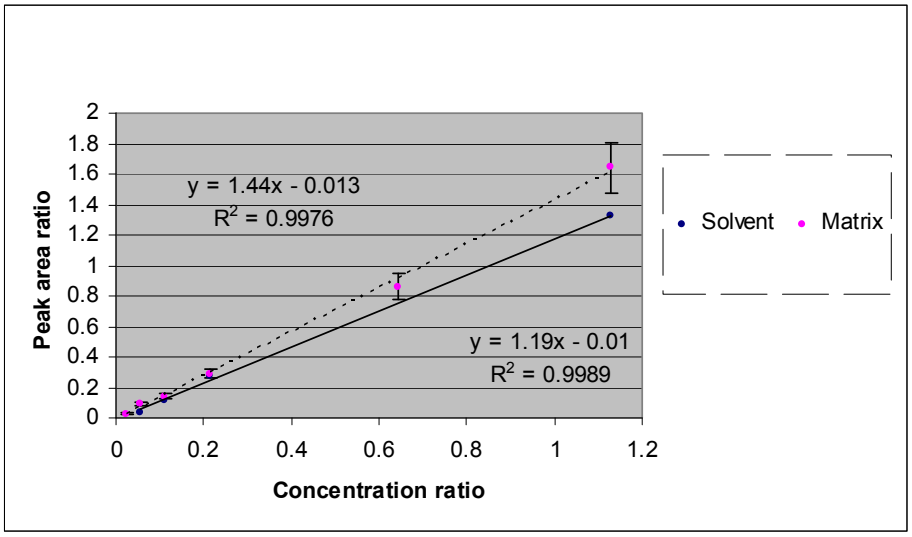


Figure 28: Calibration curve of tolylfluanid (0.1 % Hac in acetonitrile) versus calibration curve of tolylfluanid in matrix (QuEChERS extract in acetonitrile).

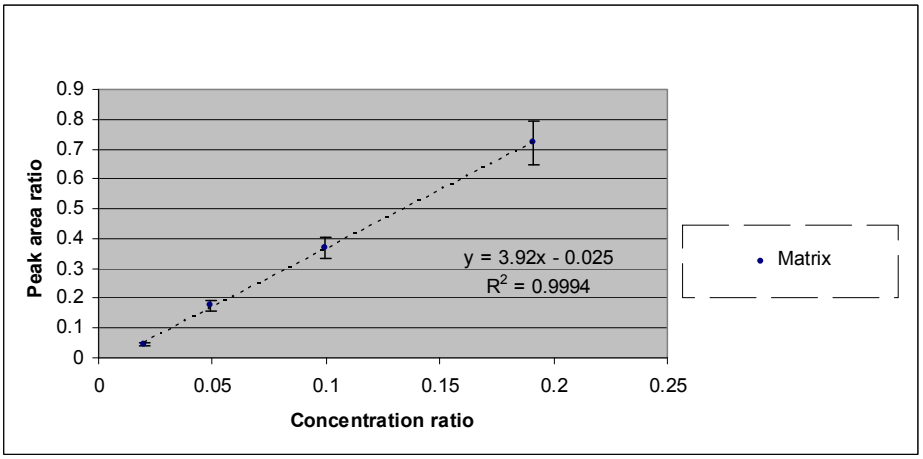


Figure 29: Calibration curve of chlorothalonil in matrix (QuEChERS extract in acetonitrile).

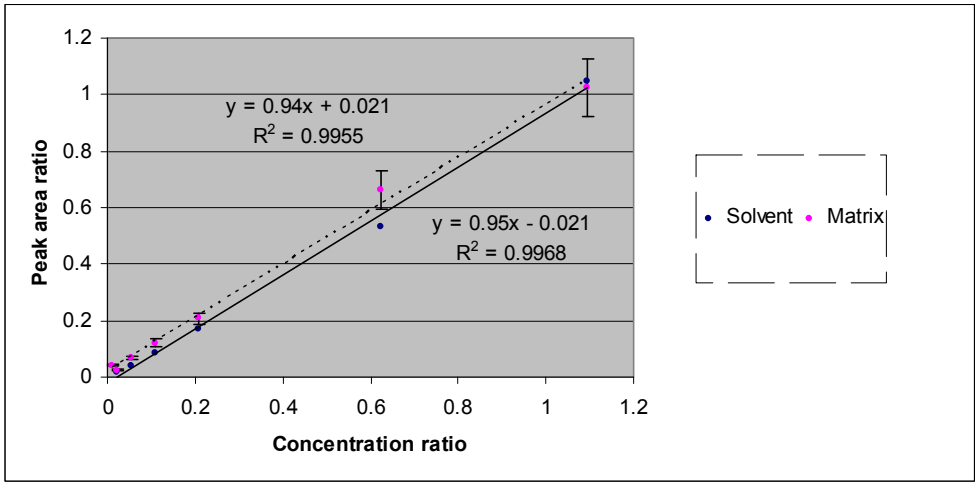


Figure 30: Calibration curve of iprodione in solvent (0.1 % Hac in acetonitrile)) versus calibration curve of iprodione in matrix (QuEChERS extract in acetonitrile).

For comparison purposes, series of pesticides solutions in acetonitrile and matrix extracts were injected in GC-MS. The pesticides for which the calibration curves are shown above, were selected to represent different analyte susceptibility to matrix-induced response enhancement in apple ased baby food. Visual inspection of the calibration curves in solvent/matrix was performed rather than a statistical study approach. A tolerance limit of ±10 of the matrix response in relation to the solvent response was set, indicating no significant difference between analyte responses in solvent and in matrix. This value of 0.90 - 1.10 of the ratio of matrix/solvent response factors represents a more stringent value then the recovery requirements (70 - 120 %) for pesticide levels <100 ng/g, at which the enhancement effect is known to be larger as compared to higher analyte concentrations. Permethrin, bromopropylate and iprodione were moderately susceptible to matrix-enhancement effects (for these the ratio of matrix/solvent response factors are within the tolerance limit for higher concentration levels but not in the lower concentration range), followed by tolylfluanid and chlorothalonil; the latter showed to be very prone to matrix effects, since it was not possible to construct a calibration in solvent only. With regard to parathion, known to be prone to matrix effect [49] and for which the correspondent labelled compound was used for quantification, the data

suggested that an isotopically labelled analogue used for calibration in IDMS was capable of fully compensating the matrix effect, and rendered calibration in solvent possible. This aspect will be further addressed in the development of an IDMS methodology for the quantification of pesticides in carrot matrix.

Calibration curves of pesticides in solvent resulted in curves with lower values for slopes and/or intercepts, as compared to the same in matrix (Table 8). This is a typical manifestation of the matrix-induced response enhancement effect, which would lead to significantly overestimated results in the analysed sample if solvent standards were used for calibration of a sample in matrix.

Table 8: Slopes (b) and intercepts (a) of the calibration curves obtained in pure solvent and matrix with and without the addition of AP for some target pesticides.

Pesticide	calibration in matrix		calibration in solvent		calibration in matrix with AP		calibration in solvent with AP	
	a	b	a	b	a	b	a	b
bromopropylate	0.02	5.32	-0.04	4.80	0.07	4.63	0.03	3.77
chlorothalonil	0.03	3.92	_	_	0.04	3.13	3.39	0.06
chlorpyrifos	-0.01	3.02	-0.11	3.98	0.04	2.04	0.06	1.71
chlorpyrifos-methyl	0.07	7.53	-0.16	6.12	0.12	6.17	0.33	4.90
diazinon	-0.03	2.19	-0.04	2.47	1.68	0.01	0.10	1.35
iprodione	0.02	0.94	-0.02	0.95	0.01	1.06	0.03	0.78
lambda-cyhalotrin	0.02	2.26	-0.01	2.38	0.02	2.26	0.01	1.96
metalaxyl	-0.02	2.77	-0.03	3.01	0.03	2.39	0.09	2.13
parathion	-0.01	1.33	-0.01	1.28	0.01	1.23	0.01	1.46
permethrin	0.06	1.89	-0.01	1.79	0.04	2.07	0.02	1.27
pirimiphos-methyl	-0.08	6.01	-0.14	6.12	0.1	4.1	0.19	3.68
propyzamide	-0.05	5.17	-0.04	5.1	-0.02	4.91	0.2	4.04

It is important to note, that discrepancies in describing the matrix effect make the comparison and utilization of published results very difficult. The use of matrix-matched calibration requires substancial work; therefore, it must be properly justified. Soboleva et al [50], in an attempt to find suitable methods to

express the magnitude and statistically evaluate the matrix effect, investigated the influence of the matrix effect on the response of various pesticides as a function of (I) the analyte concentration, (II) the matrix content of the calibration solution, and (III) different types of matrices and instrument operation conditions. The matrix effect is usually expressed by dividing the analyte responses (area or peak height) in the matrix-matched solution by the response in neat solvent and multiplying by 100 %.

In order to statistically evaluate the significance of the effect, confidence intervals of the analyte response based on the matrix matched calibration can be calculated. Matrix effect is considered significant when the analyte concentration predicted based on neat standard calibration is outside the confidence interval.This however has limitations. If there is evident curvature (goodness-of-fit of the calibration curve) in the calibration plot or it does not meet repeatability criteria, the test might fail because of too wide confidence interval. Therefore a detailed study of the matrix effect during full method validation is worthwhile because precise quantification of the analytes is required. To estimate the goodness-of-fit of the calibration plots, the correlation coefficient (r) is commonly used, to measure the degree of linear association between two variables, but it had been proven [51] that a r value very close to unity might also be obtained for a curved relationship. Other statistical tests like lack-of-fit and Mandel's fitting test, which use F-tests for statistical significance, appear to be more suitable for the validation of the linear calibration model. In addition, the evaluation of the residual plot and calculating the relative standard deviation of residuals are appropriate indicators of the linearity of the calibration function. The RSD of the residuals should be < 10 % for a truly linear calibration function. This aspect will be further addressed using matrix-matched calibration in carrot matrix in order to evaluate the linearity of the calibration curve and determine the working range.

5.1.5 Analyte Protectants (AP)

Since an effective elimination of the sources of the matrix induced response enhancement is not likely to occur in practice, analysts are required to

compensate for the effect using alternative calibration methods. The current compensation approaches include the use of: (I) matrix-matched standards, (II) standard addition method, and (III) isotopically labelled internal standards [49]. All of these techniques require extra labor and costs; moreover, they may still lead to quantitation inaccuracies because the extent of the effect depends on analyte concentration and matrix composition. It is known to be larger at lower analyte concentrations as compared to higher analyte concentrations. This is the reason why it was not possible to obtain constant response factors for the same analyte over the whole range of calibration standard concentrations in solvent or in matrix (section 5.3 and 5.4).

In order to investigate alternative approaches for pesticide quantification, the same series of standards in solvent and in blank matrix were injected with the addition of analyte protectants (APs). This was done by adding a high concentration of APs with multiple hydroxyl groups to sample extracts and calibrations standards in solvent alike. In general, hydrogen–bonding capability and volatility (to achieve a wide retention time coverage) of the AP compounds were found to be the most important factors in the enhancement effect [21].

Analyte protectants have been shown to provide accurate results, better peak shape, lower LOQ and also in providing increased rugedness of the analysis by continuing to work even in a very dirty GC system [21]. Another potential problem in routine GC analysis of pesticide residues is the gradual accumulation of non volatile matrix components in the GC system, resulting in formation of new active sites and gradual decrease in analyte responses. This effect called “matrix-induced diminishment effect” impacts ruggedness (e.g. long-term repeatability of peak responses, shape and retention times). It is another important factor to be taken into consideration in routine analysis of pesticides.

A mixture of ethylglycerol, gulonolactone, and sorbitol (at 10, 1, and 1 mg/mL, respectively) in the final sample extracts and matrix-free standards alike was found to be most effective in minimizing losses of susceptible analytes [21], and was employed in the experiments. Ideally, the analyte protectants should provide the same degree of protection (signal enhancement) regardless of whether the solution contains matrix components or not.

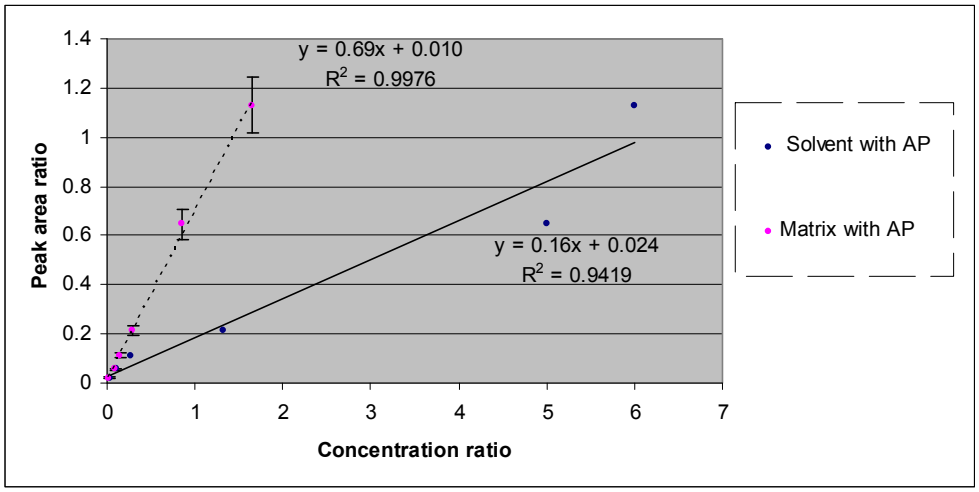


Figure 31: Calibration curve of permethrin in solvent containing AP (0.1 % Hac in acetonitrile) versus calibration curve of permethrin in matrix containing AP (QuEChERS extract in acetonitrile).

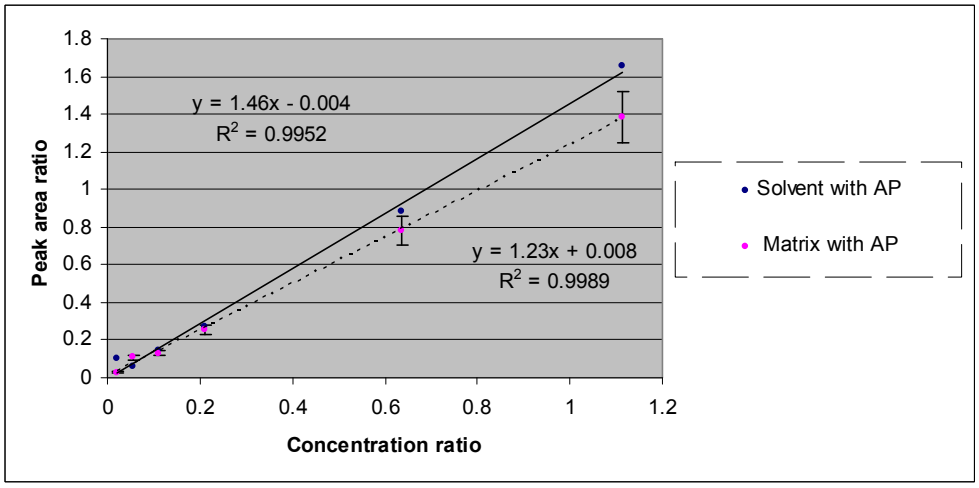


Figure 32: Calibration curve of parathion in solvent containing AP (0.1 % Hac in acetonitrile) versus calibration curve of parathion in matrix containing AP (QuEChERS extract in acetonitrile).

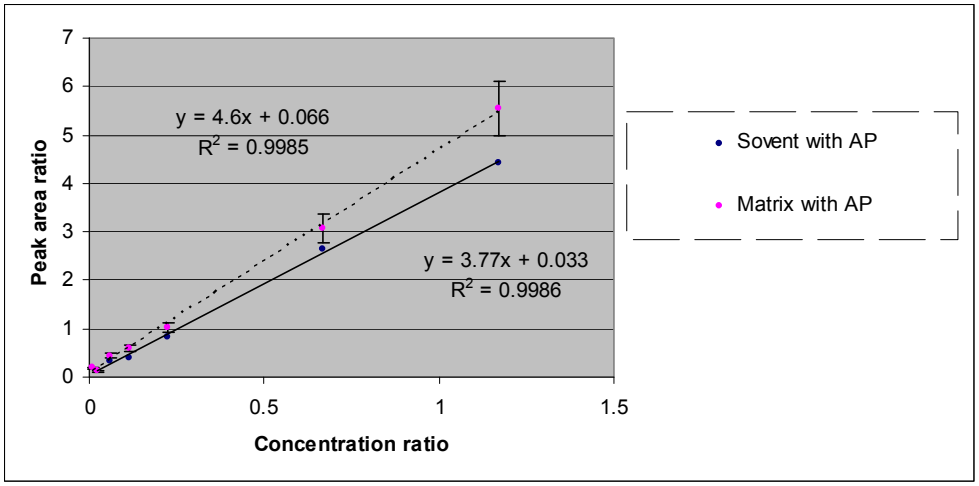


Figure 33: Calibration curve of bromopropylate in solvent containing AP (0.1 % Hac in acetonitrile) versus calibration curve of bromopropylate in matrix containing AP (QuEChERS extract in acetonitrile).

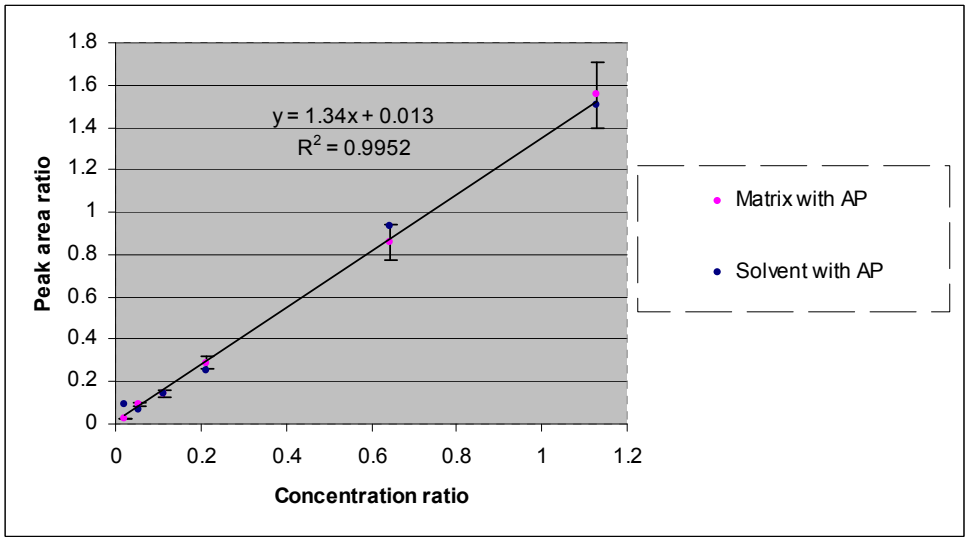


Figure 34: Calibration curves of tolylfluanid in solvent containing AP (0.1 % Hac in acetonitrile) versus calibration curve of tolylfluanid in matrix containing AP (QuEChERS extract in acetonitrile). Note: the two curves are identical.

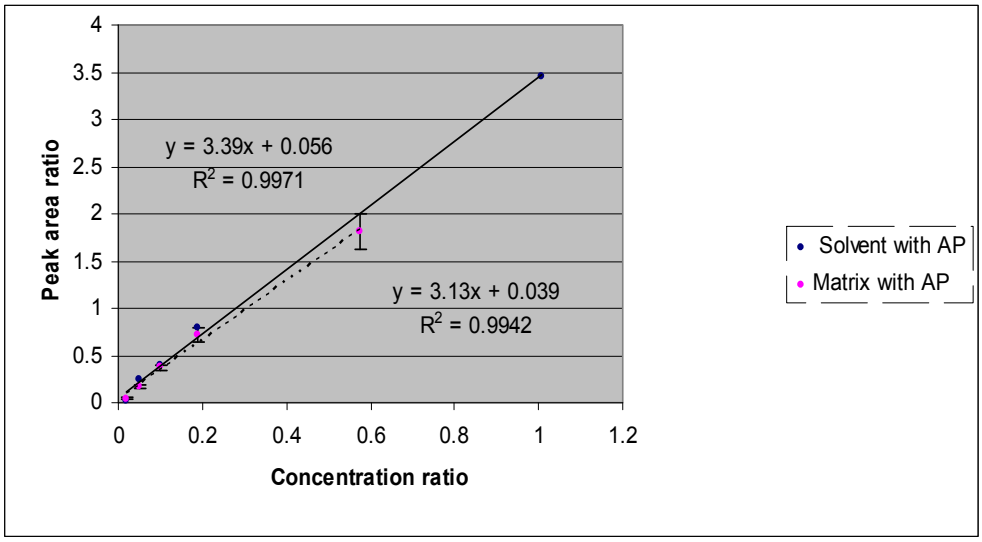


Figure 35: Calibration curve of chlorothalonil in solvent containing AP (0.1 % Hac in acetonitrile) versus calibration curve of chlorothalonil in matrix containing AP (QuEChERS extract in acetonitrile).

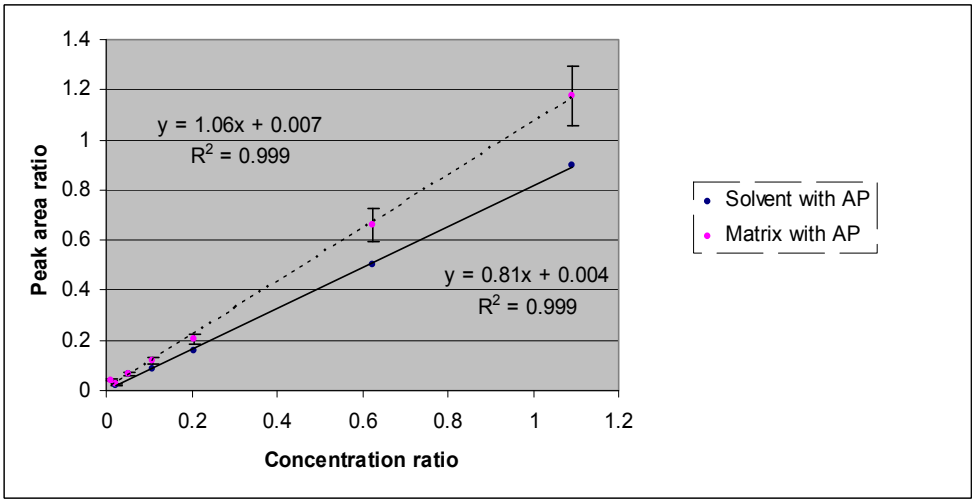


Figure 36: Calibration curve of iprodione in solvent containing AP (0.1 % HaC in acetonitrile) versus calibration curve of iprodione in matrix containing AP (QuEChERS extract in acetonitrile).

The assumption that equalization between calibrations obtained in matrix versus solvent could be obtained ($a_{\text{solvent with AP}}/a_{\text{matrix with AP}}$ or $b_{\text{solvent with AP}}/b_{\text{matrix with AP}}$ = unity) when APs were added, was not observed for most of the target analytes, except tolylfluanid and chlorothalonil (Fig. 31-36).

The addition of APs to compensate for matrix effects in pesticide analysis added one more variable to the analytical process and did not show the expected equalization effect between calibration in solvent and in matrix for most of the target analytes. Also, in this case standards can only be injected in acetonitrile, as the mixture of APs (ethylglycerol, gulonolactone, and sorbitol) is not soluble in toluene; toluene gives better sensitivity in GC and sensitivity is a key element in the present study to attain the MRLs.

For regulatory enforcement of pesticide residues limits in foods, the guidelines for residue monitoring in the European Union (EU) require the use of matrix-matched standards or an alternative approach that provides equivalent or superior accuracy [26].

In the case of matrix matched standards and if a blank material is available full compensation of matrix effects occurs. Isotopically labelled internal standards are very well suited to this purpose, but their use is rather expensive, especially for multiresidue analysis, where a separate internal standard for each analyte is required. Moreover, isotopically labelled pesticides are in many cases unavailable or of prohibitive cost.

Therefore, the use of the laborious matrix matching approach appeared unavoidable and was used in all further experiments.

5.1.6 LOQ/LOD

Series of matrix-matched standards (prepared in blank extract of apple based baby food) at the following levels (1/4 MRL, 1/5 MRL, 1/6 MRL and 1/10 MRL) were prepared by adding a proper amount of a mixed pesticide solution (containing all GC amenable analytes) and ISTD (d_{10}–parathion) to a blank extracted material. This was made to verify if the chosen analytical method could detect and quantify target analytes at ¼ MRL. It resulted in 26 analytes being detected and quantified at ¼ MRL by GC- MS.

It is important to note that varying the matrix type this detection and quantification parameters may change.

5.1.7 In-House Method Validation

The method was fully validated for 21 EU priority pesticides in an apple/pear based baby food, namely azinphos-methyl, azoxystrobin, bromopropylate, chlorpyriphos, chlorpyriphos-methyl, cypermethrin, diazinon, endosulfan (α+β), iprodione, lambda-cyhalotrin, malathion, mecarbam, metalaxyl, parathion, permethrin, phorate, pirimiphos-methyl, procymidone, propyzamide, triazophos and vinclozolin at mass fraction values corresponding to the MRL and 0.5 MRL for each pesticide. It was not possible to validate this methodology for the analysis of deltamethrin. The in-house validation was done according to internationally agreed protocols [42, 43, 44].

The analyses were performed with the selected analytical procedure described in Materials and Methods. IDMS was used for the quantification of certain analytes (parathion, malathion, phorate and cypermethrin). For the remaining pesticides where no isotopically labeled standards were available the one eluting closest served as ISTD; TPP was used as a "syringe" ISTD to isolate the GC analytical step variability. This method is supposed to correct for losses during extraction, clean-up and to compensate instrument variations. A crucial assumption in IDMS is that the analyte and the isotope spike are in thermodynamic equilibrium.

5.1.7.1 Performance criteria

The method was tested in order to fufill the performance criteria listed in Table 9.

Table 9: Target criterion and specification for the in-house method validation.

Criterion	Specification
Calibration curves	The uncertainty for an interpolated analyte quantity value using the matrix matched calibration functions should be less than 5 % at the MRL
Instrumental LOQ / LOD	The analytes should be accurately detected and quantified at ¼ MRL. S/N>3 for detection and S/N>10 for quantification
Linearity and working range	Correlation coefficients between 0.988 to 0.999 and working range between 0.25 MRL and 2 MRL of each analyte (in exceptional cases the working range reduced to 4 calibration levels)
Identity	Deviation of relative retention time of a target analyte in a sample <1 % from target analyte in standard solution. Likewise, deviation of ions ratios (quantitative, qualitative and confirmation ions) <10 %.
Repeatability	Less than 10 % RSD at 0.5 MRL and MRL (using ANOVA evaluation)
Reproducibility	Less than 10 % RSD at 0.5 MRL and MRL (using ANOVA evaluation)
Recovery	Mean recovery between 70 to 110 %
Robustness	Minor changes in the concentration of acetic acid in the extraction solvent (0 %, 0.8 %, 1 % and 1.2 %), should have no influence on recovery (using ANOVA evaluation).
Stability of extracts	Stored extracts shall remain stable at -20 °C (90 -115 %, when compared to day of preparation)

5.1.7.2 LOD/ LOQ

Since it was already verified during the method optimisation phase that all target analytes could be detected (signal/noise ratio>3) and quantified (S/N>10) at 1/6 MRL, it was assumed that LOQ is significantly below the desired working range (1/4 MRL). Therefore, no further efforts to a precise determination at the LOQ value were made. The target analytes can be detected and quantified in the range ¼ MRL to 2 MRL.

5.1.7.3 Calibration

Calibration functions for each analyte were obtained by plotting the peak area ratio $PR^{cal\ mix}$ of each calibration level against the mass fraction ratio of the standard solution.

A complete list of the calibration curves (Y=a + b X) obtained in the validation experiments is presented in Table 10. Correlation coefficients were between 0.988 to 0.999 depending on the analyte. Visual inspection (equal distribution of points on the calibration line, narrow concentration range, homogeneity of variances) and regression parameters of the curve (e.g. high r^2) underpinned linearity of the calibration models.

Table 10: Slopes (b) and Y-intercepts (a) for the linear calibration curves of the target analytes obtained in matrix extract.

Pesticide	Calibration in matrix		
	a	b	r^2
azinphos-methyl	4.01 E-3	4.53 E-1	0.994
azoxystrobin	4.84 E-2	1.63	0.988
bromopropylate	4.30 E-3	1.13	0.999
chlorpiriphos	1.48 E-2	8.44 E-1	0.997
chlorpiriphos-methyl	6.66E-2	4.82	0.997
cypermethrin	9.75 E-2	8.49 E-1	0.997
diazinon	2.55 E-3	1.27	0.997
endosulfan (α+β)	1.40 E-3	9.13 E-2	0.998
iprodione	1.79 E-3	3.76 E-1	0.996
lambda-cyhalothrin	4.72 E-3	7.26E-1	0.998
malathion	2.44 E-1	8.58 E-1	0.998
mecarbam	1.41 E-3	4.50 E-1	0.999
metalaxyl	6.31 E-2	2.03	0.997
parathion	-3.54 E-3	9.30 E-1	0.998
permethrin	-2.57 E-3	1.72	0.991
phorate	1.46 E-2	9.8E-1	0.996
pirimiphos-methyl	2.84 E-2	9.54E-1	0.998
procymidone	9.82 E-3	1.11	0.998
propyzamide	-2,8 E-2	3,02	0.997
triazophos	2.53 E-2	9.13 E-1	0.990
vinclozolin	1.77E-2	1.33	0.997

Calibration curves of all analytes resulted in linear curves within the working range of 0.25 MRL to 2 MRL level of each pesticide. In excepcional cases the working range was reduced to four calibration levels, and it was found out in subsequent analysis that as the GC system gets dirty, for some analytes like lambda-cyhalotrin, a second order calibration curve better met the need of the calibration. These facts altogether suggest that calibration of the GC system must be properly evaluated and routinely done before each analytical run to meet the repeatability criteria.

Because of the uncertainty in the values for the slope and intercept, there is a corresponding uncertainty in the best straight line that is fitted to the data. The formal calculation of the uncertainty in an analyte quantity value, interpolated from a regression line, uses the following mathemathical expression (2):

$$Sx = (rsd/a^{cal})\sqrt{1/N + 1/n + \frac{(yo - y)^2}{a^{cal\,2}(n-1)S^2(x)}} \qquad (2)$$

Where:

Sx-standard uncertainty of the interpolated analyte quantity value for the sample being analysed

rsd-residual standard deviation

a ^{cal}-slope of the regression line

N-number of replicate measurements made on sample being analysed

n-number of points in the regression line

Yo-mean value of the instrument signal for the L replicates measurements of the sample being analysed

Y-mean value of the instrument signal for the n calibration points.

S(x)-standard deviation of the x data (analyte quantity values) for the n points of regression line

$$rsd = Sy\sqrt{((n-1)/(n-2))(1-r^2)} \qquad (3)$$

rsd-residual standard deviation

Sy-standard deviation of the measured instrument signals (y values)

r-correlation coefficient of the regression line

n-number of points in the regression line

Standard and relative standard uncertainties for an interpolated analyte quantity value at the MRL value are presented in Tables 11 and 12.

Table 11: Standard uncertainties for the 21 analytes under study.

Analyte	Standard uncertainty (ng/g sample)
phorate	0.015
propyzamide	0.008
diazinon	0.004
vinclozolin	0.006
chlorpiriphos-methyl	0.042
metalaxyl	0.042
pirimiphos-methyl	0.027
malathion	2.875
chlorpiriphos	0.024
parathion	0.018
mecarbam	0.014
procymidone	0.016
endosulfan	0.009
triazophos	0.020
iprodione	0.002
bromopropylate	0.002
azinphos-methyl	0.007
lambda cyhalotrin	0.001
permethrin	0.008
cypermethrin	0.020

Table 12: Simulation of relative standard uncertainty of an interpolated analyte quantity (at the MRL value (sample) for each pesticide under study.

Pesticide	MRL value (ng/g sample)	relative standard uncertainty (%)
azinphos-methyl	42.94	0.016 %
azoxystrobin	46.88	0.006 %
bromopropylate	51.38	0.004 %
chlorpyriphos	50.85	0.047%
chlorpyriphos-methyl	47.54	0.088%
cypermethrin	47.29	0.042%
diazinon	10.44	0.038%
endosulfan a+b	50.33	0.017%
iprodione	18.83	0.011%
lambda-cyhalotrin	19.65	0.005%
malathion	493.09	0.583%
mecarbam	48.42	0.029%
metalaxyl	46.76	0.089%
parathion	49.29	0.036%
permethrin	50.31	0.016%
phorate	49.07	0.031%
pirimiphos-methyl	48.09	0.056%
procymidone	20.33	0.078%
propyzamide	20.77	0.038%
triazophos	18.73	0.107%
vinclozolin	48.47	0.012 %

5.1.7.4 Recoveries

Spiked samples were extracted with the QuEChERS method on five different days to determine recoveries of each analyte. Also on each day a set of calibration curves was obtained for each analyte using matrix matched calibration by means of spiking the blank extract.

Recoveries were calculated using the calibration curve obtained on the same day.

Calibration curves were compared between days by visual inspection, rather then statistically. A summary of the recovery results during 5 days obtained for the 21 pesticides, for two fortifications levels (0.5 MRL and MRL) is given in tables 13-14.

The recovery (%) was obtained by the average of two injections, and 3 replicates for each concentration level (a replicate denotes an independent sample with similar concentration). On one occasion (day 3) there was an error in the preparation of the MRL standard. Two replicates were affected and therefore it was not possible to calculate an average recovery. For day 3 and 5 of 0.5 MRL and day 5 of MRL the averages were obtained with two replicates, due to experimental deficiencies in the solvent exchange step of the method for one replicate.

Table 13: Recovery data (%) and RSDs, for the 21 pesticides at the fortification level of 0.5 MRL (ng/g sample).

	Fortification level				
	0.5 MRL	**0.5 MRL**	**0.5 MRL**	**0.5 MRL**	**0.5 MRL**
Pesticide	**day 1**	**day 2**	**day 3**	**day 4**	**day 5**
azinphos-methyl	89.4±11.3 %	78.8± 7.8 %	**116.6±3.17 %**	88.1±3.7 %	94.5±6.7 %
azoxystrobin	104.3± 2.3 %	98.9± 2.6 %	107.4±3.4 %	95.±2.1 %	95.9±1.7 %
bromopropylate	90.8±14.6 %	100.3± 1.1 %	95.7±1.3 %	98.0±1.3 %	103±3.9 %
chlorpyriphos	96.2±1.5 %	100.9± 1.3 %	101.1±2.0 %	105.1±0.2 %	98.1±2.3 %
chlorpyriphos-methyl	104.4±1.6 %	97.5± 3.6 %	100.9±0.9 %	99.9±2.3 %	96.6±0.2 %
cypermethrin	98.3±3.8 %	89.1± 3.3 %	103.2±2.8 %	104.3±5.6 %	91.4±0.5 %
diazinon	103.8±1.6 %	96.5± 0.6 %	99.9±0.7 %	97.7±2.3 %	98.6±1.2 %
endosulfan a+b	95.4±1.4 %	97.0± 2.0 %	104.0±4.1 %	104.3±0.3 %	95.8±0.4 %
iprodione	89.3±12.1 %	92.4± 2.2 %	101.8±3.2 %	92.1±1.5 %	96.46±3.4 %
lambda-cyhalotrin	98.8±13.5 %	96.9± 1.6 %	103.6±2.3 %	99.2±0.7 %	98.3±3.5 %
malathion	103.4±1.4 %	101.1± 0.7 %	109.5±2.7 %	98.9±3.5 %	100.1±5.2 %
mecarbam	103.9±1.4 %	98.7± 1.8 %	103.5±0.9 %	108.6±2.1 %	99.7±0.3 %
metalaxyl	**117.5±1.5 %**	110.5± 3.5 %	**116.1±1.2 %**	**125.6±0.9 %**	92.2±2.9 %
parathion	103.0±0.4 %	95.5± 0.6 %	104.3±4.5 %	103.8±0.1 %	100.4±1.2 %
permethrin	94.2±14.4 %	100.7± 0.5 %	97.5±1.3 %	100.6±0.7 %	102.9±0.9 %
phorate	101.2±0.6 %	98.3± 1.5 %	98.5±2.6 %	100.7±1.7 %	100.7±0.6 %
pirimiphos-methyl	101.6±0.51 %	103.1± 1.2 %	106.5±3.0 %	97.9±3.0 %	97.9±6.7 %
procymidone	97.4±2.6 %	98.7± 1.8 %	103.5±1.3 %	106.5±3.1 %	100.7±0.5 %
propyzamide	100.7±2.5 %	100.6±2.6 %	97.3±3.6 %	102.7±1.67 %	96.0±0.24 %
triazophos	97.6±2.1 %	93.0± 1.2 %	**114.95±1.2 %**	105.8±7.7 %	83.8±2.1 %
vinclozolin	105.7±1.8 %	99.4± 3.5 %	106.7±1.9 %	101.9±0.52 %	99.0±2.0%

Table 14: Summary of recovery (%) data and RSDs, for the 21 pesticides at the at the MRL level.

	Fortification level			
	MRL	**MRL**	**MRL**	**MRL**
Pesticide	**day 1**	**day 2**	**day 4**	**day 5**
azinphos-methyl	96.5±1.7 %	70.1±13.4 %	89.8±4.7 %	79.6±13.9 %
azoxystrobin	105.1±5.1 %	96.7±3.8 %	94.4±0.7 %	90.3±6.4 %
bromopropylate	96.6±1.8 %	97.7±2.68 %	95.5±1.6 %	102.7±2.1 %
chlorpyriphos	92.3±0.9 %	98.4±1.3 %	105.02±1.4 %	101.3±1.7 %
chlorpyriphos-methyl	107.1±3.4 %	96.8±1.2 %	99.6±0.5 %	96.5±0.3 %
cypermethrin	99.8±3.7 %	96.1±3.4 %	93.4±2.4 %	96.3±0.4 %
diazinon	106.6±2.8 %	101.8±1.1 %	99.8±0.6 %	99.8±1.1 %
endosulfan a+b	91.4±1.1 %	96.9±1.5 %	100.9±2.30 %	104.1±2.8 %
iprodione	94.8±1.8 %	86.6±6.4 %	92.2±1.8 %	92.1±2.9 %
lambda-cyhalotrin	98.3±2.2 %	95.9±2.2 %	95.2±1.8 %	96.6±2.7 %
malathion	104.2±1.4 %	104.1±2.3 %	95.3±1.6 %	100.3±0.3 %
mecarbam	93.8±1.8 %	96.5±1.2 %	105.1±1.4 %	98.3±0.4 %
metalaxyl	**114.7±5.8 %**	105.3±4.9 %	109.1±2.9 %	92.8±1.2 %
parathion	98.4±2.2 %	98.1±0.5 %	97.9±0.8 %	101.9±1.7 %
permethrin	100.9±0.6 %	99.2±2.3 %	96.3±1.9 %	100.9±0.9 %
phorate	100.5±0.7 %	102.8±0.30 %	98.2±1.7 %	100.3±1.9 %
pirimiphos-methyl	102.2±0.8 %	105.6±3.1 %	94.4±1.3 %	100.9±1.1 %
procymidone	96.9±1.5 %	96.9±0.7 %	101.4±2.1 %	103.5±0.03 %
propyzamide	103.1±4.4 %	100.8±2.5 %	101.9±1.65 %	97.7±0.03 %
triazophos	98.5±3.9 %	91.9±6.3 %	102.03±2.0 %	94.7±0.8 %
vinclozolin	107.0±4.2 %	98.3±0.9 %	99.7±1.7 %	97.5±1.1 %

Note: the result of recovery for each level in 1 day is obtained using the average value of 3 replicates.The results indicated that the performance of the method met the set requirements (mean recovery 70-110 %) with only a few exceptions. Recoveries outside the requirements, which are attributed mostly to errors in the quantitative step (e.g. the GC-MS integration), are shown in bold in Tables 13 and 14.

In order to evaluate the significance of the differences of average recoveries between the two concentration levels, recoveries of the 21 pesticides at each spiking level were compared using one way ANOVA for each day.

An example of summary of ANOVA calculation for day 1 of diazinon, comparing two levels of spiking, and 3 replicates each, is shown below.

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	3	311.58	103.86	2.9341
Row 2	3	319.87	106.6233	8.814533

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.45402	1	11.45402	1.949847	0.235108	7.708647
Within Groups	23.49727	4	5.874317			
Total	34.95128	5				

The summary of ANOVA shows that between group variance (one group consists of recovery data obtained for one analyte at one spiking level) is not significantly different than the within group (average of 6 replicate analysis) at a 95% confidence level.

The average recoveries obtained for each studied analyte did not show any concentration relationship. Consequently, their average could be calculated as a typical value for the tested matrix.

ANOVA was also used to evaluate if there was a significantly difference of recovery values between days. Replicate is used to denote a % Recovery including two spiking levels (0.5 MRL and MRL).

Between group variance (one group consists of recovery data obtained for 1 analyte in one day) was not statistically different (Fcal < F critical), showing no difference between recovery averages between days. This was done for every analyte, showing the same conclusion (Fcal < F critical).

5.1.7.5 Method Repeatibility and Intermediate Precision

Repeatibility is defined as the precision under repeatability conditions, *i.e.*, when independent test results are obtained with the same method on identical test items in the same laboratory using the same equipment within short intervals of time .

The repeatability of the method is shown in Table 15 and was calculated as RSD according to the following mathemathical expression (4):

$$RSD_{repeatibility} = \frac{\sqrt{MS_{\text{Within Groups}}}}{y_{mean}} \cdot 100$$

The intermediate precision (ip) is the precision where at least one of the conditions for repeatability does not apply. It was calculated using the following mathemathical expression (n = 6) (5):

$$RSD_{ip} = \frac{\sqrt{\frac{MS_{\text{Between Groups}} - MS_{\text{Within Groups}}}{n_{\text{per group}}}}}{y_{mean}} \cdot 100$$

In the case of chlorpyriphos, chlorpyriphos–methyl, and permethrin the RSD_{ip} could not be calculated since $MS_{\text{within group}} > MS_{\text{between group}}$. Instead the

u^*_{bb} was calculated according to [50] to give the upper limit of intermediate precision.

As shown in Table 9, the repeatability and intermediate precision was always <10 % for all target analytes as required in the validation plan, except for chlorpyriphos-methyl (repeatability) and chlorpyriphos-methyl (repeatability) and azinphos-methyl (intermediate precision). This is likely to be related to a dirty GC system (column, injector) giving a poor peak shape or related matrix enhancement effects.

Table15: RSD repeatability [%] and RSD within-laboratory reproducibility [%] for the 21 pesticides under study.

Pesticide	$RSD_{rep.}$	RSD_{ip}
azinphos-methyl	2.00	14.04
azoxystrobin	3.33	5.83
bromopropylate	4.98	2.61
chlorpyriphos	8.76	U_{bb}*=0.49
chlorpyriphos-methyl	42.69	U_{bb}*=0.51
cypermethrin	4.60	3.72
diazinon	2.20	2.69
endosulfan a+b	3.11	3.91
iprodione	5.19	3.84
lambda-cyhalotrin	4.77	1.56
malathion	2.16	3.45
mecarbam	3.27	4.18
metalaxyl	6.08	8.21
parathion	2.59	1.31
phorate	1.93	0.77
pirimiphos-methyl	2.77	4.00
procymidone	2.22	2.82
propyzamide	2.51	2.09
triazophos	4.13	8.94
vinclozolin	2.66	3.39
permethrin	5.03	U^*_{bb}=1.25

5.1.7.6 Robustness

Robustness testing evaluates how small changes in the method conditions affect the measurement result. The aim is to identify factors that could match possible deviations usually encountered in the laboratory and choose those factors that could influence the results.
In the present validation study the concentration of the acetic acid in the extraction solvent was changed around the ideal value (0 %, 0.8 %, 1 % and 1.2 %).

For each pesticide using the replicate values of two spiking levels (0.5 MRL and MRL) one way ANOVA was used to evaluate if there is a significant difference between mean recoveries due to a variation of % acetic acid. Between group variance (one group consists of recovery data obtained for 1 analyte with one acetic acid concentration) was not statistically different from within group variance (variance of replicate analyses). This was done for every analyte, showing the same conclusion (F cal < F crit).

This factor serves more as a confirmation of literature findings that acidification of the extracts will not be needed in future experiments when no base/acid sensitive pesticides are under the scope of the analysis, and this was the case.

5.1.7.7 Stability of the extracts

The stability of extracts obtained with the ideal concentration of acetic acid in the extraction solvent (1 %) was evaluated over 4 days by storing the extracts in the freezer at -20 °C before and after each day of analysis. On the day of extraction (day 1) samples were fortified and the % recoveries obtained from the stored samples were given as a % of day 1.

Table16: Recoveries of day 2 expressed as a percentage of the day 1, i.e. 100 %.

Pesticide	DAY 2	REC (%)	REC (%)	REC (%)	REC (%)	REC (%)
	0.5 MRL 1	0.5 MRL 2	0.5 MRL 3	MRL 1	MRL 2	MRL 3
phorate	106.0	103.5	100.5	95.3	95.9	100.5
propyzamide	102.5	98.4	99.3	100.4	98.3	99.7
diazinon	102.2	100.2	107.3	99.1	98.7	99.6
vinclozolin	99.6	97.5	110.9	100.5	97.6	98.4
chlorpiriphos-methyl	106.5	101.1	105.1	99.9	98.1	99.5
metalaxyl	106.9	92.2	101.6	100.4	96.5	100.1
pirimiphos methyl	97.2	99.9	103.8	101.2	100.2	100.1
malathion	98.9	98.7	105.7	100.9	100.6	100.7
chlorpiriphos	101.6	102.1	**126.7**	99.3	102.6	99.9
parathion	100.2	100	109.1	98.2	100.6	99.9
mecarbam	99.7	94.8	**123.8**	100.4	99.7	100.1
procymidone	97.9	94.9	**120.5**	101.4	99.1	100.3
endosulfan	100.6	101.9	114.6	99.3	100	100.2
Triazophos	96.5	113.3	**77.6**	99.1	98.8	101.3
Iprodione	104.4	104.6	93.9	99.7	99.5	100.1
bromopropylate	103.0	99.4	100.6	100.1	100.9	100.2
azinphos-methyl	103.4	**72.1**	83.3	99.3	95.2	100.5
lambda cyhalotrin	107.5	101.2	99.6	100	99.1	99.9
permethrin	101.6	98.7	103.2	100.1	98.	100.4
cypermethrin	107.9	**78.0**	105.1	99.6	95.6	99.5
azoxystrobin	99.4	111.3	94.8	96.4	96.2	96.6

Table 17: Recoveries of day 3 expressed as a percentage of the day 1, i.e. 100 %.

Pesticide	DAY 3	REC(%)	REC(%)	REC(%)	REC(%)
	0.5 MRL 1	0.5 MRL2	0.5 MRL3	MRL 1	MRL 3
phorate	97.1	96.0	98.5	97.4	96.5
propyzamide	91.3	93.2	89.8	98.0	94.9
diazinon	98.8	102.6	94.0	85.6	99.3
vinclozolin	93.5	100.2	91.7	**142.7**	97.0
chlorpiriphos-methyl	95.7	95.5	90.3	108.2	95.2
metalaxyl	93.6	91.2	85.4	**134.2**	97.7
pirimiphos methyl	101.1	101.1	106.4	97.3	99.2
malathion	101.7	100.3	103.8	**129.7**	100.3
chlorpiriphos	111.1	110.7	102.4	**219.6**	**123.9**
parathion	107.3	103.6	103.0	103.7	110.9
mecarbam	114.9	107.8	103.9	**145.6**	**124.1**
procymidone	112.8	108.8	99.2	**193.0**	**130.6**
endosulfan	102.5	105.2	99.0	96.2	88.6
triazophos	113.2	114.0	97.7	**123.8**	**125.4**
Iprodione	102.7	101.0	102.9	93.4	98.5
bromopropylate	94.7	93.1	92.9	84.8	94.5
azinphos-methyl	117.7	124.3	110.2	**79.5**	110.7
lambda cyhalotrin	100.3	94.8	97.8	94.8	94.1
permethrin	98.7	98.2	100.8	101.2	101.5
cypermethrin	107.1	**197.6**	103.3	**87.6**	98.7
azoxystrobin	109.4	110.4	**89.8**	**76.4**	101.8

Table 18: Recoveries of day 4 expressed as a percentage of the day 1 i.e. 100 %.

Pesticide	DAY 4 (% REC)					
	0.5 MRL 1	0.5 MRL 2	0.5 MRL 3	MRL 1	MRL 2	MRL 3
phorate	100.7	93.3	91.9	95.7	90.9	92.5
propyzamide	101.5	97.7	95.2	103.3	100.9	101.5
diazinon	104.4	105.4	99.1	103.5	101.4	101.9
vinclozolin	100.5	101.6	95.8	99.6	102.0	99.8
chlorpiriphos-methyl	105.3	103.1	96.0	101.3	101.0	99.1
metalaxyl	101.2	94.8	88.4	98.8	98.8	98.7
Pirimiphos-methyl	99.8	98.1	97.7	97.6	94.2	92.5
malathion	98.7	100.0	97.6	100.3	95.5	95.12
chlorpiriphos	112.1	110.6	102.3	107.9	110.1	104.8
parathion	101.6	99.2	96.7	99.9	104.3	100.3
mecarbam	101.5	101.8	90.4	100.4	102.5	99.7
procymidone	101.8	102.9	89.5	100.1	98.4	101.1
endosulfan	98.6	102.4	99.1	102.6	103.0	99.3
triazophos	96.3	109.3	98.1	108.8	106.8	107.8
Iprodione	106.1	106.4	109.6	100.8	96.7	99.9
bromopropylate	103.0	98.8	99.4	101.	98.3	99.8
azinphos-methyl	115.7	**121.6**	**116.6**	113.4	103.1	**117.1**
lambda cyhalotrin	104.3	98.8	99.5	98.4	94.9	93.8
permethrin	86.0	100.7	104.7	102.2	95.4	99.1
cypermethrin	109.1	**193.0**	103.4	94.4	90.20	103.6
Azoxystrobin	104.2	108.2	104.2	97.9	99.5	105.5

Recoveries that are not in the acceptable range when compared to day 1 (100±15 %) are shown in bold, but since these variations are not consistent (no trend observed), these errors were attributed to the integration step in GC-MS, since no signs of degradation of the analyte were found during the GC analysis in the subsequent days.

The summarized results indicate that pesticides are stable in matrix extracts for 4 days after storage in a freezer at -20 °C.

5.1.7.8 Stability in solvent

Currently available data [52] show that stock standards of the large majority of pesticides in toluene are stable for at least 5 years in the freezer when stored in tightly closed glass containers. This parameter was out of the scope of the present validation exercise.

5.1.7.9 Selectivity

The selectivity of GC is primarily determined by the ability to separate the target compounds from matrix interferences. Under the specific GC conditions used the retention time will remain constant for each peak. Also the ratios between quantitative, qualitative and confirmation ion (Tgt, Q1, Q2), are particular for each analyte and serve as an additional confirmatory measure.
In the present study, a reagent blank (to check for solvents and column interferences) and a matrix blank (to check for matrix interferences) were evaluated to check if the identification of the target analyte and its quantification is hindered by the presence of one or more of the interferences.

Table 19: Retention time (R_t), quantitation ion (Tgt), confirmation ions (Q1, Q2), MS dwell time and respective interferences for the 21 analytes under study.

Pesticide	R_t (min)	Tgt, Q1, Q2 (m/z)	Dwell time (ms)	Interference Reagent blank	Interference Matrix Blank
labelled phorate(ISTD)	7.21	264, 125, 235	40		
phorate	7.21	260, 75	40		At ion 75
propyzamide	8.07	173, 175	40		
diazinon	8.24	304, 137, 179	30		At ions 137,179
vinclozolin	10.66	212, 214	30		At ion 214
chlorpyrifos-methyl	9.33	286, 290	30		
metalaxyl	9.63	206, 249, 279	30		
pirimiphos-methyl	10.04	290, 305	40		
labelled malathion (ISTD)	10.17	183, 132	40		
malathion	10.27	173, 158	40		
chlorpyrifos	10.55	197, 314, 258	40		
labelled parathion (ISTD)	10.47	301, 115, 99	25		
parathion	10.58	291, 109, 97	25		At ion 97
mecarbam	11.70	159, 329, 296	25		
procymidone	11.92	283, 285	25		
endosulfan (α+β)	12.45,14.14	339, 341	40		At ion 341
triazophos	15.03	161, 162	40		
iprodione	16.28	314, 316	40		
bromopropylate	16.42	341, 343	40		
azinphos-methyl	16.96	160, 132	40		
lambda-cyhalotrin	17.25	181, 197	40		
Permethrin (1+2)	17.79,17.90	183, 163	40		
labelled cypermethrin (ISTD)	18.65,18.75,18.85	187, 207	40		
cypermethrin	18.56,18.65,18.72	181, 163,209	40		At ion 163 in all isomers
Azoxystrobin	20.81	344, 345	40		
TPP (ISTD)	15.90	325, 326, 233	40		

According to reagent blank analysis there were no notable interferences at the retention times of the target analytes.

The interferences due to matrix components are presented in Table 19 above. These interferences did not hinder the quantification of the analytes, and the % of interference in the confirmation ions became more evident as concentration of the pesticide in the standard decreased.

Performance criteria for the ratios of the ions were met and are presented in Annex 5.

5.2 Uncertainty Budget

Since a typical chemical measurement consists of a number of measurement steps, it requires a careful design of the measurement procedure to keep the traceability chain to the SI unit. To make a measurement result traceable to the SI unit, it is also necessary to evaluate the uncertainty of every step in the measurement procedure (gravimetric and IDMS calibration) and combine them to meet the principles of the internationally agreed guide Quantifying Uncertainty in Analytical Measurements, GUM, 1995.

The uncertainty was calculated using the top-down approach taking into account the uncertainty of the preparation of the standards (purity given on the certificate of analysis by the producer, and weights), the method repeatability, the intermediate precision, the calibration and the recovery (as a measure of trueness). For the latter the total number of independent samples used in the recovery experiments was taken into consideration. A coverage factor of k=2 was chosen to result in a confidence level of approximately 95 %.

The expanded uncertainty was calculated from the different contributions found in the validation study. As a CRM was not available, recovery served as a measure of trueness.

The values obtained from the different contributions as well as the final uncertainty value of the measurements for the different pesticides analysed are shown in Annex 5.

5.3 General conclusions

- ✓ The method is applicable within the analytical range of 0.25 MRL to 2 MRL of each pesticide in an apple /pear based baby food
- ✓ The repeatability and intermediate precision fulfilled the requirements listed in the validation plan
- ✓ The recovery values are within the acceptable range of 70 -110 % for EU pesticide legislation. Therefore recoveries were not corrected
- ✓ Measurement uncertainty was less than 10 % for all analytes except for azinphos-methyl and chlorpyrifos-methyl
- ✓ The method is fit for the intended purpose, which is the analysis of EU priority pesticides in apple/pear based baby food

5.4 Remarks–In house validation

With regard to the validation procedures four method performance parameters are reviewed here: the determination of LOD/LOQ, the repeatability/within-laboratory reproducibility, the trueness (recovery) results and the linearity/working range of the calibration curves. The evaluation of these and other parameters is an integral part of the validation of an analytical method, which can be defined as the process which allows to demonstrate the *accuracy* (*trueness* and *precision*) of the results produced by the method in question and therefore its suitability for the intended application. It can be performed within (i) an intralaboratory study (in-house validation*) or (ii) an interlaboratory (collaborative study).

In trace analysis, where analytes are often present at very low concentrations, it sometimes becomes difficult to decide whether the signal emerges from the component to be determined or from the inevitable noise produced by the procedure "chemical" noise from coeluting interfering compounds) or the instrument (" electronic" or " detector" noise). This uncertainty gives rise to the so-called limit of detection (LOD). In general, the limit of detection is the smallest observed signal that with a specified reliability can be considered as being caused by the component to be measured [43].

In residue analysis, the LOD is usually expressed not as the smallest signal but as the smallest content of the analyte in the sample (corresponding to the signal Y_{LOD}), which can be detected with reasonable statistical certainty (at least 95 %).

It can be determined by repetitive measurements of at least 20 representative blank samples, (6) [43]:

$$Y_{LOD} = Y_0 + 3\, s_0 \quad (6)$$

Where Y_0 is the average signal of the blank sample (at the elution time of the analyte) and s_0 is the standard deviation of the blank sample signals. However, this determination is rather impractical and time-consuming. In practice, the LOD can be estimated from the matrix-matched calibration curves by extrapolating the signal/noise (S/N) ratios to determine the concentration at which S/N =3 (Annex 5). The limit of quantification (LOQ) is the lowest content of the analyte in the sample, which can quantitatively be determined with the specified reliability. According to the QA/QC guidelines for pesticide residue analysis [43], the LOQ is the lowest calibrated level (LCL) at which the method was validated. As the lowest calibration level was ¼ of the MRL level specific for each analyte, the S/N ratio for this concentration was evaluated (Annex 2, Validation report).

The analyte can be accurately quantified when S/N ratio =10. Naturally, both the LOD and LOQ are analyte dependent; however, they also vary with sample type and with time (e.g. in GC analysis they depend on the current conditions of the GC-system–the GC inlet and column contamination, etc). In practice, the regular re-evaluation of these performance characteristics is therefore required.

Precision is the closeness of agreement between independent test results obtained under stipulated conditions [43]. The measure of precision is usually expressed in terms of imprecision and computed as a (relative) standard deviation of the test results. Quantitative measures of precison critically depend on the stipulated conditions. It is necessary to distinguish between: (I) *repeatability* which is precision under repeatability conditions (independent test results are obtained with the same method in the same laboratory by the same

operator using the same equipment within short intervals of time and (II) *reproducibility* which is precision under reproducibility conditions (when independent test results are obtained with the same method, but in which at least one repeatability component does not apply).

The precision data was assessed using one way ANOVA, which allows the separation of between–days variation and method repeatability influences. For some analytes $RSD_{reproducibility}$ was lower than $RSD_{repeatability}$ and this could be explained by differences in the batch samples and reagents, and different GC conditions (e.g. liner change during a long run).

The determination of *trueness* is much more complicated, because trueness, contrary to precision, relates to the true value. Thus, it strongly depends on the determination of the *accepted reference value*. The use of CRMs would be undoubtedly the best approach. In the absence of a CRM, trueness is therefore mostly expressed as recovery and determined by analysis of spiked samples (blank samples with addition of the known amount of analytes). The problem with the use of spiked samples is that pesticides are not incorporated as strongly into the matrix, so higher recoveries may be achieved for the spiked samples than for real-world samples with incurred residues. An alternative is to use a sample previously characterized in a proficiency test, which often contains naturally incurred residues in addition to those spiked into the matrix. A comparison with a different method is also helpful.

Accuracy is a term which involves a combination of random components (*precision*) and a commom systematic or bias component (*trueness*). It is defined as the closeness of agreement between a test result and the *accepted reference value*.

ISO, IUPAC and AOAC International, have co-operated to produce agreed guidelines, on the use of recovery information in analytical measurement [53]. Such protocols aim to outline minimum recommendations on quality control procedures, to the best estimation of the true value and to contribute to the comparability of the analytical result. However, at present, there is no single well defined approach to estimating, expressing and applying recovery information, which leads to difficulties while comparing results or in verifying the fitness of the data for an intended purpose. This is of special importance in pesticide residue analysis in complex matrices like foodstuffs.

Most of the analytical methods employed in pesticide analysis, from the sampling until the instrumental measurement, result in the loss of analyte, whether it remains in the matrix after extraction or it is due to incomplete transfers during the procedure. Consequently, the measurement gives a lower value than the true concentration in the original sample.

There are different procedures for assessing recovery values. When certified matrix reference materials are available, recovery is the ratio of the concentration of the analyte found in a sample to that stated in the CRM certificate. If the recovery is statistically different from 100 %, results obtained on a test material of the same matrix type can be corrected if:

(I) there is no matrix mismatch
(II) the concentration range in the sample is equivalent to the CRM available

In the absence of CRMs, recovery values can be estimated in several ways using a surrogate [3]. Regarding the nature of the assumptions at least three types of surrogates are defined, namely:

(I) Isotpe dilution
(II) Spiking, and
(iii) Internal Standard

As far as isotope dilution is concerned, an isotopically labelled version of the native analyte is used. The assumptions include that an effective equilibrium between native and spiked analyte is achieved, since the chemical properties of those are very close. As explained before this can be difficult when, for instance, a pesticide residue may be partly chemically bound to the matrix and a vigorous extraction method might not be possible to be used without the danger of destroying it.

In this case the recovery of the surrogate is likely to be greater than that of the native analyte.

Spiking is normally used when a matrix blank is available; the analyte can be spiked into it and its recovery determined after application of the normal

analytical procedure. An allowance for sufficient equilibration time has to be made to ensure proper distribution of the spike added to the matrix. When no matrix blank is available, spiking is still possible (standard addition method). Again an allowance for equilibration has to be made.

The use of an internal standard includes the use of an entity chemically distinct of the analyte(s), but of close chemical behaviour.

After these considerations, it is easy to argue that, especially in the context of enforcement analysis where an estimate of the true value is required, there are implications in the interpretation of analytical data that can affect seriously the credibility of science applied to risk assessment. Several arguments in favour and against correcting analytical results for recovery have been put forward.

The main reason for recovery correction is the fact that in case of significantly low recoveries of analyte the true analyte content can only be estimated if results are recovery corrected.

It is also argued that a correction factor often has a high relative uncertainty, when compared with the relative small deviations from unity, which could arise largely due to random errors rather than a systematic loss of the analyte.

In conclusion, the strategy commonly employed, and which was also used in the whole study, was to assess recovery during the process of method validation. The obtained values can then be applied during the subsequent use of the analytical method for the characterisation of a material, which in this case may become a candidate RM. This would help to ensure that the analytical system does not change in a significant way that would invalidate the original estimates of the recovery.

[3] **Note**: Surrogate, denotes a compound added to the analysis, that behaves quantitatively, in the same way as the native analyte, specially in regard to its partition between the various phases of the analytical method. In practice similarities are often difficult to demonstrate and assumptions are made.

Instrumental analytical methods do not deliver directly analytical result in well defined properties (e.g. mass), but a response described by complex empirical algorithms. These emprirical methods are based upon the measurement of standards with known values of the measurand (e.g. concentration) in a procedure called calibration. Most of the time, the instrumental equipment is very complex and sensitive to small variation of experimental parameters, difficult to control and therefore must be calibrated before analysis. Usually, the analyst demands to define a linear relationship between the instrumental signal and the quantity of analyte in the sample. When this is not done correctly the quantification result might be subjected to significant systematic errors, or in case these are detected, it would be necessary to estimate an additional uncertainty associated to the simplified model used. The function by which the mathematic relation between the instrumental signal and the quantity of analyte is described is called the *calibration curve*. Independently of the mathematical model used to describe the calibration curve, some rules apply:

1. The working range must be adequate for the expected value of the sample (e.g. the resultant interpolation value must fall within this range)
2. The calibration must include a “zero calibration” or “blank calibration”, meaning a sample that does not contain the analyte in question, but that has undergone the same procedure as the samples (contains solvent, reagents, matrix, etc.). Many times this response is not equal to “zero” but dictates sensitivity of the calibration method
3. The analyte levels applied during calibration must be equidistant

Two aspects must be taken into consideration when describing the analytical instrumental response:

1. The trend followed by the instrumental signal as a function of the analyte content;
2. The dispersion behaviour (variance) of the analytical signal in the calibration range (a constant dispersion is called homoscedastic and when it is variable across the all calibration range it is called heterocedastic). Generally, when the

response is heterocedastic, its variance increases with increasing content of the analyte.This is best described mathematically when the instrument response reflects the relation between the independent variable, X (e.g. concentration of the analyte), and the dependent variable (Y) (e.g. instrumental response) as follows:

$$Y = a + b X \qquad (7)$$

Where **a** and **b** represent the intercept and the slope of the linear curve, respectively.

This model assumes that the errors associated with X are negligible in relation to the precision of the instrumental response, and its looks for the line that minimize the deviations between the experimental points and the estimates of Y. These deviations are called residuals. The regression model minimizes the square sum of the residuals, and that is why this model is usually mentioned in literature as “method of least squares”.

The linear correlation coefficient (r) is used to test the linear tendency of two variables in a data pool. This is simple but, as mentioned previously, it is not a convenient methodology. One of the disadvantages of this tool is the fact that even if r is a high value (near unity), it is possible that the data do not present a linear tendency.

Generally, the strategy for performing proper instrument calibration involves the following steps:

1. Statistical tests for the evaluation of outliers at each concentration level
2. Selection of the regression model according to the analysis of homogeneity of variances and
3. Statistical tests to evaluate the quality of the chosen mathematical model (e.g. using a residual plot)

During the in house-validation experiments of the QuEChERS method the data was fitted to straight lines using the Validata software and tested for linearity according to Mandel [54]. The residual standard deviations of the first and second order calibration functions were examined for significant differences (99 %). If such a difference existed, the working range was reduced as far as necessary to receive a linear calibration curve.

6. Trace analysis of EU priority pesticides in carrot/potato baby food by isotope dilution mass spectrometry: (matrix effects) and uncertainty evaluations.

The use of matrix matched calibration recommended by EU quality control tools [20], requires substancial work, therefore it must be properly justified. A reference matrix could be perfectly suitable for routine screening where a small uncertainty may not be critical, but may not be suitable for law enforcement and risk assessment. This report provides methodology to evaluate the extent of matrix effects in carrots based baby food matrix, by comparing calibration in solvent with calibration in blank matrix.

This section also describes the advantages of an IDMS method for the determination of pesticides in a vegetable matrix via GC-MS, in particular the benefical effect of the istopocially labelled surrogates for reducing the influence of the matrix on quantitation.

Quantification of the target pesticides was done using the QuEChERS procedure described in previous sections. The internal standard consisted of a mixture of 7 isotopically labelled pesticides. For each compound integration was performed using the corresponding labelled congener (Table 21).

An adapted version (appendix 3) of the one proposed by Gonzalez et al., [66] was used for the statistical analysis of matrix effects assessment, using Validata software. Table 20 shows that from the target list of analytes only, chlorpyrifos-methyl and phorate do not show significant matrix enhancement effect in carrots baby food. The slopes and the intercepts of the calibration curves in matrix and solvent do not differ statistically ($t_{calc} < t_{crit}$) which means that the quantification of these analytes are not affected by the presence of matrix. This is in accordance with previous findings and also it is possible to predict such results from their chemical structure [49]. Organophosphorous pesticides with a (-P=S) group are not as susceptible to matrix-induced enhancement as those with a (-P = O) group.

Most of the compounds prone to matrix enhancement effect are polar and/or strong hydrogen- bond acids and/or bases exemplified by the presence of phosphate (-P=O), hydroxyl, amino, imidazole, benzimidazole, carbamate (-O- CO-NH-) and urea (-NH-CO-NH-) functional groups. The same way it also shows that almost all target analytes are affected by the matrix enhancement phenomena well described in previous references [49] and that matrix matched calibration should be used for quantification purposes.

For the other analytes, when the slopes are not statistically different but intercepts are, the matrix effect introduces a constant systematic bias. On the other hand, when slopes are statistically different but not the intercepts, the matrix effect introduces a proportional systematic bias. When both the slopes and the intercepts are statistically different, the matrix effect introduces a constant and proportional systematic bias and that fact justifies the use of matrix matched standards for calibration purposes.

Table 20: Comparison of slopes and intercepts of calibration in solvent/calibration in matrix for 21 EU priority pesticide analytes using t statistics and TPP as IS.

Pesticide	t_{calc} **slope**$_{solvent/matrix}$	t_{calc} **intercept** $_{solvent\ /matrix}$
azinphos methyl	15.77	3.22
azoxystrobin	14.92	1.96[a]
bromopropylate	11.61	1.89[a]
chlorpyrifos	9.73	1.40[a]
chlorpyrifos-methyl	0.57[a]	0.61[a]
cypermethrin(sum of isomers)	3.90	0.16[a]
diazinon	5.57	0.02[a]
endosulfan (α+β)	11.86	1.00[a]
iprodione	9.22	0.43[a]
lambda-cyhalotrin	6.32	0.36[a]
malathion	1.76[a]	7.05
mecarbam	3.60	0.23[a]
metalaxyl	4.19	4.45
parathion	9.8	0.505[a]
permethrin	0.79[a]	3.96
phorate	1.18[a]	0.07[a]
pirimiphos-methyl	9.55	4.05
procymidone	42.52	6.9
propyzamide	9.60	1.07[a]
triazophos	9.43	0.64[a]
vinclozolin	11.03	4.9

[a]- slopes and /or intercepts do not differ statistically

The experimental data on table 20 suggest that matrix matched calibration should be used for quantification of a sample (or quantification using calibration in solvent will result in biased values of the concentration of the sample matrix), owing to notable differences between calibration in solvent and calibration in carrots matrix.

However, when applying the IDMS calibration (using 7 labelled internal standards) in solvent to the test samples, the concentration results showed that, the obtained values were statistically similar ($t_{cal} < t_{tab}$) to the ones obtained with IDMS calibration in matrix (using 7 labelled internal standards). This demonstrates that IDMS fully compensate the matrix effects.

Table 21: Ions used for quantification of pesticide analytes by gas chromatography-isotope dilution-mass spectrometry (IS, denotes isotopically labelled standard).

Compound	Quantification ion (m/z)	Confirmation ion (m/z)	Internal standard used for quantification
phorate- $^{13}C_4$ (IS1)	264	235, 125	-
phorate	260	75	IS1
propyzamide	173	175	IS1
diazinon	304	179,137	IS1
vinclozolin	212	214	IS1
chlorpyrifos-methyl	286	290	IS1
metalaxyl	206	249,279	IS1
pirimiphos-methyl D6 (IS2)	206	249,279	IS1
pirimiphos-methyl	290	305	IS2
malathion-D10 (IS3)	183	132	-
malathion	173	158	IS3
chlorpyrifos-D10 (IS4)	324	198	-
chlorpyrifos	314	258,179	IS4
parathion-D10 (IS5)	301	115,99	-
parathion	291	109,97	IS5
mecarbam-D10 (IS6)	339	116,99	-
mecarbam	329	296,159	IS6
procymidone	283	285	IS6
endosulfan (α+β)	339	341	IS6
triazophos	161	162	IS6
iprodione	314	316	IS6
bromoproylate	341	343	IS6
azinphos-methyl	160	132	IS6
lambda-cyhalotrin	181	197	IS6
permethrin	181	197	IS6
cypermethrin- D6 (IS7)	187	207,163	-
cypermethrin	181	163,209	IS7
azoxystrobin	344	345	IS7

The labelled spike solutions (pirimiphos-methyl-D6, mecarbam dietoxy D10, cypermethrin, mix of sterioisomers, phenoxy 13C6, phorate dietoxy-$^{13}C_4$, parathion-ethyl diethyl-D10; chlorpyriphos diethyl D10 and malathion D10) and the calibration solutions of the correspondent natural congeners were examided by GC/MS at the same conditions as described above in SCAN mode to test their cross contamination, which can lead to bias in the final results. The spike solution of each labelled compound did not show a peak used for the quantification of the native above the noise level on their ion chromatogram.

Based on this signal-to-noise ratio, the contamination level by the unlabelled compound was considered to be negligible (estimated to be less than 0. 01 % of the labelled). Also, the GC/MS measurement of the calibration solution of the native compounds showed that they were free from contamination by the labelled compound.

6.1 Recoveries native/labelled compound

Isotope dilution mass spectrometry had clearly a positive effect on the truness of the analysis (Fig. 37). The deviation of the obtained results from the target values was much smaller compared to the conventional internal standard procedure.

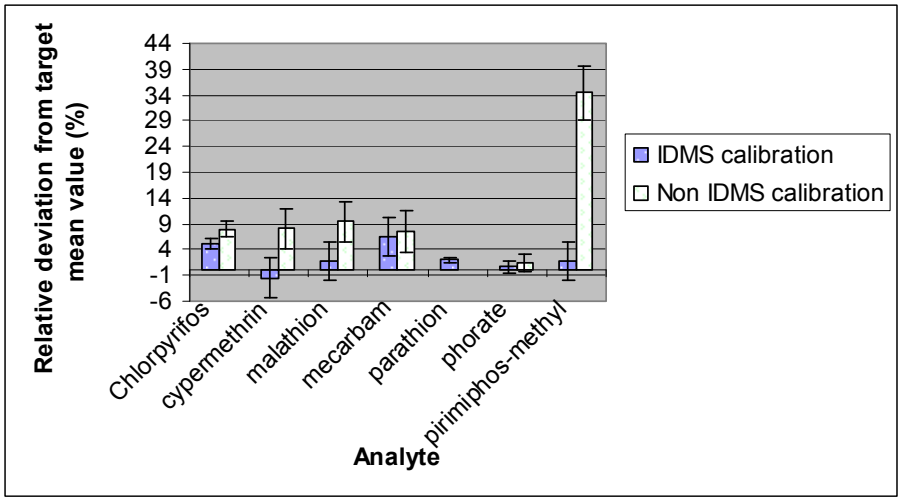


Figure 37: Comparison of relative deviation form target mean value of spiking (%) for the 7 analytes using IDMS and non IDMS calibration (conventional internal standardization calibration).

Unfortunately the isotopically labelled analogs are only available for a limited number of pesticides. On top labelled compounds are rather expensive, which renders this option unattractive for routine application. Currently, matrix matched calibration is the preferred option in routine multiresidue analysis.

6.2 Conclusions

The results of the comparisons showed the superior accuracy of IDMS over conventional calibration procedures. Although IDMS is generally expensive for routine analysis, its accuracy and precision makes it a reliable analytical tool for the certification of reference materials.

The described methodology will give reliable results and will be suitable for new users after being subjected to inter laboratory validation exercises. Full validation must take place to ensure that any other major potential sources of error have been detected.

7. A natural matrix (carrot/potato baby food) candidate Reference Material

7.1 Introduction and characterization

This section provides an overview of the feasibility study for the production of a (certified) reference material for 21 EU priority pesticides in products of plant origin. It describes the re-validation parameters for the new matrix under study.

Heat treated, homogenized carrots baby food (Olvarit Brand), purchased on the local market (Geel, Belgium) spiked with the target analytes at the specific MRL level (the MRL for the specific analyte/matrices combinations of the EU 2002-2005 monitoring scheme), was selected as the candidate reference material representing a root crop of high water content. Carrot belong to the EU list of priority matrices for pesticide analysis (Table 3). When producing matrix CRMs for the verification of method accuracy (trueness and precision) one must bear in mind that a perfect match between the CRM matrix composition and the sample composition is not always achievable, which calls for a cautious evaluation owing to matrix differences.

The method's repeatability for the new matrix under study (carrot/potato based baby food, Olvarit, Belgium) was evaluated and it is provided in Table 22.

Table 22: Method repeatability of 21 EU priority pesticides in carrots baby food.

Pesticide	$RSD_{repeatability\ (\%)}$
azinphos-methyl	3.89
azoxystrobin	10.91
bromopropylate	1.47
chlorpyriphos	1.07
chlorpyriphos-methyl	2.37
cypermethrin	3.76
diazinon	8.59
endosulfan a+b	8.07
iprodione	4.86
lambda-cyhalotrin	7.32
malathion	2.95
mecarbam	1.17
metalaxyl	9.31
parathion	2.39
permethrin	1.43
phorate	1.33
pirimiphos-methyl	1.20
procymidone	1.65
propyzamide	2.50
triazophos	9.67
vinclozolin	2.61

According to the validation report (previous section) and the results presented in Table 22, it was concluded that the method repeatability for the new matrix was within the target performance criteria, which means that performance criteria were still met when using processed matrices for the quantification of the target analytes ($RSD_{repeatability} < 10\ \%$), and recoveries of spiked material, as a measure of trueness, were verified to be between 70 and 110 %. Only azoxystrobin gave a $RSD_{repeatability} > 10\ \%$. These results indicated that the other performance characteristics established during method validation were maintained for the analysis of pesticides in carrots baby food.

In the present study, a reagent blank (to check for solvents and column interferences) and a matrix blank (to check for matrix interferences) were evaluated to check if the identification of the target analyte was hindered by the

presence of one or more of the interferences, or the quantification was notably influenced, considering the new matrix of carrots.

Figures 38, 39 and 40 represent GC-MS chromatograms of a reagent blank, an extract of blank carrots baby food and an extract of carrots baby food spiked with target pesticides at the specific MRL level.

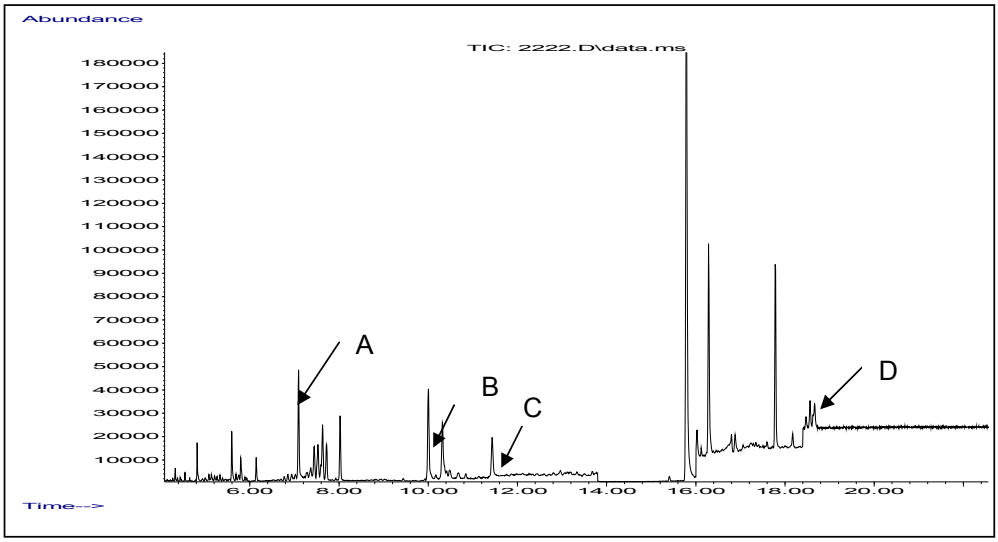


Figure 38: Total ion chromatogram of a reagent blank (water was used instead of a food sample) in GC-MS.
ISTD is represented by A-(labelled phorate, R_t-7.06 min), B-(labelled parathion, R_t-10.26min), C-(labelled mecarbam, R_t-11.48 min), and D- (labelled cypermethrin sum of α, β, γ isomers, R_t-18.40; 18.50; 18.60 min). Total run analytical run time was 27.7 min.

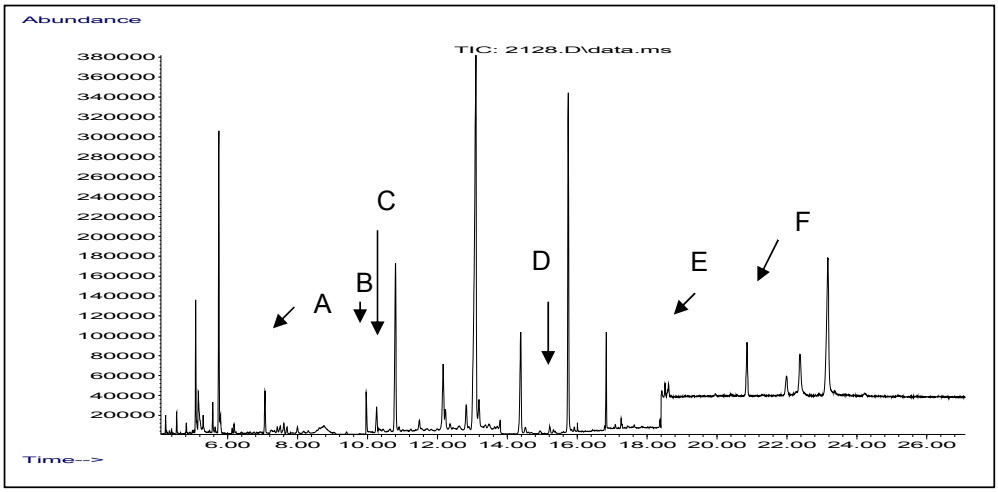


Figure 39: Total ion chromatogram of a blank extract of carrots baby food, injected in GC-MS, total analytical run time was 27.7 min.

ISTD is represented by A- (labelled phorate, R_t-7.06 min); B-(labelled malathion, R_t-10.06 min), C-(labelled parathion, R_t-10.26 min), and E-(labelled cypermethrin (mix of α, β, γ isomers, R_t-18.40;18.50;18.60 min). D is a false positive of triazophos (R_t-14.93 min) and F is a false positive of azoxystrobin (R_t-20.86 min).

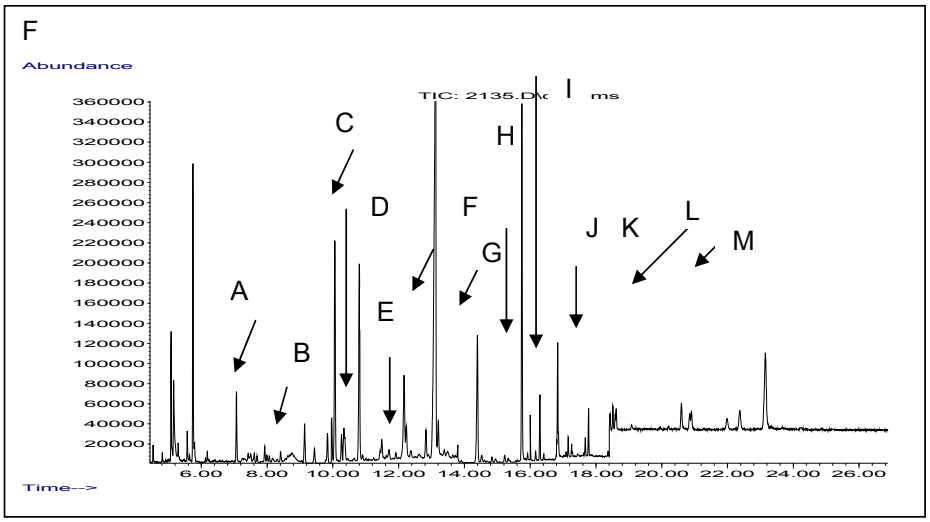


Figure 40: Total ion chromatogram of an extract of carrots baby food spiked at the MRL level in GC-MS. Total analytical run time was 27.7 min.

A represents ISTD (labelled phorate, R_t-7.069 min,and phorate R_t-7.07 min); B (propyzamide, R_t-7.92 and diazinon R_t-8.07 min, vinclozolin R_t-9.13 min, chlorpyrifos -methyl R_t-9.14 min, metalaxyl R_t-9.43 min and pirimiphos–methyl, R_t-9.83 min); C (malathion. R_t-10.06 min); D (ISTD labelled parathion R_t- 10.26 min chlorpyrifos R_t-10.33 min and parathion R_t-10.26 min); E (mecarbam R_t-11.49 min and procymidone R_t-11.71 min); F (α-endosulfan R_t-12.20 min); G (β endosulfan R_t-13.90 min); H (triazophos R_t-14.83 min); I (iprodione R_t-16.16 min and bromopropylate R_t-16.29 min); J (azinphos-methyl R_t-16.85 min and lambda-cyhalotrin R_t-17.15 min); K (permethrin (mix of isomers 1+2) R_t-17.67. 17.77 min); L (ISTD–labelled cypermethrin (mix of α, β, γ, isomers) R_t-18.42,18.51,18.61 min) and M (azoxystrobin R_t-20.59 min).

Two other matrices were considered as potential matrices for the feasibility study of producing a candidate RM namely spinach and orange (commercially based baby food), but those matrices were not further used in the feasibility study. Carrot/potato was selected because of its good freeze drying behaviour. Nevertheless the method repeatability for the two new wet matrices was within the target performance criteria ($RSD_{repeatability} < 10$ %) and recoveries of spiked material, as a measure of trueness, verified to be between 70 - 110 %. The same conclusions were obtained with spiking experiments of freeze-dried, frozen and sterilized matrix of carrots/potato mixture and wet/freeze dried spinach. These results indicate that the other method performance characteristics were maintained for the wet/freeze-dried matrices with the exception of freeze dried orange based baby food (Olvarit, Belgium).

8. Evaluation of the suitability of different processes (freezing, freeze–drying and sterilization) for the stabilization of a candidate reference material.

8.1 Introduction

The stability of CRMs can be divided into two aspects: stability of the matrix and stability of the analyte(s).

A basic recommendation for ensuring the stability of any sample of biological origin is the storage at low temperatures (e.g. -20 °C).This is done because their stability might strongly affect the ruggedness of the analytical technique employed and also because of appropriate transport of samples between and to laboratories.

This work aimed to ensure that the generated data are valid and the measurands remain accurately quantifiable from the time of sampling to analysis, for each process/ specific time frame the sample was submitted to.

8.2 General guidance for the experiments

Experiments were carried out to evaluate the effect of three different physical processes, freezing, freeze drying and sterilization, on the stability of 21 target pesticides in carrots baby food. These investigations converge to the preservation of analytes, linked to the preparation and storage of a natural matrix CRM. Organic analytes are subject to degradation by different modes: biological (e.g enzymatic hydrolysis and microbial growth), chemical (e.g hydrolysis, oxidation) and physical (e.g photolysis or volatilization). The temperature and the matrix in which the analytes of interest are contained constitute a major factor for their stability.

It is known that the behaviour of residues during storage and processing can be rationalised in terms of the physico-chemical properties of the pesticide

(solubility, hydrolytic rate constants, volatility, octanol-water partition coefficients, matrix pH, etc.).

In practice, however, the lack of detailed data, particularly on the interactions with food components, results in a more empirical approach. More research is required on some of these fundamental physico-chemical processes in the context of food processing [56].

This section examines the effects of processing on pesticides residues with a view to find a process /storage type to stabilize target analytes in a matrix of plant origin.

8.3 Freezing

The effect of sample freezing at -70 °C, -30 °C and -20 °C, in terms of stability and degree of homogeneity, was studied.

The raw material used in this study was a commercial carrots based baby food (Olvarit, GB Geel). A pesticide mixture solution in acetonitrile, was spiked at the specific MRL for analyte/matrix combinations of the EU monitoring programme 2002-2005. The spiking of the material (1 kg) was done by weight. The mixture of pesticides at the MRL level was diluted appropriately to ensure that 10 mL (approx. 10 g) of the spiking mixture was added to 100 g of blank material. The amount of the spiking solution was maintained around 10 % (volume of spiking solution/weight of baby food), to ensure proper homogenization using a blender at a velocity of 4000-10000 rpm for 10-15 min (Fig. 41).

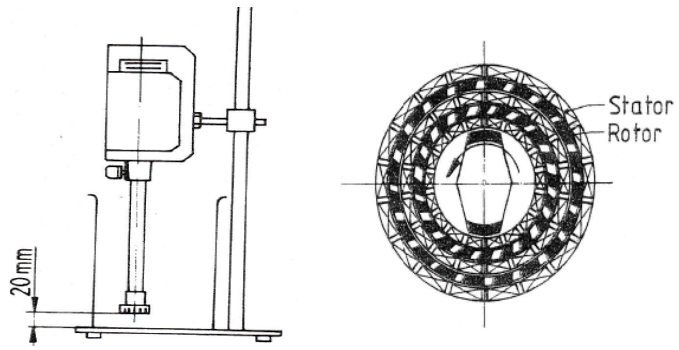


Figure 41: Schematic diagram of the blender (Ultra Turrax T 50, Jahnke & Kunkel, Staufen, Germany) used for the homogenization of the samples along with the rotor used in the same operation.

Jars (100 mL) with metal screw caps were filled with 60 g of blank and spiked material. Six jars of blank and spiked material were spread over the three processing temperatures (-70 °C, -30 °C and -20 °C) and left in the respective freezers for a period of 8 days.

Samples were defrosted and equilibrated at room temperature. They were extracted and measured via GC-MS using the in-house validated QuEChERS method. Matrix-matched calibration standards were prepared with processed blank material. Detailed conditions of the method set-up are described in the above section.

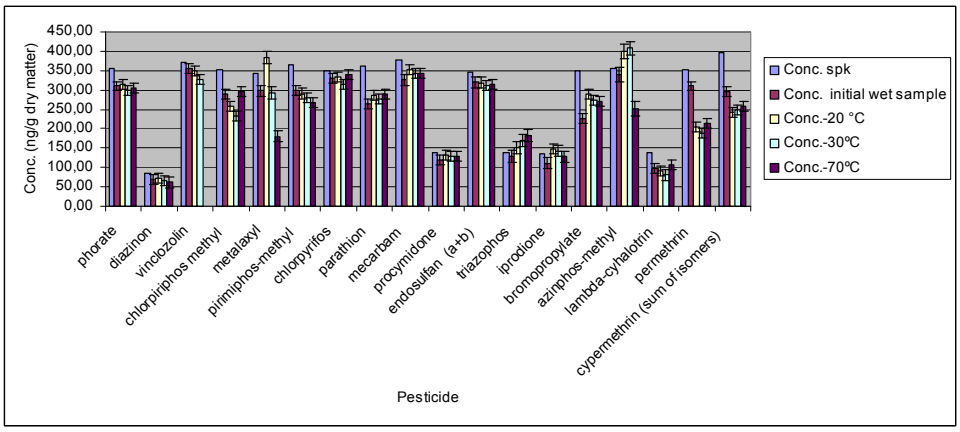


Figure 42: Mass ratios (ng/g dry matter) for the target pesticides before and after processing at (-70 °C, -30 °C and -20 °C). Malathion is not represented due to a much higher MRL (500 ng/g wet carrots baby food) compared with the other pesticides, and recoveries obtained for malathion were on average 150 % for all three temperatures tested.

Firstly, six samples of the spiked bulk sample were analysed, and results expressed in ng/g dry matter. This is referred in the graphical form as concentration initial wet sample. Method repeatability was within method validation criteria (<10 % RSD) except for chorpyrifos-methyl, triazophos, lambda-cyhalotrin, permethrin, cypermethrin and azoxystrobin. This could be due to a dirty GC injection system.

Samples were then left during 8 days at -70 °C, -30 °C and -20 °C. Processed blank material was used to construct the matrix-matched calibration curves and 6 samples (2 for each jar) were analysed and the average of two

injections was obtained. The results are represented in graphical mode (Fig. 42) as concentration (ng/g dry matter) at -70 °C, -30 °C and -20 °C. During a time span of 8 days all pesticides remained stable in the frozen samples at all tested temperatures, with recoveries values after processing of 100±20 % except for permethrin, which was 70±10 %.

The uncertainty of the results after freezing/thawing process was evaluated taking into consideration the method validation uncertainty budget, the method's repeatability obtained from the current experiments, the uncertainty of the water content determination and the uncertainty of the spiking mixture (which was negligible). The combined uncertaityn was expanded using a coverage factor of 2, resulting in a confidence level of approximaetly 95 %.

All experimental data presented here refer to MRL (ng/g dry matter) taking into consideration the sample's water content. This was done to ensure data comparability for the three processes under study (freezing, freeze drying and sterilization).

The experimental set-up ensured that the errors resulting from measurement, sampling and sample treatment were similar for all samples; only the degree of homogeneity may vary.

Method repeatability was better than 10 % RSD, meeting the methods repeatability validation criteria, for all analytes except for some late eluting compounds for the reasons mentioned above. Between bottle variation could not be detected for all compounds, therefore u*bb can be adopted as potential hidden inhomogeneity contribution. It is also to note that after thawing of samples, irrespective of the storage temperature, propyzamide, vinclozolin and azoxystrobin showed bad peak shape at all tested temperatures.

This experimental data shows that freezing is a good process for stabilizing these target analytes in the carrots based baby food matrix during the time frame of 8 days. Long term stability needs to be evaluated. Most high moisture unprocessed foods must be held in refrigerators (0 to 5 °C) for short to medium storage or deep frozen (-10 to -20 °C) for longer periods. Studies on a variety of pesticides on whole foodstuffs under cool or frozen storage have shown that residues are stable or decay only slowly [56]. The temperature of storage is important for less stable or more volatile compounds [56].

8.4 Freeze-Drying

The effect of freeze-drying on homogeneity and stability of a pesticides spiked into baby food material was studied.

Blank (verified to be pesticide free) and carrot baby food (approx. 1.5 kg) spiked at the specific MRL level of the target analytes, were homogeneized with a blender and processed in a pre-cooled freeze-dryer(Epsilon 2-85D, Martin Christ, Osterode, Germany). The process of freeze-drying for carrot baby food was developed internally at IRMM, RM unit (see its description in processing section). The dried material was ground and sieved before any analysis.

Samples were prepared and measured via GC-MS using the in-house validated QuEChERS method. Matrix-matched calibration standards were prepared with processed blank material. For samples having a water content below 80 % cold water (to avoid degradation of volatile pesticides) must be added leading to a total water content in the extraction tube of approximately 10 g. Freeze-dried products can be rehydrated (reconstituted) much more quickly and easily because it leaves microscopic pores. The pores are created by the ice crystals that sublimate, leaving gaps or pores in its place.

The water content of wet carrots baby food was 86.4 %, which resulted in 13.6 % dry matter; to maintain the same sample intake in terms of dry matter for the wet/freeze dried sample, the sample intake was adjusted to 1.4 ± 0.1 g for both matrix-matched calibration standards and samples of the freeze dried material.

Reagent blank, matrix blank, and spiked (specific MRL level) freeze dried material were extracted and analysed in GC-MS (scan and SIM mode) to check for interferences at the Rt of the analytes of interest, which might have resulted form the physical process itself.

Particle size analysis by laser light diffraction after milling the freeze dried sample was carried out at IRMM, RM unit according to RM WI/0042. The particle size distribution is given as a volume fraction or equivalent sphere diameter in µm. According to the cumulative distribution (Q (x)/ % vs particle size/µm), the particle size of the freeze dried powder was less than 515 µm, with an apex of the distribution at 55.7 µm ((50 % of the particle size was below

55.7 µm, and the other 50 % above 55.7 µm). From the density distribution curve (q*(x)), which was derived from the cumulative curve, the mean particle size was estimated to be 50 µm.

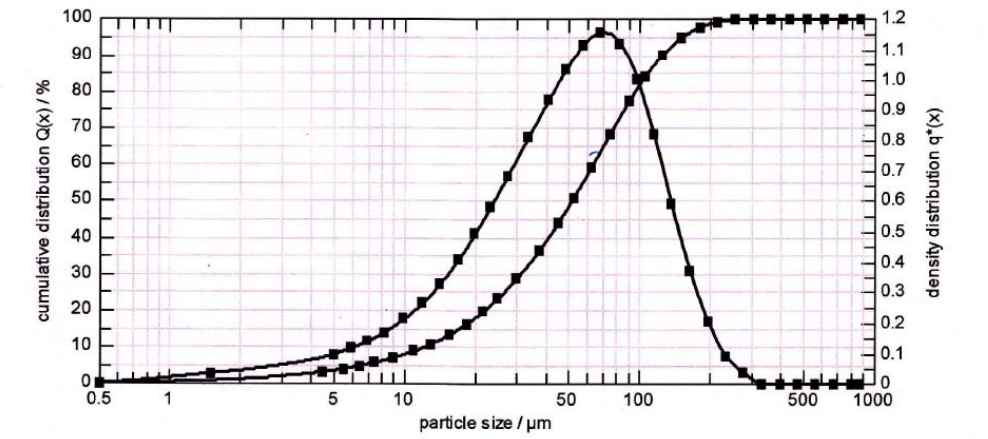


Figure 43: Average particle size distribution curves for three replicates of sample ID 6315 of carrot/potato powder of the units allocated for additional characterisation, each measured twice using RM WI/0042. Optical concentration was 20.3 % on average using the cuvette and 2-propanol as dispersant using a Sympatec Helos laser light scattering instrument (Clausthal-Zellerfeld, Germany).

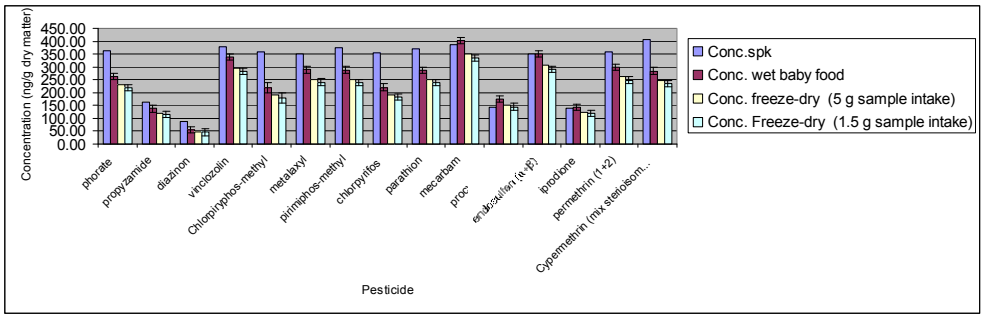


Figure 44: Mass ratios (ng/g dry matter) for the target pesticides before and after the freeze drying process for the target analytes. Malathion is not represented because of a much higher MRL (500 ng/g wet carrots baby food) compared with the other pesticides, and concentrations obtained for malathion were on average 3672 ng/g dry matter for sample intake of 1.5 g and 3855 ng/g dry matter for sample intake of 5 g.

The results presented in Figure 44 were obtained by analysing 10 samples of wet and milled freeze dried material. Triazophos, azinphos-methyl,

azoxystrobin, bromopropylate and lambda-cyhalotrin gave inconsistent recoveries. This is most probably due to a dirty ion source at the time the samples were injected.

From figure 44 one can conclude that the average recovery (freeze dried sample/ wet sample) was 133 % using 5 g sample intake and 117 % using 1.5 g sample intake.

The higher amount of dry matter in a 5 g sample intake of freeze dried sample (3 % water) in comparison with approximately 1.4 g of dry matter in a sample intake of 10 g wet material with 86 % water, was sufficient to cause a noticeable matrix enhancement effect. Also different susceptibilities of pesticides to matrix effects were confirmed (e.g procymidone vs parathion) since thematrix effect is both compound and matrix dependent (quantity/type) [49]. These findings suggest to pay especial attention to sample intake for the matrix calibration standards, when comparing samples before and after the freeze drying process. Data comparability to a dry matter basis before and after processing is ensured when equal amounts of sample are used for extraction.

The experimental batch set-up ensured that the errors resulting from measurement, sampling and sample treatment were similar for all samples; only the degree of homogeneity of the wet material in comparison to a milled freeze dried material could vary.

Method repeatability for the target analytes in the dried material was below 10 % RSD, meeting the methods repeatability validation criteria for all analytes except for some late eluting compounds for the reasons mentioned above. The experimental data (average recoveries 117 %) showed that freeze-drying is a suitable physical process for stabilizing pesticides in a carrot matrix, because the process did not degrade the targeted pesticides to a great extent.

8.5 Sterilization in autoclave

The effect of autoclaving on the homogeneity and stability of pesticides spiked into baby food material was studied.

Blank (verified to be pesticide free) carrot baby food (approx. 1.5 kg) spiked at the specific MRL level of the target analytes was homogeneized with a blender and processed in an autoclave (Matachana B-4023 autoclave, Webeco, Ober-Ramstadt, Germany). The sterilization process was set at 121 °C for 15 min (total run time 1 hour). 3 jars (120 mL glass vials with screw caps) were filled with blank and 3 jars with spiked material (1 jar contained 60 g of material) and were processed completely closed to avoid evaporation. Preliminary experiments were done with the jars slightly open. The details of time, temperature, degree of moisture loss and whether the system was open or closed were important to minimize losses of pesticides. The rates of degradation/volatilization were dependent on the heat load involved in the process.

Samples were equilibrated at room temperature. Matrix-matched calibration standards were prepared with processed blank material according to the QuEChERS sample preparation and were injected in GC-MS. From each of the three spiked jars, 3 samples of 10 g of processed material were taken for analysis giving a total of 9 samples; 6 samples of initial wet bulk sample were analysed by the same procedure.

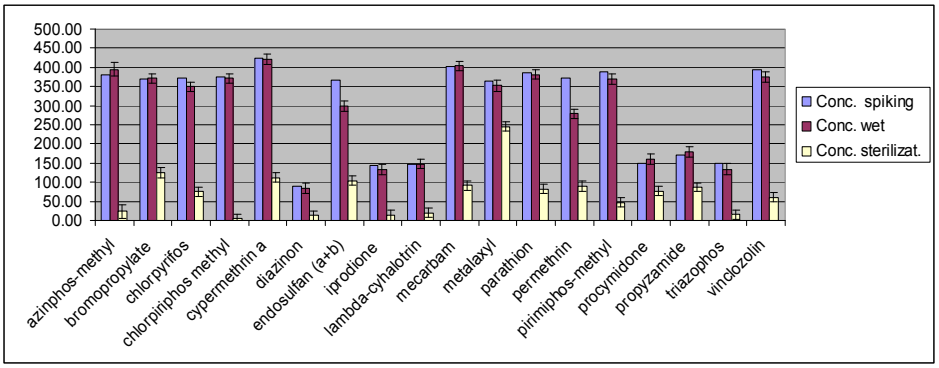


Figure 45: Mass ratios (ng/g dry matter) for the target pesticides before and after the sterilization process for the target analytes. Jars were completely closed. Malathion is not represented due to a much higher MRL (500 ng/g wet carrot/potato baby food) compared with the other pesticides, and recovery (wet/after process) obtained for malathion was 21 % (182 ng/g dry matter).

Processes involving heat can increase volatilization or chemical degradation and thus reduce residue levels. The analysis of the sterilized samples showed bad irregular peak shapes for triazophos, iprodione, endosulfan (a+b) and azoxystrobin. Phorate was completely eliminated in the autoclaved material.

The following pesticides were quantified at their limit of quantification: azinphos-methyl, chlorpyrifos-methyl, iprodione, lambda-cyhalotrin, pirimiphos-methyl and triazophos.

When bottles were closed during processing, the average recovery obtained for the target pesticides was 45,5 %. An average of 25,5 % recoveries was obtained when the bottles were left slightly open (due to autoclave operational conditions).

Figure 45 relates to the sterilization process with closed jars, which reduced to a great extent the evaporation during sterilization in an autoclave. If only degradation due to heat is considered, different pesticides show different degradation rates. For example, phorate has a high vapor pressure (Vp = 85 mPa) at 25 °C, and it is therefore expected to volatilize easily, when compared to the other pesticides on the target list. Phorate hydrolysis occurs at rates dependent upon the temperature and pH [57]. Chlorpyrifos-methyl is referred to be stable only at room temperature storage conditions, so reduced stability at 121 °C is expected. Diazinon decomposes at >120 °C [57].

The average recovery for the target analytes obtained during the sterilization process with closed jars was 45.5 %. Method repeatability was below 10 % for all target analytes. The method validation performance criterion for repeatability was therefore met for all pesticides except for azinphos-methyl, cypermethrin and triazophos. Although compared with the previous processing methods lower recoveries of pesticides were obtained, these are still in a quantifiable range and further discussion is needed in order to consider whether or not the sterilization process to stabilize pesticides in carrots baby food is indeed a viable option and to design proper long term storage conditions.

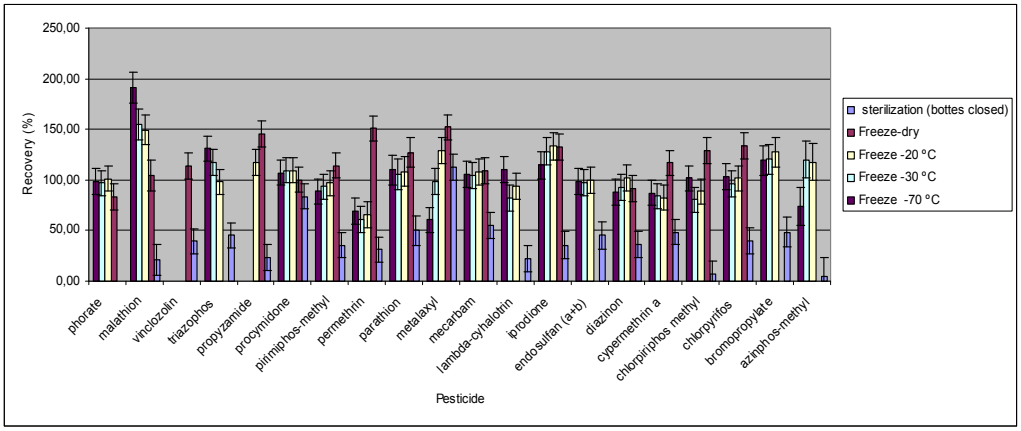


Figure 46: Analytes recovery (%)-processed/ wet initial sample-for the freeze, freeze drying and sterilization processes of carrot/potato baby food. Recoveries (%) of malathion were on average 120 % for freeze and freeze–drying processes and 21 % for the sterilization process.

It is seen from Figure 46 that for several pesticides and especially with the freeze-dried and freezen samples, recoveries higher than 100 % were encountered. As sample intake was carefully controlled to ensure data comparability, this fact could not be due to inacurracies in sample preparation. Taking into consideration the time frame necessary to perform all analyses, it is expected that increasing contamination of the analytical system has occurred leading to formation of new active sites and inacurracies of the measurements in time.Nevertheless, the main objective was achieved, because among the three tested methods for stabilizing the analytes in the matrix significant differences in recoveries of the pesticides were observed. Sterilisation appeared to be less suitable to stabilize the target pesticides, while freezing and freeze-drying preserved almost all the target pesticides and did not generate processing artifacts interefering with the analytical method applied.

9. Feasibility study for the production of candidate reference materials of plant origin containing pesticides

In the feasibility study three different matrices were investigated for the development of appropriate freeze-drying programs as mentioned above. Spinach, orange and a carrot/potato mixture were tested. It was found that the two matrices based on vegetables were easier to freeze-dry than the fruit based material due to their lower sugar content. In addition, one matrix (carrot/potato) was freeze-dried as a blank as well as spiked. The resulting dry matrices were milled and the powder was homogenised and checked for water content and PSA. Initial GC-MS experiments were also carried out on the spiked matrix.

9.1 Selection of raw material

After the initial experiments with optimisation of the freeze drying process it was decided to use the carrot/potato matrix from Olvarit/Nutricia (Bornem, Belgium) for further studies. The material packed in glass jars was bought at a local supermarket and brought to the IRMM by car. The material used for the feasibility study was slightly different from the material used for the method validation, as the supplier had changed the composition. The water content was raised by 0.5% and the rice content was reduced by 0.5 % (m/m). This resulted in a slightly different colour of the matrix as shown in Figure 47.

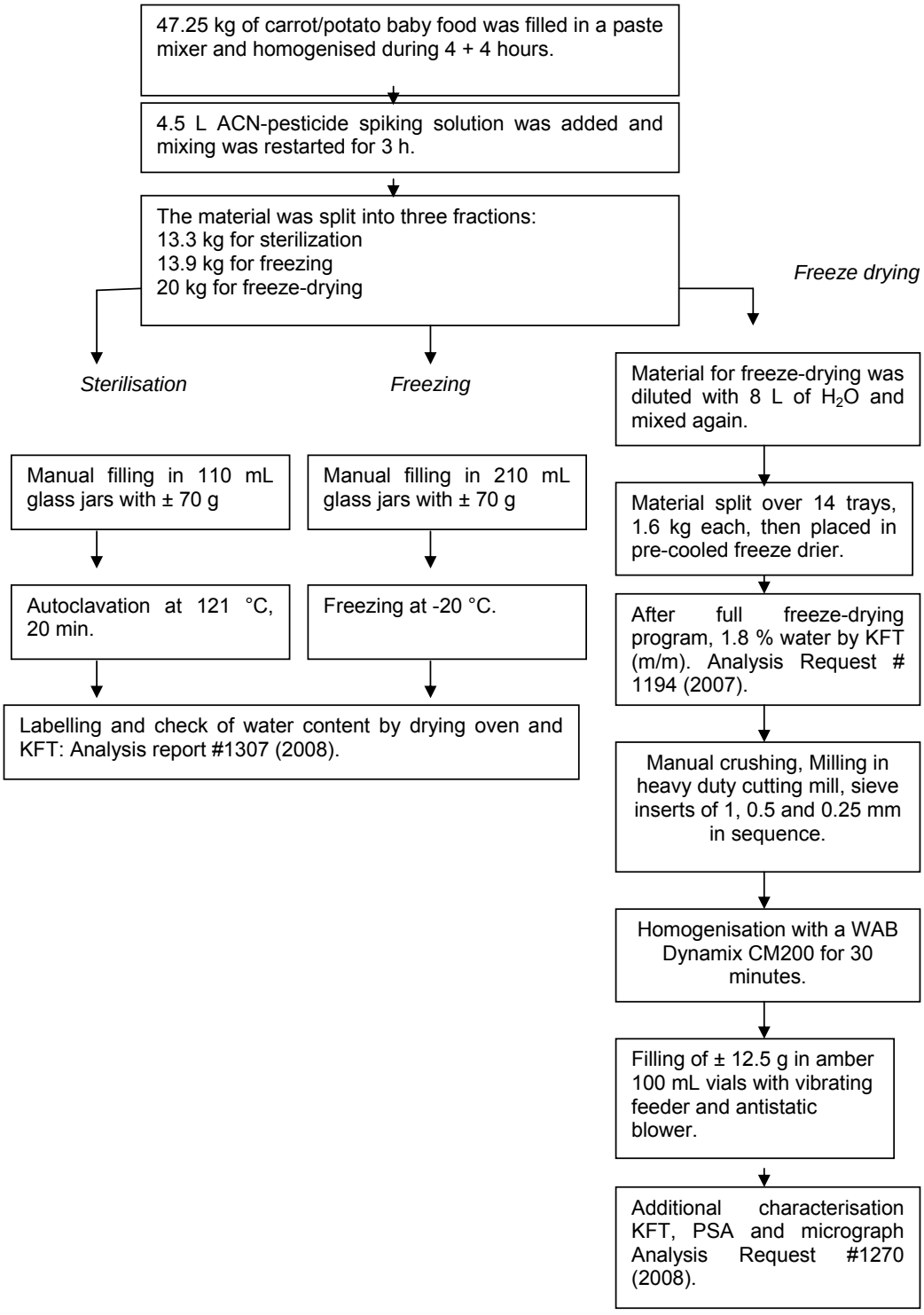
Figure 47: Different colour between the two carrot/potato batches.

9.2 Preparation of the bulk raw material

Six kg and 75 g of the "old" batch and 40.5 kg of the "new" batch of the carrot/potato mixture were used in this study. The baby food was placed in a stainless steel mixing vessel which is part of a mixer for paste assembly (IKA-Janke Kunkel, Staufen, Germany) and mixed at full speed with a change of direction every 15 minutes to ensure good homogenisation. Mixing was done for 4 hours, stopped over night and then mixed for 4 hours the next day. Subsequently 4.5 L of acetonitrile spiking solution, containing the 21 pesticides in acetonitrile, was added. Thereafter the stirring continued for 3 hours in the same manner as described above. After the addition of the spike and after through mixing the bulk material was split into three parts:

1. Fraction of 13.3 kg for sterilization
2. Fraction of 13.9 kg for freezing
3. Fraction of 20.0 kg for freeze-drying

9.3 Flow chart for the preparation of carrot with potato candidate RMs.



9.4 Freeze-drying

Fraction three which was destined for freeze drying had to be diluted with 8 L of demineralised H_2O and homogenised further before it was spread over 14 freeze-drying trays. Thereafter they were placed into the pre-cooled freeze-dryer; model Epsilon 2-85D (Martin Christ, Osterode, Germany). Fraction one and two were kept over night in a fridge at +4 °C. Each of the 14 trays was filled with 1.6 kg of the homogenised slurry. The freeze drying programme developed during the initial studies was used. Two Pt100 sensors and one lyo-control sensor were placed in the material contained in the trays placed high, in the middle and low in the drying chamber. Care was taken that the probes did not touch the bottom of the trays (as to give an erroneous temperature read-out). Thereafter the freeze-drying program with duration of about 5 days was started with the typical sequence: Freezing, sublimation, and secondary drying as depicted in Fig 48. The water content was checked after the freeze drying cycle before further manipulation and water content was1.97 and 1.66 % (m/m), using Karl Fischer titration measurements.

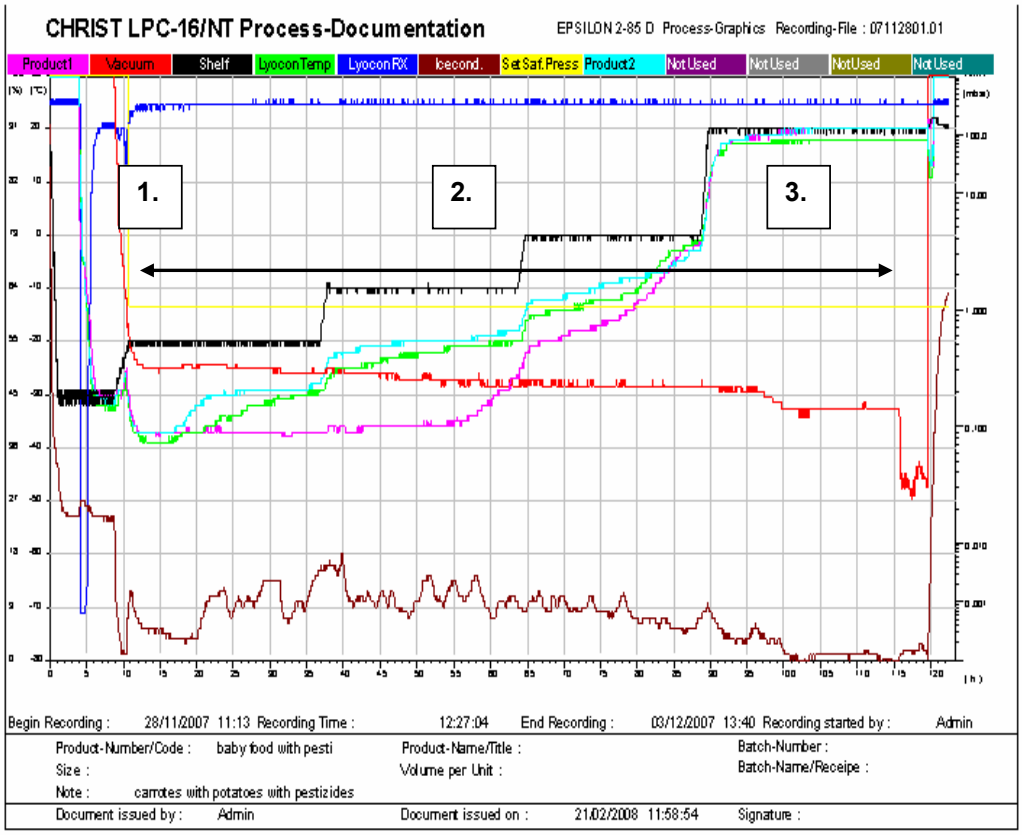


Figure 48: Graphical representation of freeze-drying cycle with explanation of the coloured traces at the top. Number 1 depicts the pre-freezing step, number 2 the sublimation step and number 3 the secondary drying step, respectively.

9.5 Milling

The freeze-dried material was manually crushed with a PFTE pestle and then it was milled with a Retsch (Haan, Germany) heavy duty cutting mill with 1.0, 0.5 and subsequently a 0.25 mm sieve insert. A total amount of 2 kg was available after milling. To prevent inhalation of fine dust particles with pesticides an FFP3 breathing mask was used when manipulating the dry spiked material.

9.6 Homogenisation

The homogenisation with a three-dimensional mixing action was performed in one run of 0.5 h in the Dyna-MIX CM200 mixer (WAB, Basel, Switzerland).

9.7 Filling

Filling of about 12.5 g of the carrot/potato powder into 100 mL amber glass vials was performed using a vibrating feeder and an antistatic blower. A total number of 156 units were filled in this way and additionally 3 units with 12, 19 and respectively 20 g were obtained.

9.8 Capping and labelling

Capping of the material, using Teflon screw caps, was done automatically in a capping machine from Bausch & Ströbel (Ilshofen, Germany). The capping machine was operated at 10 vials per minute which is an appropriate speed for the on-line water measurement as well as for the operators who manually loaded and unloaded the vials from the assembly.

9.9 Freezing and sterilization

The fractions kept for freezing and sterilization were manually filled in 100 mL glass jars with about 70 g per jar. For freezing as well as for the sterilization 156 jars were filled, respectively. The material to be kept frozen was stored at -20 °C and the material to be sterilized (autoclaved), was treated in a Matachana B-4023 autoclave (Webeco, Ober-Ramstadt, Germany) and thereafter stored at
+4 °C.

10. Online measurement of water by AOTF-NIR

10.1 Introduction

A Luminar 4030 Acusto-Optical Tunable Filter Near Infrared Spectrometer (AOTF-NIR, Applitek, Nazareth, Belgium) was placed in the capping machine which provided a suitable measurement frequency of 10 vials/min. Each measurement commences with a trigger signal for reproducible collection of spectra as soon as a vial passes in front of a sensor placed next to the AOTF-NIR instrument. From each vial one hundred spectra were obtained in the range 1300 nm to 2100 nm with a 2 nm increment. The transmittance spectra were then mathematically transformed, first to absorbance spectra and then translated to Unscrambler® files (CAMO, Oslo, Norway). In Unscrambler® the water content in each sample was predicted by using a PLS model, with three principal components. The model was developed using calibrants prepared in meat powder in the range from about 1 % water (m/m) to 8 % water (m/m). Kestens et al. has described the AOTF-NIR setup in detail [58].

10.2 Results of water content for the carrot/potato powder

The water content in the carrot/potato material was measured with high accuracy using Karl Fischer titration (KFT) operated under ISO 17025 as given in Table 23 and Table 24. The AOTF-NIR results are in good agreement with the KFT results as shown below.

Table 23: Comparison of results between volumetric-KFT and AOTF-NIR with the number of replicates mentioned in parenthesis. Note that for the AOTF the spread given is ± one standard deviation. For the KFT measurements the spread is expanded uncertainty (k=2).

MATRIX	% H_2O (m/m) AOTF-NIR	% H_2O (m/m) V-KFT
carrot/potato	2.4 ± 0.4 (156)	2.3 ± 0.3 (5)

In graphical mode is expressed the water content in the carrot/potato material for the overall samples analysed (Figure 49).

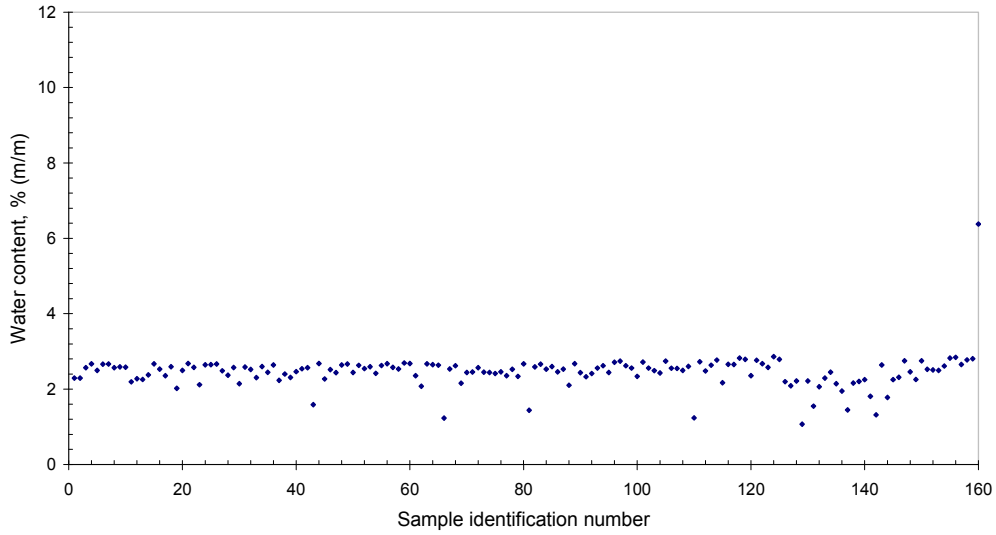


Figure 49: Typical results for the water content in the carrot/potato material. On the Y axis the unit is % H_2O (m/m) and the overall result is 2.4 ± 0.4 %.

10.3 Micrographs

Micrographs are a valuable complement to sieve analysis and particle size distribution measurements because they reveal different fractions due to shape and colour differences and they provide an accurate estimate of the

particle size based on direct comparison with a certified length scale for individual particles.

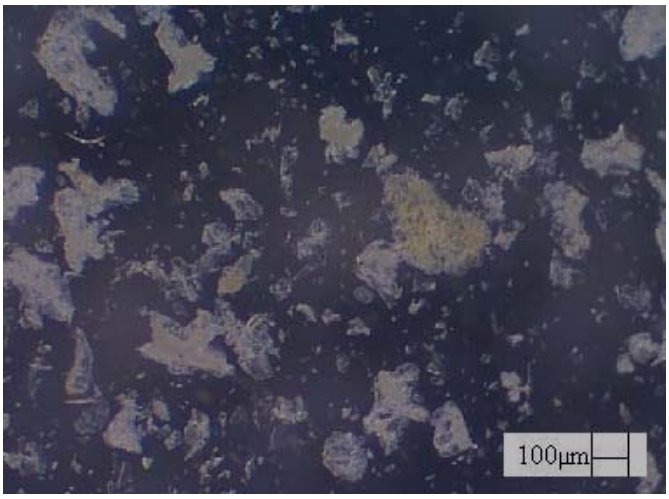


Figure 50: Micrograph of the final product, baby food carrot/potato.

As can be seen in the micrograph (Fig. 50), major particles are in the range of 250 µm which coincide with the results of the particle size analysis. In this way micrographs are also very useful in confirming the PSA results.

10.4 Comparison KFT and oven drying

From Table 24 it can clearly be seen that KFT is rather imprecise at high water concentrations whereas the oven method is more precise. Although the KFT is selective for water only the oven method would also detect remaining solvent from the spike solution. A small difference exists between the averages for KFT and drying oven which could be interpreted as if the amount of remaining solvent is in the range of 2-3 % (m/m). Unfortunately the the KFT data is not precise enough to allow an unambiguous assessment about the remaining amount of solvent. Based on the oven drying data it is nevertheless clear that no major difference between sterilised and frozen matrix has been found with respect to water content.

Table 24: Comparison between KFT and oven drying of the frozen (F) and the sterilised material (ST), the first ten results are given for KFT for which an uncertainty is also reported. The last ten results show the drying oven data. All data come from Analysis report 1307 (2008).

Unique RM Sample ID / Vial number / Treatment	Water content % (m/m), ± expanded uncertainty	Average water content per technique and treatment, (n = 5)
8325 / 0038 / F	88.5 ± 13.1	87.8
8326 / 0003 / F	88.3 ± 13.1	
8327 / 0077 / F	88.6 ± 13.1	
8328 / 0154 / F	89.9 ± 13.3	
8329 / 0059 / F	83.8 ± 12.4	
8330 / 0043 / ST	84.4 ± 12.5	86.0
8331 / 0028 / ST	79.7 ± 11.8	
8332 / 0003 / ST	90.7 ± 13.4	
8333 / 0010 / ST	84.3 ± 12.5	
8334 / 0021 / ST	91.0 ± 13.5	
8325 / 0038 / F	90.1	89.2
8326 / 0003 / F	89.1	
8327 / 0077 / F	88.9	
8328 / 0154 / F	89.1	
8329 / 0059 / F	88.9	
8330 / 0043 / ST	89.0	89.1
8331 / 0028 / ST	89.0	
8332 / 0003 / ST	89.1	
8333 / 0010 / ST	89.2	
8334 / 0021 / ST	89.1	

10.5 Particle size analysis, PSA

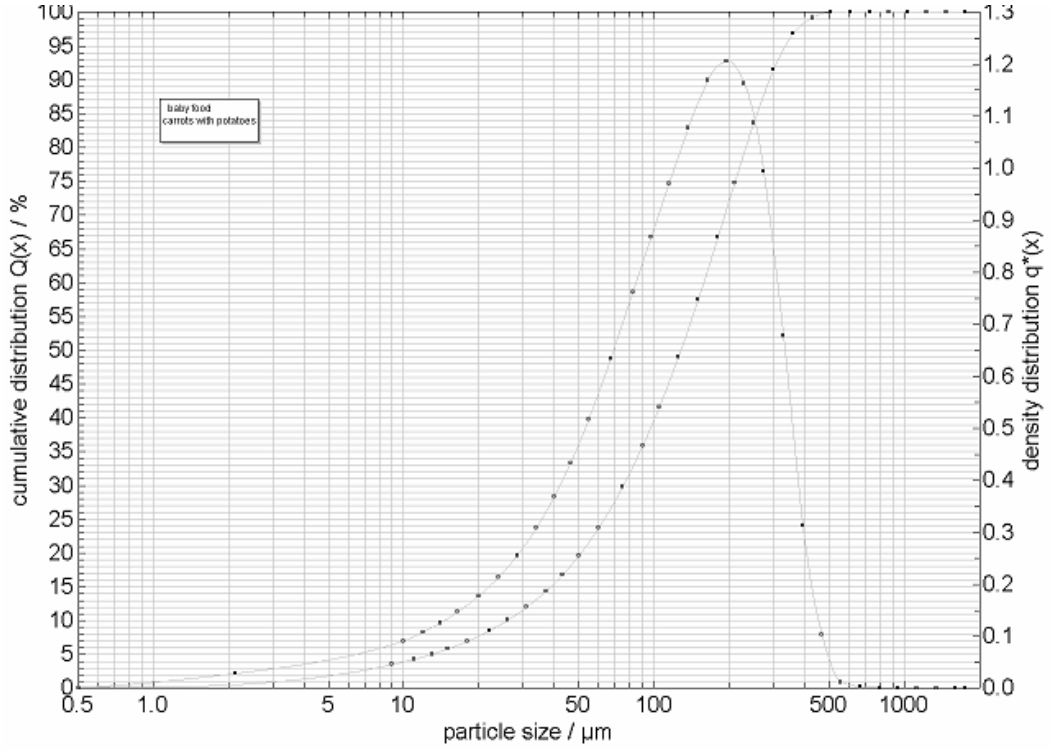


Figure 51: Average particle size distribution curves for five different samples of carrot/potato powder of the units allocated for additional characterisation (bottle 0003; 0010, 0021, 0035, and 0055) each measured twice using RM WI/0042. Optical concentration was 21 % on average using the cuvette and 2-propanol as dispersant using a Sympatec Helos laser light scattering instrument (Clausthal-Zellerfeld, Germany).

Table 25: Average particle size, absolute and relative standard deviations for the predefined cumulative distributions X_0. Highlighted average values are used for the calculation of deviation in % between the cumulative particle size distribution curves (n=5).

Upper band limit	Average particle size / µm, (n = 5)	Standard deviation / µm	Relative standard deviation/ %
X_{10}	26.08	1.04	0.04
X_{16}	41.34	1.39	0.03
X_{50}	128.30	3.53	0.03
X_{84}	253.42	5.95	0.02
X_{90}	291.23	6.44	0.02

As an overall assessment of comparability between the different units, the average deviation in % for X_{10}, X_{50} and X_{90} can be calculated in comparison with the average particle size for all measurements. When scrutinizing the data for the five measurements, it can be concluded that the average deviation for X_{10}, X_{50} and X_{90} from the average particle size was varying as given in Table 26. Generally if the result stays below 20 % average deviation for the X_{10}, X_{50} and X_{90}, the result is acceptable. This quality criterion is based on the experience acquired in the processing sector over many years and what can be observed for many different kinds of materials. It should be pointed out that if X_{10} has a negative deviation, X_{50} and X_{90} are also very likely to have a negative deviation from the average. As can be seen from the data here the result is below 20 %. To calculate the values in Table 26 equation 9 was used. Here an example for X_{10} is shown:

$$((X_{10\_repl1} - X_{10\_average}) / X_{10\_average})*100 \quad (9)$$

Table 26: Average deviation in % of X_{10}, X_{50} and X_{90} from the average of all measurements. (n = 5,) calculated with eq. 9. See analysis report 1278 (2008) for details.

Sample ID number and replicate	**Average deviation of X_{10}, X_{50} and X_{90} from average of all measurements, %**
8153 rep a	-3.7
8153 rep b	-2.1
8154 rep a	0.25
8154 rep b	-2.3
8155 rep a	1.5
8155 rep b	0.3
8156 rep a	-0.9
8156 rep b	1.2
8157 rep a	5.2
8157 rep b	0.6

10.5.1 Final product and number of units produced

In total 156 units were produced for each of the technological processes. The units containing the freeze-dried materials contained 10 g, while the wet materials contained 70 g per unit. The content of pesticides in the processed matrices was determined by the validated QuECHERS method (Table 27). Suspicious results were found for azinphos-methyl, azoxystrobin, mecarbam, procymidone and triazophos in the frozen batch, and propyzamide in the sterilized batch, which could be due to integration erros or interactions in the chromatographic system (injector).

Table 27: Results of screening measurements on the content (ng/g dry matter) of the target pesticide analytes in the test materials (frozen, freeze dried and sterilized matrices), using the average results on 3 replicates. The ratio wet/dry mass is 10 % (dry mass wet batch/dry mass freeze dried batch*100).

	Spiking level (ng/g dry matter)	Mass fraction (ng/g dry matter)		
		Frozen	Freeze dried	Sterilized
azinphos-methyl	499.3	**560.5**	287.8	At LOQ
azoxystrobin	486.2	419.8	405.2	**505.7**
bromopropylate	487.4	386.3	362.4	426.4
chlorpyrifos	488.5	399.7	330.3	365.7
chlorpyrifos-methyl	492.2	382.7	206.7	At LOQ
cypermethrin	556.3	316.6	421.9	486.9
diazinon	118.9	104.8	69.7	45.9
endosulfan (a+b)	482.9	357.9	302.6	330.2
iprodione	189.4	170.3	120.6	At LOQ
lambda-cyhalotrin	191.9	115.3	145.2	128.7
malathion	4479.3	4499.8	2845.8	612.8
mecarbam	528.9	**635.6**	409.8	234.9
metalaxyl	479.2	440.2	354.6	457.2
parathion	508.5	453.6	357.9	300.2
phorate	499.0	487.3	125.5	Not detected
permethrin	491.1	300.7	347.3	367.9
pirimiphos-methyl	511.5	487.3	321.7	220.4
procymidone	196.5	**231.6**	167.8	170.2
propyzamide	226.9	200.9	176.9	220.7
triazophos	195.3	**231.3**	170.5	At LOQ
vinclozolin	517.6	450.12	360.2	189.4

Figure 52: The final product of carrot/potato baby food

10.6 Conclusions

It may be argued that the large quantity of solvent added to the bulk matrix radically changes the matrix in comparison with naturally contaminated samples. First of all one must realise that no naturally contaminated samples should reach the market (which is the case for PCBs in mackerel). Indeed, the absence of pesticides in the blank material was verified analytically. Secondly, in order to achieve a homogeneous distribution of the target pesticides with a reasonable effort of work it is better to keep the dilution factor low implying a rather lower volume of solvent. Thirdly, one may also anticipate that part of the solvent actually escapes during mixing before further manipulation of the material although it is not known exactly to which extent. Results obtained by Karl Fischer titration and drying oven suggest that 2-3 % (m/m) of solvent remains in the sterilised and frozen matrices.

11. Homogeneity of the candidate reference material

A homogeneity study was carried out for the three candidate reference materials. A minimum sample intake for all test materials has also been defined. The minimum sample intake obtained is equal to 10 g regarding frozen and sterilized batches and 1.5 g in the freeze dried batch, taking into account the water content in the wet and freeze dried test materials. Although for some analytes a sample intake of about 8 g did not introduce a significant variability of the within-jar measurements, one must bear in mind that a multiresidue extraction method is employed and therefore the minimum sample intake should be the same for all target analytes.

11.1 Planning of homogeneity assessment

The planning was based on the envisaged uncertainty of homogeneity (u_{bb}). Although the actual degree of homogeneity is a material property that cannot be assessed on beforehand, it is possible to plan the homogeneity study in a way that allows detecting a certain degree of inhomogeneity. Therefore, planning of the number of replicates per unit should be based on the maximum degree of inhomogeneity that can be hidden by method variation (u^*_{bb})–see experimental protocol for detailed calculations (Annex 1).

For each processed batch of samples (frozen, freeze dried and sterilized matrices), 10 jars and 3 replicates of each jar were analysed for the target pesticides with the in-house validated QuEChERS method. A random stratified sampling was done covering the whole batch (156 jars).

A matrix matched calibration curve, and the three labelled internal standards parathion-ethyl (diethyl-D10, 100 µg/mL in nonane), phorate (dietoxy-^{13}C4, 100 µg/mL in acetonitrile), pirimiphos-methyl (D6 100 ng/µLin acetone) were used to quantify the analytes of interest.

The extraction of the samples and the respective measurements were performed under repeatability conditions.

To minimize matrix effects, blank extracts were used to construct the matrix matched calibration. Only for the freeze dried batch, the processed blank matrix was used. For the other processed batches (frozen and sterilized), blank wet carrots was used for the calibration curve. This and the fact that only 3 internal standards were used for quantification might compromise the accuracy of the results but the overall objective was to assess the relation between the sample measurements. This is achieved by using repeatability conditions (e.g a calibration curve and extractions/measurements done in the same day/short interval of time) for each batch of measurements.

11.2 Data Evaluation

The aim of this evaluation was to determine if the variation between jars of each batch would significantly influence the certified uncertainty of a future matrix reference material containing pesticides at the MRL (mg/kg) level. Evaluation of homogeneity studies for each batch (frozen, freeze dried and sterilization) was done by means of evaluating the following parameters using SoftCRM software:

- outliers
- trends in the analytical sequence
- trends in the filling sequence
- the distribution of individual results using histograms and the evaluation of individual/ sample means using normal probability plots.

Single and double Grubbs-tests were performed to detect potentially outlying individual results as well as outlying jar averages.

- For the frozen batch, no outlying individual result was found, but one to two outlying jars average were found for bromopropylate, chlorpyrifos, chlorpyrifos-methyl, endosulfan (a+b) and propyzamide
- (jar 78, was common to all except propyzamide and jar 142 was an outlier for propyzamide at a 95 % level of confidence).

- Concerning the freeze dried batch the SoftCRM analysis showed that outliers in jar averages were found for chlorpyrifos-methyl (jar 105 and jar 32 at 95 % confidence level), pirimiphos methyl (jar 97 at 99 and 95 % level of confidence), parathion (jar 105 at 99 and 95 %), endosulfan (jar 105 at 95 %). Outlying individual results were found for diazinon (jar 8 at 95 % confidence level), pirimiphos–methyl (jar 97 at 95 and 99 % level of confidence), malathion (jar 8 at 95 and 99 % level of confidence), chlorpyrifos (jar 8 at 95 and 99 % level of confidence), mecarbam (jar 16 and 24 at 95 %), triazophos (jar 8 at 95 % level of confidence), azoxystrobin (jar 8 at 95 % confidence level), metalaxyl (jar 8 at 95 %) and parathion (jar 105 at 95 % level of confidence).
- With regard to the sterilized batch, Grubbs tests indicated average jar outliers for chlorpyrifos (jars 62 and 55 at a 95 % level of confidence) and diazinon (jars 62 and 48 at a 95 % level of confidence). Individual outliers were also found for the following pesticide analytes: azoxystrobin (jar 24), chlorpyrifos (jars 55 and 62), cypermethrin (jar 44), diazinon (jar 62), endosulfan (a+b) (jar 55), malathion (jar 62), parathion (jar 48), pirimiphos-methyl (jar 55) and vinclozolin (jar 55) (see Table 35).

As no technical reason for the outliers could be found, all the data were retained for statistical analysis.

Regression analysis was performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence.

- For the frozen batch some trends in the analytical sequence were visible (Table 28), for azinphos-methyl (at a 95 and 99 % level of confidence), iprodione (at 95 % and 99 %), lambda-cyhalotrin (at 95 %) and triazophos (at 95 %), pointing for the instability of the analytical system (e.g dirty injection system) for the quantification of these analytes. In the sample means a trend was found for azoxystrobin and triazophos at 95 and 99 % level of confidence.
- Concerning the freeze-dried batch of samples, iprodione showed a trend in the analytical sequence at both levels of confidence and azinphos-

methyl showed a trend in the analytical sequence at 95 but not at 99 % level of confidence. A filling trend was observed for azoxystrobin, diazinon and metalaxyl at 95 % level of confidence but not at 99 %.

- Analysis of the occurrence of trends in the analytical sequence or filling sequence for the sterilized batch showed an analytical trend at 95 % level of confidence but not at 99 % level, for bromopropylate, chlorpyrifos-methyl, diazinon, propyzamide, and vinclozolin. A filling trend for metalaxyl was detected at 95 and 99 % level of confidence.

Furthermore it was checked whether the individual data and bottle averages followed a normal distribution using normal probability plots and whether the individual data are unimodally distributed using histograms. Because all individual values and sample means of the three batch samples followed unimodal distributions, the results could be evaluated using analysis of variance (ANOVA).

The results of the descriptive evaluation are given in Tables 28, 29 and 30.All data was used for the homogeneity calculations. Although no potential outliers have been excluded from the calculations the uncertainty contribution of homogeneity in all test batches had an average below 7%.

Table 28: Results of the descriptive evaluation of the homogeneity study for the content (ng/g dry matter) of pesticides in the **frozen batch.**

Pesticide	Outliers		Significant trends (95% confidence)		Distribution of individual results		Distribution of bottle means	
	Individual values	Bottle average	Analytical sequence	Filling sequence	Normal	Unimodal	Normal	Unimodal
azinphos-methyl	No	No	yes	No	yes	yes	approx.	yes
azoxystrobin	No	No	No	yes	No	yes	No	yes
bromopropylate	No	2	No	No	yes	yes	yes	yes
chlorpyriphos	No	1	No	No	yes	yes	Approx.	yes
chlorpyriphos-methyl	No	1	No	No	yes	yes	yes	yes
cypermethrin	No	No	No	No	yes	yes	yes	yes
diazinon	No	No	No	No	yes	yes	yes	yes
endosulfan a+b	No	1	No	No	yes	yes	approx	yes
iprodione	No	No	Yes	No	yes	yes	approx	yes
lambda-cyhalotrin	No	No	yes	No	yes	yes	yes	yes
malathion	No	No	No	No	yes		Approx.	
mecarbam	No	No	No	No	yes	yes	yes	yes
metalaxyl	No	No	No	No	yes	yes	yes	yes
parathion	No	No	No	No	yes	yes	yes	yes
permethrin	No	No	No	No	yes	yes	Approx	yes
phorate	No	No	No	No	yes	yes	yes	yes
pirimiphos-methyl	No	No	No	No	yes	yes	yes	yes
procymidone	No	No	No	No	yes	yes	Approx.	yes
propyzamide	No	1	No	No	yes	yes	yes	yes
triazophos	No	No	yes	yes	yes	yes	Approx.	yes
vinclozolin	No	No	No	No	yes	yes	yes	yes

Table 29: Results of the descriptive evaluation of the homogeneity study for the content (ng/g dry matter) of pesticides in the **Sterilized batch.**

Pesticide	Outliers		Significant trends (95% confidence)		Distribution of individual results		Distribution of bottle means	
	Individual values	Bottle average	Analytical sequence	Filling sequence	Normal	Unimodal	Normal	Unimodal
azoxystrobin	1	No	No	No	yes	yes	yes	yes
bromopropylate	No	No	yes	No	yes	yes	yes	yes
chlorpyriphos	1	1	No	No	Approx.	yes	Approx.	yes
chlorpyriphos-methyl	No	No	yes	No	yes	yes	yes	yes
cypermethrin	1	No	No	No	yes	yes	yes	yes
diazinon	1	2	yes	no	Approx.	yes	Approx.	yes
endosulfan a+b	1	No	No	No	yes	yes	yes	yes
lambda-cyhalotrin	No	No	No	No	yes	yes	yes	yes
malathion	1	No	No	No	yes	yes	yes	yes
mecarbam	No	No	No	No	yes	yes	yes	yes
metalaxyl	No	No	No	yes	Approx.	yes	Approx.	yes
parathion	1	No	No	No	yes	yes	yes	yes
permethrin	No	No	No	No	yes	yes	yes	yes
pirimiphos-methyl	1	No	No	No	yes	yes	yes	yes
procymidone	No	No	No	No	yes	yes	yes	yes
propyzamide	No	No	yes	No	yes	yes	yes	yes
vinclozolin	1	No	yes	No	yes	yes	yes	yes

Table 30: Results of the descriptive evaluation of the homogeneity study for the content (ng/g dry matter) of pesticides in the **Freeze dried batch.**

Pesticide	Outliers		Significant trends (95% confidence)		Distribution of individual results		Distribution of bottle means	
	Individual values	Bottle average	Analytical sequence	Filling sequence	Normal	Unimodal	Normal	Unimodal
azinphos-methyl	No	No	yes	No	yes	yes	approx.	yes
azoxystrobin	No	No	No	yes	No	yes	No	yes
bromopropylate	No	2	No	No	yes	yes	yes	yes
chlorpyriphos	No	1	No	No	yes	yes	Approx.	yes
chlorpyriphos-methyl	No	1	No	No	yes	yes	yes	yes
cypermethrin	No	No	No	No	yes	yes	yes	yes
diazinon	No	No	No	No	yes	yes	yes	yes
endosulfan a+b	No	1	No	No	yes	yes	approx	yes
iprodione	No	No	Yes	No	yes	yes	approx	yes
lambda-cyhalotrin	No	No	yes	No	yes	yes	yes	yes
malathion	No	No	No	No	yes		Approx.	
mecarbam	No	No	No	No	yes	yes	yes	yes
metalaxyl	No	No	No	No	yes	yes	yes	yes
parathion	No	No	No	No	yes	yes	yes	yes
permethrin	No	No	No	No	yes	yes	Approx	yes
phorate	No	No	No	No	yes	yes	yes	yes
pirimiphos-methyl	No	No	No	No	yes	yes	yes	yes
procymidone	No	No	No	No	yes	yes	Approx.	yes
propyzamide	No	1	No	No	yes	yes	yes	yes
triazophos	No	No	yes	yes	yes	yes	Approx.	yes
vinclozolin	No	No	No	No	yes	yes	yes	yes

The standard deviations within jars (s_{wb}) and between jars (s_{bb}) as well as the maximum heterogeneity that could be hidden by the method repeatability (u^*_{bb}) were calculated. The (s_{wb}) is equivalent to the analytical variation if the individual subsamples were representative for the whole jar.

The s_{bb} expressed as a relative standard deviation is given by the following equation (1):

$$S_{bb} = \frac{\sqrt{\frac{MS_{among} - MS_{within}}{n}}}{\bar{Y}} \qquad (1)$$

Where:

MS_{among}-mean square among bottles from an ANOVA

MS_{within}-mean square within a bottle from an ANOVA

n- average number of replicates per bottle

$\bar{Y}$ - average of all results of the homogeneity study

The u^*_{bb} is defined as follows:

$$u^*_{bb} = \frac{RSD_{method}}{\sqrt{n}} * \sqrt[4]{\frac{2}{\gamma_{MSwithin}}} \qquad (2)$$

$\gamma_{MSwithin}$ -degrees of freedom of MS_{within}

Where:

$$RSD_{method} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad (3)$$

The results of the evaluation of the between–unit variation are summarized in the following tables for the frozen, freeze dried and sterilized batch. The larger value of S_{bb} or u^*_{bb} were used as uncertainty contribution for homogeneity.

Table 31: Results of homogeneity studies for the content (ng/g dry matter) of pesticide analytes in the frozen batch.

Pesticide	Average	S_{wb}		$\mathbf{S_{bb}}$		$\mathbf{u^*_{bb}}$	
	[ng/g dry matter]	[ng/g dry mater]	[%]	[ng/g dry matter]	[%]	[ng/g dry matter]	[%]
azinphos-methyl	578.2	62.8	10.9	n.c.	n.c	20.4	**3.5**
azoxystrobin	427.0	29.7	7.4	140.9	**33**	9.7	2.3
bromopropylate	394.9	28.1	7.1	8.8	2.2	9.1	**2.3**
chlorpyriphos	416.2	30.9	7.4	14.7	**3.5**	10.1	2.4
chlorpyriphos-methyl	391.7	24.6	6.3	n.c.	n.c	7.9	**2.0**
cypermethrin	324.1	28.6	8.8	6.1	1.9	9.3	**2.9**
diazinon	110.3	5.2	4.7	n.c.	n.c.	1.7	**1.5**
endosulfan a+b	366.4	20.6	5.6	9.32	**2.5**	6.7	1.8
iprodione	181.9	16.7	9.2	n.c	n.c	5.4	**3.0**
lambda-cyhalotrin	117.3	10.8	9.2	n.c.	n.c	3.5	**3.0**
malathion	4514.7	108.8	2.4	57.1	**1.3**	35.3	0.8
mecarbam	662.8	22.8	3.4	n.c.	n.c.	7.4	**1.1**
metalaxyl	450.8	31.8	7.0	n.c.	n.c.	10.2	**2.3**
parathion	540.9	23.4	4.3	4.2	0.8	7.6	**1.4**
permethrin	309.2	26.1	8.4	9.1	**2.9**	8.5	2.7
phorate	490.1	34.9	7.1	n.c	n.c	11.4	**2.3**
pirimiphos-methyl	495.6	22.6	4.6	5.92	1.2	7.3	**1.5**
procymidone	243.5	7.5	3.1	2.45	1.0	2.5	**1.0**
propyzamide	216.8	13.2	6.1	n.c	n.c	4.3	**2.0**
triazophos	262.4	20.3	7.7	n.c.	n.c	6.6	**2.5**
vinclozolin	469.5	19.9	4.3	n.c.	n.c	6.5	**1.4**

n.c. not calculated as MSB<MSW.

Table 32: Results of homogeneity studies for the content (ng/g dry matter) of pesticide analytes in the **freeze dried** batch.

Pesticide	Average	S_{wb}		$\mathbf{S_{bb}}$		$\mathbf{u^*_{bb}}$	
	[ng/g dry matter]	[ng/g drymater]	[%]	[ng/g dry matter]	[%]	[ng/g dry matter]	[%]
azinphos-methyl	293.5	19.6	6.7	15.6	**5.3**	6.4	2.2
azoxystrobin	418.1	35.1	8.4	12.5	**3.0**	11.4	2.7
bromopropylate	366.9	16.6	4.5	n.c.	n.c.	5.4	**1.5**
chlorpyriphos	334.8	35.9	10.7	18.0	**5.4**	11.7	3.5
chlorpyriphos-methyl	211.5	7.4	3.5	3.0	**1.4**	2.4	1.1
cypermethrin	428.04	24.6	5.7	n.c.	n.c	7.9	**1.9**
diazinon	64.8	3.4	5.2	1.1	1.6	1.1	**1.7**
endosulfan a+b	303.1	11.8	3.9	5.6	**1.9**	3.8	1.3
iprodione	123.7	12.1	9.8	n.c.	n.c	3.9	**3.2**
lambda-cyhalotrin	152.3	6.71	4.4	2.1	1.4	2.2	**1.4**
malathion	2858.2	313.1	11	110.4	**3.9**	101.6	3.6
mecarbam	415.6	16.7	4.0	6.2	**1.5**	5.4	1.3
metalaxyl	360.5	54.8	15.2	22.7	**6.3**	17.8	4.9
parathion	377.1	11.6	3.1	8.2	**2.2**	3.8	1.0
permethrin	354.7	15.6	4.4	5.5	**1.5**	5.1	1.4
phorate	120.8	9.3	7.7	n.c.	n.c	3.0	**2.5**
pirimiphos-methyl	327.8	10.2	3.1	n.c.	n.c	3.3	**1.0**
procymidone	160.3	6.8	4.2	n.c.	n.c	2.2	**1.4**
propyzamide	175.6	11.5	6.6	5.5	**3.1**	3.7	2.1
triazophos	176.6	11.9	6.7	4.8	**2.7**	3.8	2.2
vinclozolin	368.2	12.16	3.3	6.49	**1.8**	3.9	1.1

n.c. not calculated as MSB<MSW.

Table 33: Results of homogeneity studies for the content (ng/g dry matter) of pesticide analytes in the **sterilized batch** of samples.

Pesticide	Average	S_{wb}		$\mathbf{S_{bb}}$		$\mathbf{u^*_{bb}}$	
	[ng/g $_{dry\ matter}$]	[ng/g $_{dry\ mater}$]	[%]	[ng/$_{g\ dry\ matter}$]	**[%]**	[ng/g $_{dry\ matter}$]	**[%]**
azoxystrobin	510.4	51.9	10.2	57.0	**11.2**	16.9	3.3
bromopropylate	431.5	22	5.1	19.7	**4.6**	7.1	1.7
chlorpyriphos	374	65.5	17.5	57.8	**15.4**	21.3	5.7
cypermethrin	482.2	53.4	11.1	38.2	**7.9**	17.4	3.6
diazinon	49.3	5.9	12	1.2	2.4	1.9	**3.9**
endosulfan a+b	335.9	14.3	4.3	9.3	**2.8**	4.6	1.4
lambda-cyhalotrin	131.4	6.4	4.8	8.6	**6.6**	2.1	1.6
malathion	617.3	67.1	10.9	66.3	**10.7**	21.8	3.5
mecarbam	239.3	6.9	2.9	10.5	**4.4**	2.2	0.9
metalaxyl	467.7	59.8	12.8	19.4	**4.2**	19.4	4.2
parathion	303.3	15.5	5.1	3.1	1.0	5.1	**1.7**
permethrin	387.2	20.2	5.2	23.8	**6.2**	6.5	1.7
pirimiphos-methyl	225.4	7.5	3.3	9.2	**4.1**	2.4	1.1
procymidone	171.2	6.7	3.9	3.7	**2.2**	2.2	1.3
propyzamide	221.3	14.8	6.7	n.c.	n.c.	4.8	2.2
vinclozolin	188.5	11.9	6.3	7.3	**3.9**	3.9	2.0

n.c. not calculated as MSB<MSW.

Based on the method repeatability and the set-up of the study, the average of the uncertainty contribution resulting from the homogeneity assessment for the target analytes in the carrots matrix was 6.1; 2.6 and 6.2 %, respectively, for the frozen, freeze dried and sterilized batches of samples.

In the Frozen batch azoxystrobin presented a high contribution, value of 33 %, whereas the remaining pesticides showed uncertainty contributions less than 4 %. With regard to the freeze dried batch equally most of the target analytes showed uncertainty contributions less than 4%, except azinphos-methyl, chlorpyriphos and metalaxyl. As far as the sterilized batch is concerned the opposite is true, most of the analytes showed a homogeneity uncertainty contribution equal or bigger than 4 %, except parathion,endosulfan (a+b) and procymidone.

Moreover, the determined content of all analytes in the homogeneity study was in agreement with the values (ng/g dry matter) obtaining during the screening measurements.

In many cases S_{bb} could not be calculated as MSB<MSW, this reveals that between bottle homogeneity was satisfactory. However it is important to point out, the S_{wb} parameter which includes the method and the within bottle variability, was in some cases (6) above 10%, and this occurred for different pesticides in the three batches of samples.

11.3 Minimum sample intake

Usual sample intakes for carrying out replicate measurements are 10 g for the frozen and sterilized batch and 1.5 g of freeze dried sample, considering an average water content of 90 % in the wet carrots baby food and 2 % in the freeze dried samples.

A series of independent analysis was performed (6 replicates), using decreasing amounts of sample (8, 6 and 4 g wet equivalent material). The minimum sample intake is defined as the amount of sample material before which the variability of results increases significantly when independent measurements are performed.

The guiding principle for quantifying the minimum sample intake must be that the variation of the analyte content due to the sample intake shall not contribute to the measurement uncertainty.

The relative standard deviations of the within jar measurements of the target analytes were compared using decreasing sample intakes. Figures 53-73 summarize the RSD [%] values for each processing type/analyte combination.

Although for some analytes a sample intake of about 8 g did not introduce a significant variability of the within-jar measurements, one must bear in mind that a multiresidue extraction method is employed and therefore the minimum sample intake should be the same for all target analytes. Ten gram sample was chosen as the minimum sample intake.

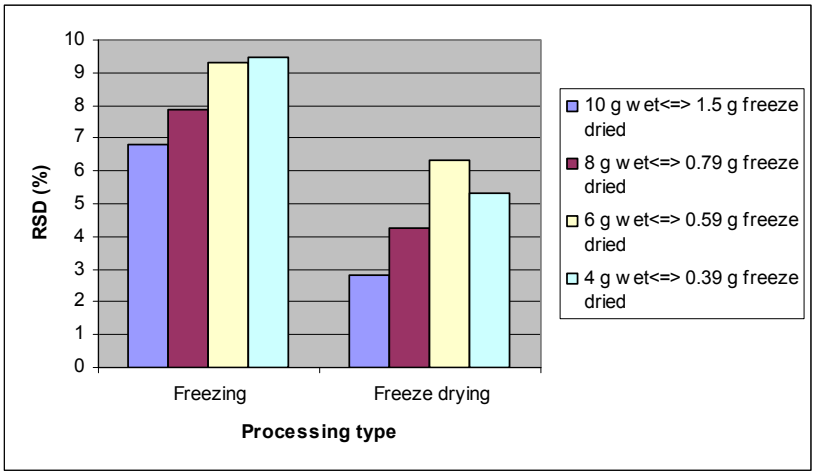


Figure 53: Relative standard deviation [%] of within-jar measurements for phorate, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

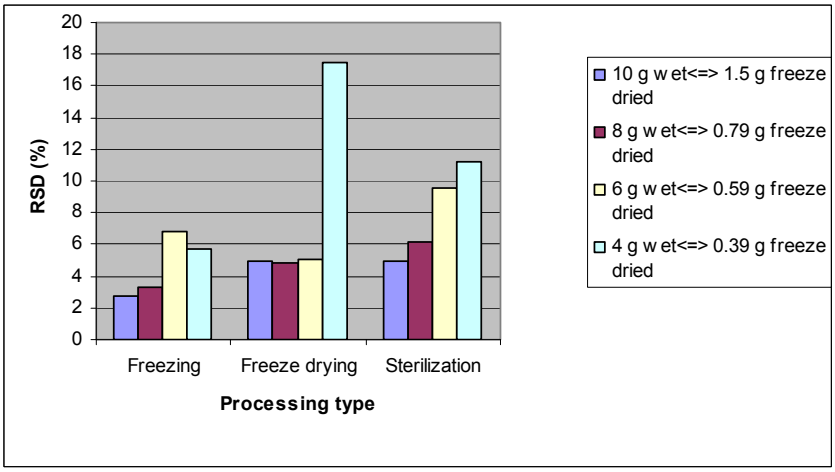


Figure 54: Relative standard deviation [%] of within-jar measurements for propyzamide, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

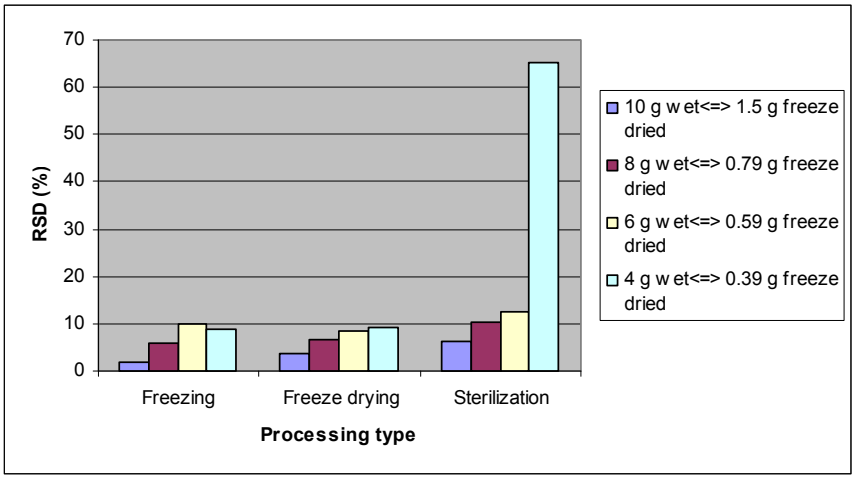


Figure 55: Relative standard deviation [%] of within-jar measurements for diazinon, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

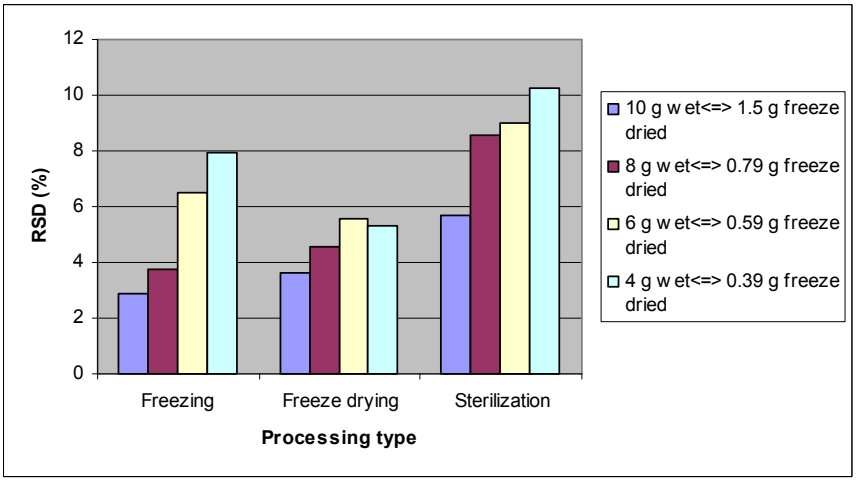


Figure 56: Relative standard deviation [%] of within-jar measurements for vinclozolin, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

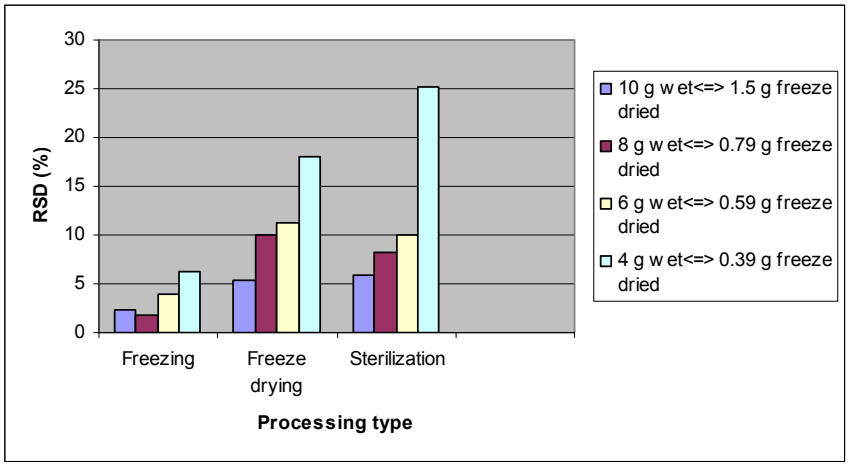


Figure 57: Relative standard deviation [%] of within-jar measurements for chlorpyrifos-methyl, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

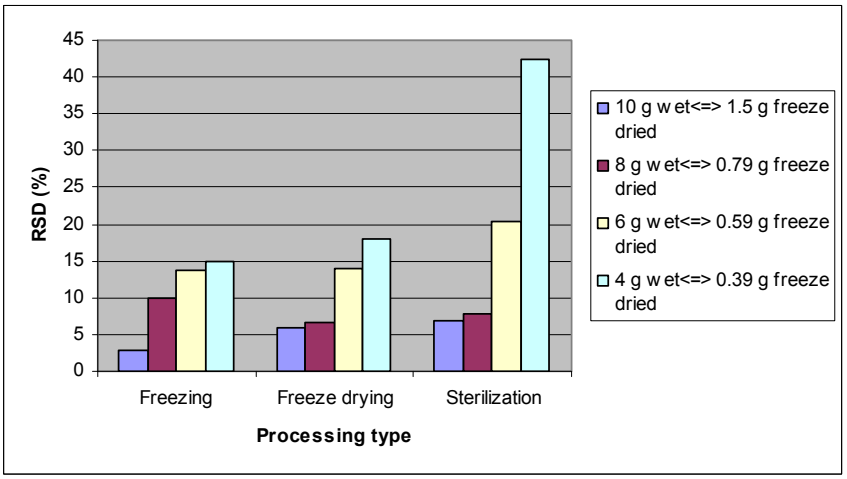


Figure 58: Relative standard deviation [%] of within-jar measurements for metalaxyl, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

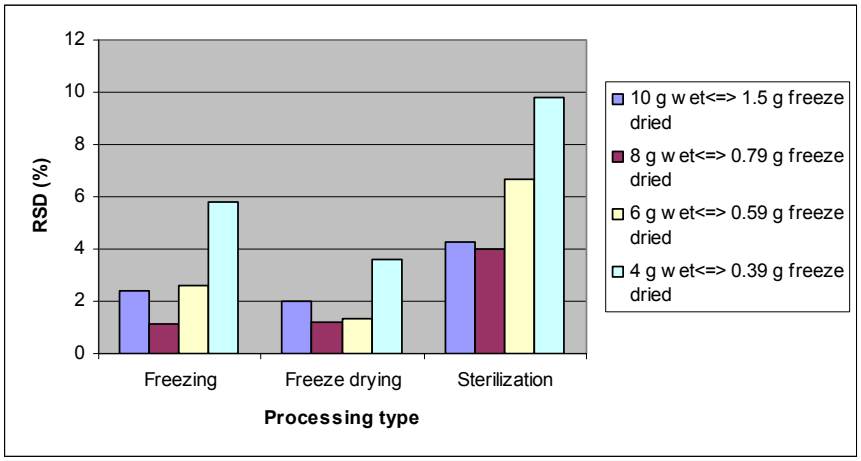


Figure 59: Relative standard deviation [%] of within-jar measurements for pirimiphos-methyl, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

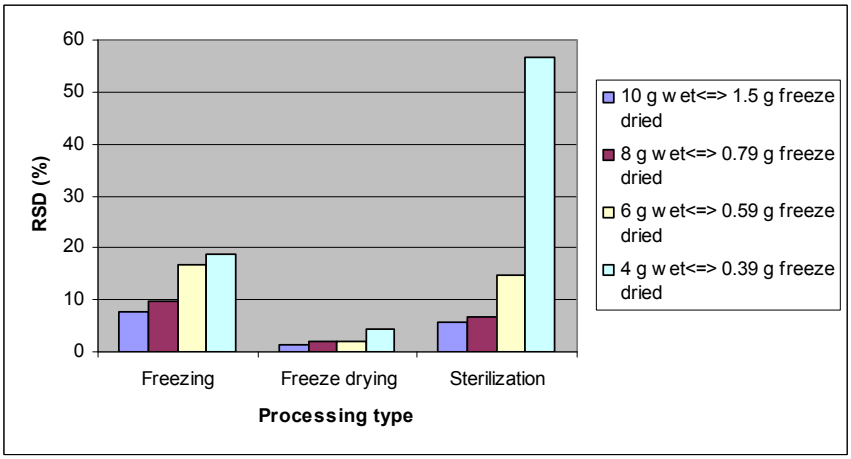


Figure 60: Relative standard deviation [%] of within-jar measurements for malathion, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

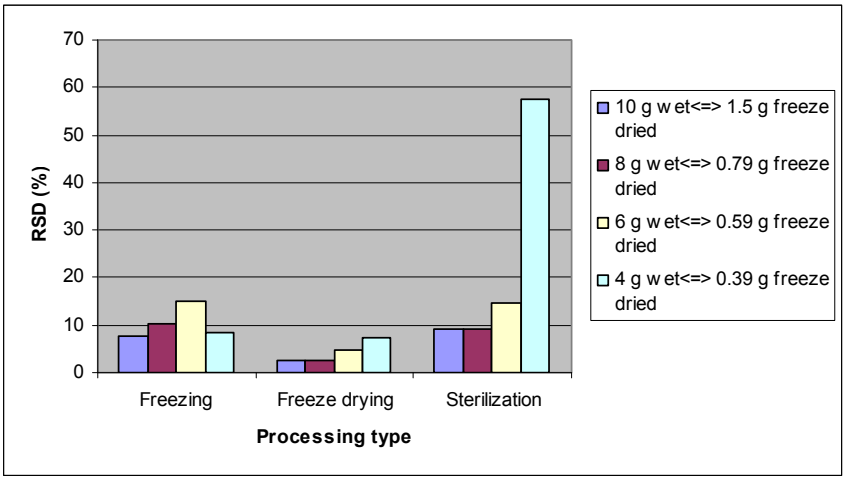


Figure 61: Relative standard deviation [%] of within-jar measurements for chlorpyrifos, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

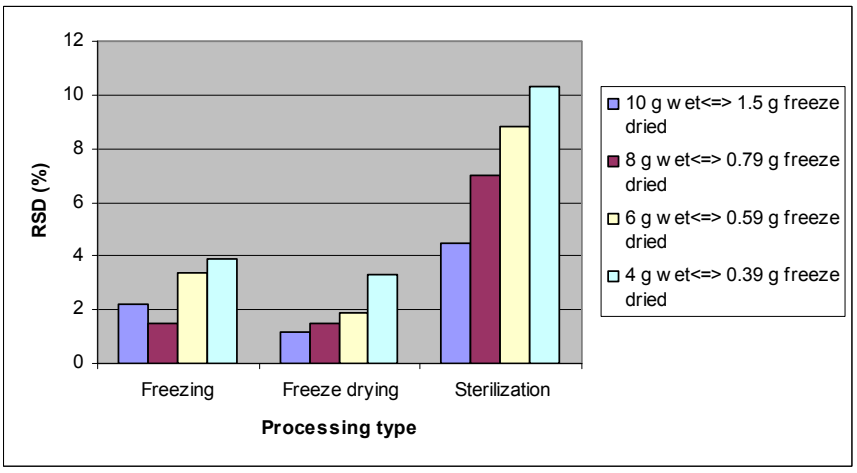


Figure 62: Relative standard deviation [%] of within-jar measurements for parathion, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

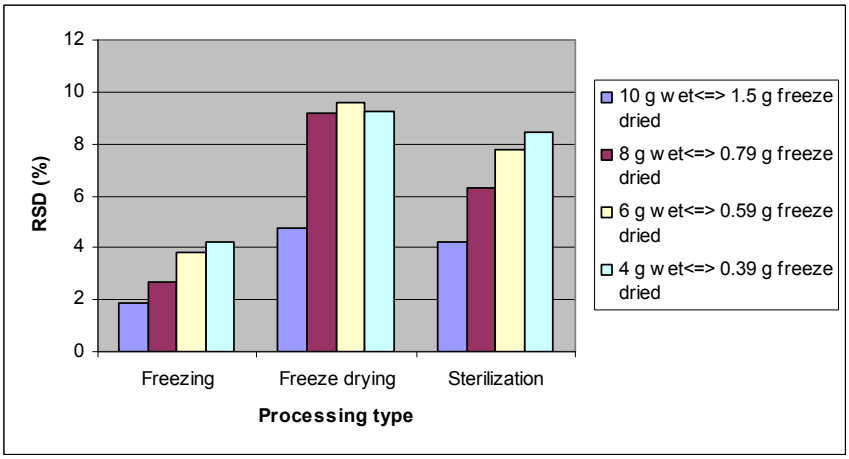


Figure 63: Relative standard deviation [%] of within-jar measurements for mecabam, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

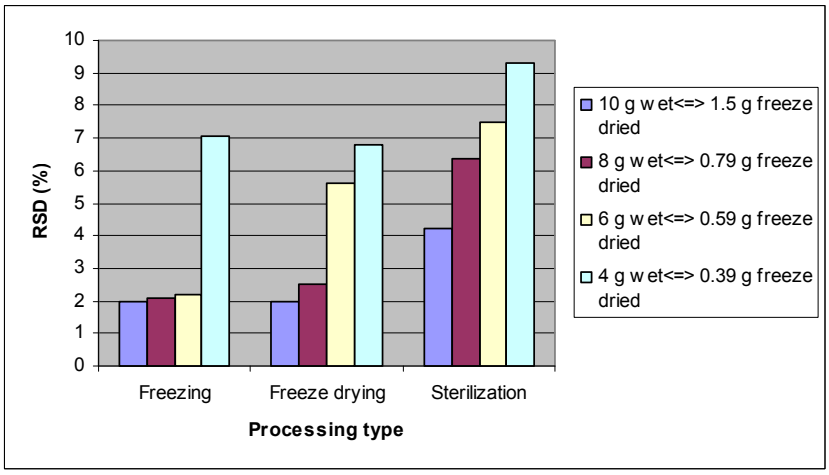


Figure 64: Relative standard deviation [%] of within-jar measurements for procymidone, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

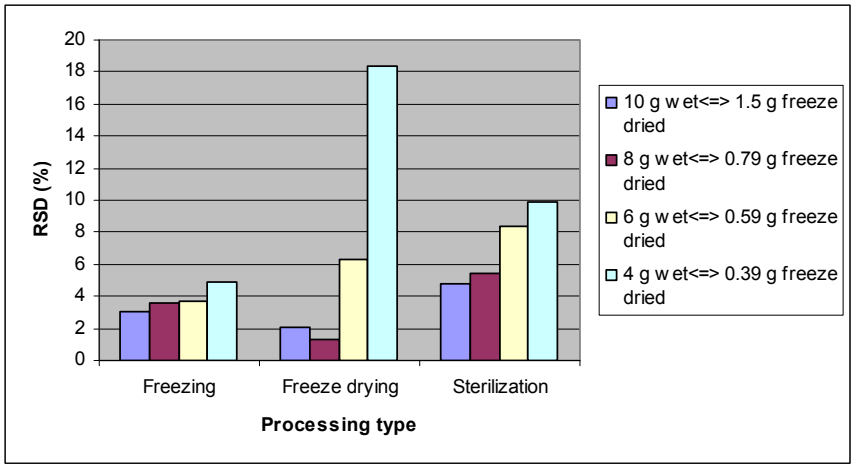


Figure 65: Relative standard deviation [%] of within-jar measurements for endosulfan (a+b), using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

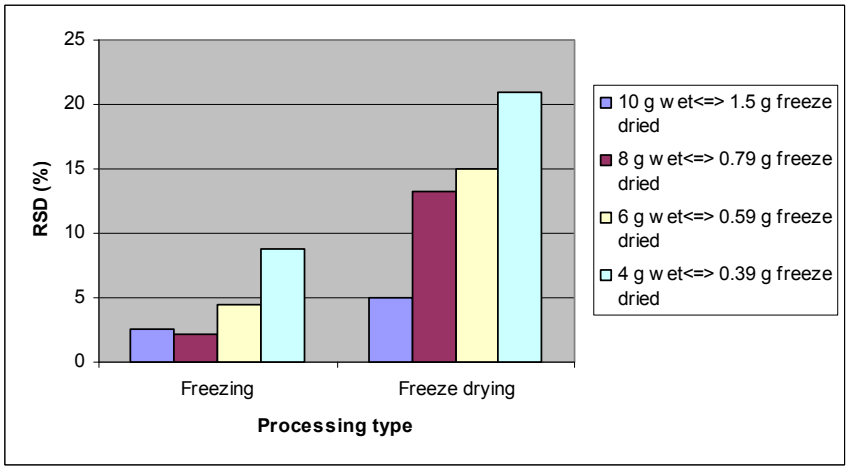


Figure 66: Relative standard deviation [%] of within-jar measurements for triazophos, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

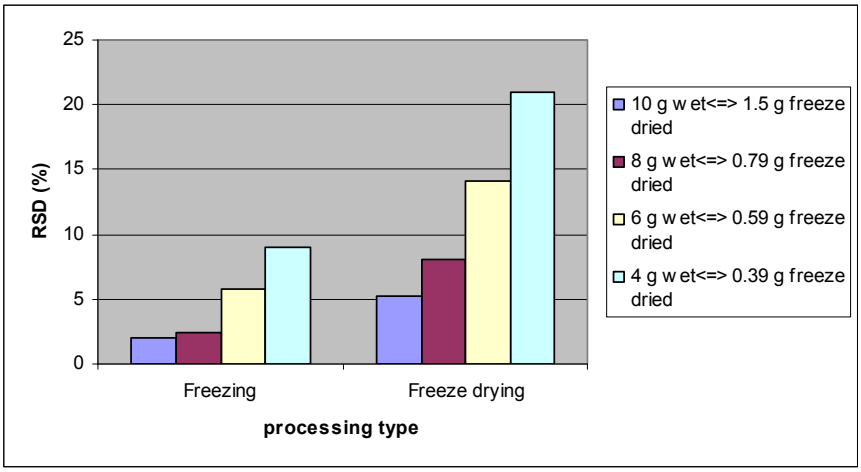


Figure 67: Relative standard deviation [%] of within-jar measurements for iprodione, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

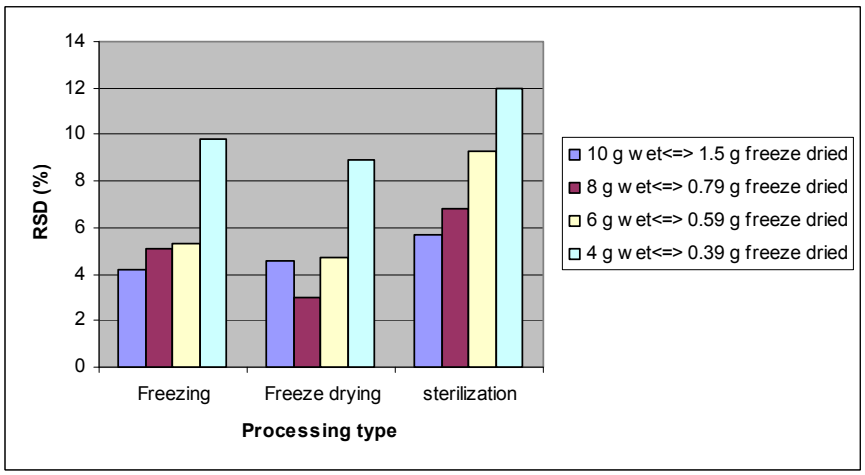


Figure 68: Relative standard deviation [%] of within-jar measurements for bromopropylate, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

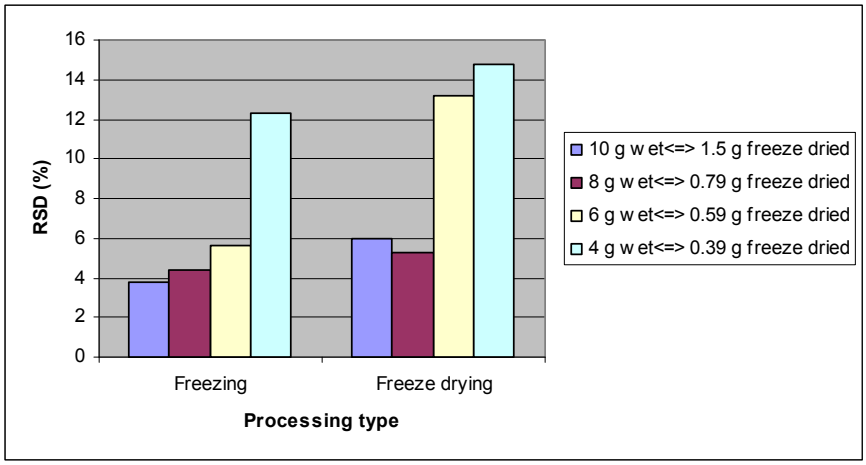


Figure 69: Relative standard deviation [%] of within-jar measurements for azinphos-methyl, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

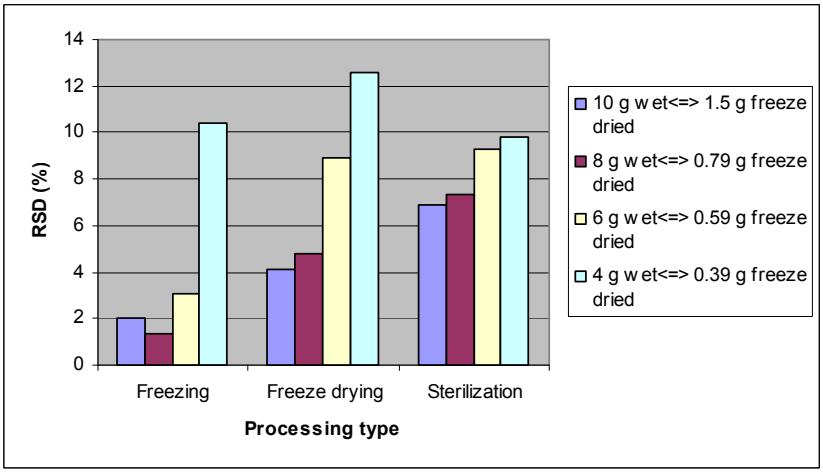


Figure 70: Relative standard deviation [%] of within-jar measurements for lambda-cyhalotrin, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

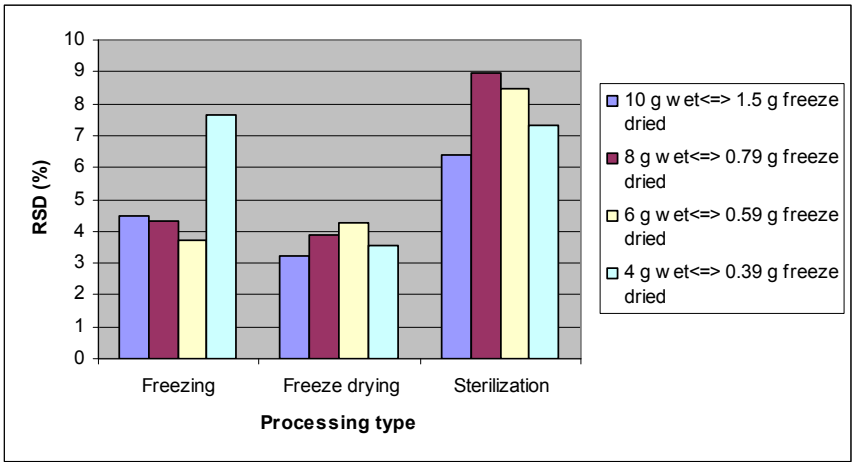


Figure 71: Relative standard deviation [%] of within-jar measurements for permethrin, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

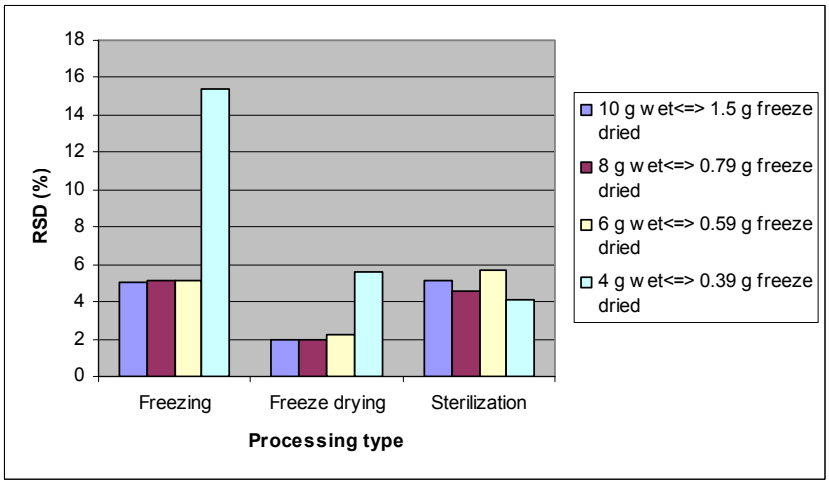


Figure 72: Relative standard deviation [%] of within-jar measurements for cypermethrin, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

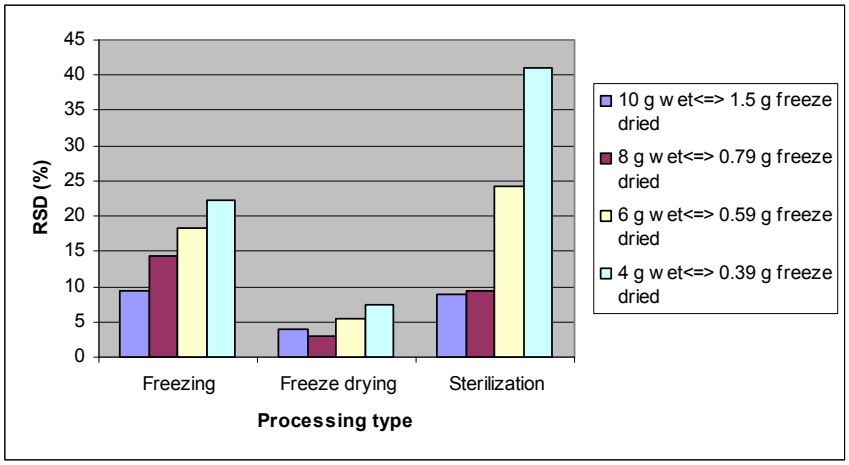


Figure 73: Relative standard deviation [%] of within-jar measurements for azoxystrobin, using decreasing amounts of sample intake (10, 8, 6 and 4 g equivalent).

12. Stability evaluation of the test materials (frozen, freeze dried and sterilization batches).

In order to asses the stability of the three tests materials (frozen, freeze dried and sterilized batches), two aspects of the stability of the materials were studied: short-term stability study and long-term stability study.

The short-term stability study design aims at determining an appropriate transport temperature for the test material. This study was designed with a duration of 4 weeks. Short-term degradation studies are carried out to simulate degradation during transport and to decide under which conditions the material, once it is certified, has to be dispatched. For this purpose storage under extreme conditions (60 °C) is compared to storage at low temperatures (-20 °C, +4 °C, +18 °C) during relatively short periods of time.

The long-term stability study evaluates a material stability at the storage conditions, and typically covers a storage period of 1 year. It shall ensure the stability of the target analytes during storage of the material and shall allow the definition of shelf life.

The temperature where stability is investigated must include at least one temperature below the envisaged storage temperature.

This allows the assessment of stability at this lower T (e.g -20 °C) if the results obtained at the higher T (e.g +4 °C) reveals signs of degradation of material.

The test material stability was evaluated using measurements based on the "isochronous" storage design. This method [48] can be used when the total duration of the stability study is known. Consequently it is applicable to the (short term) study of possible degradation during transport as well as to the (long term) study of storage conditions of the candidate RMs.

12.1 Short term stability evaluation of the test materials (frozen, freeze dried and sterilization batches)

The samples of the three test batches were stored for 0, 1, 2, and 4 weeks at -20 °C, +4 °C, +18 °C and +60 °C according to the planed isochronous study. The reference temperature was set to -70 °C. Two jars per each storage temperature were selected using a random stratified sampling scheme and analysed with respect to the target analytes content. The samples were kept at room temperature for at least one hour before opening to reach the equilibrium temperature. From each jar, three samples were taken and analysed in a randomized manner. Water content was determined for each test batch in triplicate.

To minimize matrix effects, blank extracts were used to construct the matrix matched calibration. Only for the freeze dried batch the processed blank matrix was used. For the others processed batches (frozen and sterilized), blank wet carrots were employed for the calibration curve. In any case it would be impossible to have a matrix matched calibration that simulates all the alterations that a matrix suffers at the different temperatures/storage time along with analyte stability changes. This and the fact that only 3 internal standards were used for quantification might compromise the accuracy of the results but the overall objective was to assess the quantitative relation between the sample measurements. This was achieved by using repeatability conditions during all batch measurements

A random stratified sampling was done by splitting the whole batch into blocks of equal size, and randomLy taking from each block jars for the stability study.

The results were screened for outlying results before data processing.

The data points obtained were plotted against storage time at the test temperature and the regression line was calculated. The slope of the regression line was then tested for statistical significance according to the SoftCRM Software statistics (Tables 34 to 42). More specifically, for each temperature the following calculations were performed.

I. Average, standard deviation, standard error, relative standard deviation
II. Slope and intercept of the linear regression line and the corresponding standard errors
III. A “t-test” to determine if the slope was significantly different from zero (95 % and 99 % level of significance)
IV. Single and double Grubbs test for outliers
V. Estimation of shelf life (months) in case of long-term stability studies.

Table 34: Results of the short–term stability study for the pesticides in the frozen test material. Test temperature -20 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Frozen test material				
phorate	398.1	6.3	3.6	No
propyzamide	200.6	9.4	2.7	No
diazinon	99.6	5.9	0.1	No
vinclozolin	425.3	6.5	3.2	No
chlorpyrifos-methyl	359.4	7.3	3.8	No
metalaxyl	468.6	7.3	10.2	No
pirimiphos-methyl	422.9	4.8	3.8	No
malathion	3872	3.7	-38.1	No
chlorpyrifos	340.8	8.1	6.6	No
parathion	444.9	4.6	4.8	No
mecarbam	553.8	6.4	18.2	Yes
procymidone	203.3	5.4	1.9	No
endosulfan (a+b)	360.6	9.6	14.4	Yes
triazophos	207.2	24.6	10.0	No
iprodione	154.8	12.8	2.4	No
bromopropylate	355.6	10.3	15.0	Yes
azinphos-methyl	357.4	12.7	-4.1	No
lambda-cyhalotrin	117.9	11.3	3.4	No
permethrin	287.9	10.3	9.8	Yes but not at 99 % c.l.
cypermethrin	373.6	14.4	16.6	Yes but not at 99 % c.l.
azoxystrobin	555.3	9.3	0.2	No (1 outlier)

Table 35: Results of the short–term stability study for the pesticides in the freeze dried material. Test temperature -20 ºC; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Freeze dried test material				
phorate	122.6	9.8	0.1	No
propyzamide	164.7	6.5	2.3	No
diazinon	48.9	8.6	1.1	No
vinclozolin	279.5	12.1	8.5	No
chlorpyrifos--methyl	200.2	11.9	3.9	No
metalaxyl	384.8	6.8	4.2	No
pirimiphos--methyl	336.7	9.8	3.3	No
malathion	2302.0	7.4	29.9	No
chlorpyrifos	291.9	11.7	1.7	No (1 outlier)
parathion	287.6	7.8	-5.6	No
mecarbam	378.4	12.7	9.2	No
procymidone	163.5	12.9	5.1	No
endosulfan (a+b)	214.2	14.2	6.8	No (1 outlier)
triazophos	193.2	23.8	1.6	No
iprodione	102.2	23	-1.2	No
bromopropylate	301.7	16.6	2.6	No
azinphos-methyl	216.3	11.8	5.6	No
lambda--cyhalotrin	138.9	15.15	1.1	No
permethrin	300.6	17.6	3.1	No
cypermethrin	335.7	12.1	4.0	No
azoxystrobin	370.1	19.3	17.2	No

Table 36: Results of the short–term stability study for the pesticides in the freeze dried test material. Test temperature +4 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Freeze dried test material				
phorate	At LOQ			
propyzamide	163.9	5.4	0.3	No (2 outliers)
diazinon	48.0	9.1	-0.4	No
vinclozolin	268.4	10.5	-2.4	No
chlorpyrifos--methyl	144.7	9.1	-1.7	No
metalaxyl	404.97	5.9	0.5	No
pirimiphos--methyl	232.7	15.2	4.9	No (1 outlier)
malathion	2318.9	9.3	-9.5	No
chlorpyrifos	212.9	6.8	0.4	No
parathion	297.1	7.49	-0.7	No (1 outlier)
mecarbam	383.07	14.2	-0.3	No
procymidone	164.5	14.5	0.1	No
endosulfan (a+b)	215.2	11.9	-5.1	No
triazophos	223.7	23.6	3.4	No (2 outliers)
iprodione	108.4	14.8	0.6	No
bromopropylate	198.1	14.2	-1.3	No (1 outlier)
azinphos--methyl	212.8	9.3	1.5	No
lambda--cyhalotrin	At LOQ			
permethrin	At LOQ			
cypermethrin	At LOQ			
azoxystrobin	368.6	18.3	-1.7	No

Table 37: Results of the short–term stability study for the pesticides in the freeze dried test material. Test temperature +18 °C; reference temperature -70°C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Freeze dried test material				
phorate	At LOQ			
propyzamide	159.0	9.7	-4.5	Yes but not at 99% c.l.
diazinon	44.6	17	-3.6	Yes
vinclozolin	240.9	11.0	-11.2	Yes
chlorpyrifos--methyl	134.5	19.8	-10.8	Yes
metalaxyl	392.7	8.4	-2.3	No
pirimiphos--methyl	220.6	12.9	-11.3	Yes
malathion	2150.5	11.9	-117.0	Yes
chlorpyrifos	210.3	12.3	-4.2	No
parathion	278.3	14.4	-15.4	Yes
mecarbam	351.8	14.8	-17.6	Yes but not at 99% c.l.
procymidone	156.9	12.7	-3.2	No
endosulfan (a+b)	218.2	15.3	-1.9	No
triazophos	210.5	26.3	-2.8	No
iprodione	99.9	11.5	-2.9	No
bromopropylate	At LOQ			
azinphos--methyl	196.1	14.2	-10.3	Yes
lambda--cyhalotrin	At LOQ			
permethrin	At LOQ			
cypermethrin	At LOQ			
azoxystrobin	365.2	15.47	-9.1	No

Table 38: Results of the short–term stability study for the pesticides in the freeze dried test material. Test temperature +60 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Freeze dried test material				
phorate	At LOQ			
propyzamide	139.8	13.4	-10.3	Yes
diazinon	At LOQ			
vinclozolin	236.6	11.2	-13.1	Yes
chlorpyrifos--methyl	AT LOQ			
metalaxyl	395.6	7.0	-4.4	No
pirimiphos--methyl	At LOQ			
malathion	At LOQ			
chlorpyrifos	154	20.1	-16.5	Yes
parathion	250.7	14.8	-21.7	Yes
mecarbam	328.2	16.7	-21.5	Yes
procymidone	156.2	14.6	-1.4	No
endosulfan (a+b)	At LOQ			
triazophos	At LOQ			
iprodione	102.6	8.6	0.5	No
bromopropylate	At LOQ			
azinphos--methyl	At LOQ			
lambda--cyhalotrin	At LOQ			
permethrin	339.0	8.3	0.4	No
cypermethrin	At LOQ			
azoxystrobin	333.1	21.5	-0.5	No

Table 39: Results of the short–term stability study for the pesticides in the sterilized test material. Test temperature -20 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Sterilized test material				
phorate	Not detected			
propyzamide	260	6.2	0.9	No
diazinon	47.5	11.9	1.5	Yes but not at 99% c.l.
vinclozolin	186.7	8.2	3.1	No
chlorpyrifos--methyl	At LOQ			
metalaxyl	505.9	7.8	9.4	No
pirimiphos--methyl	212.6	5.7	-1.9	No
malathion	547.7	9.4	2.8	No
chlorpyrifos	329.2	9.9	-10.6	Yes but not at 99% c.l.
parathion	298.9	8.41	-1.5	No
mecarbam	263.8	8.8	-3.5	No
procymidone	206.13	8.7	-4.5	No
endosulfan (a+b)	At LOQ			
triazophos	At LOQ			
iprodione	At LOQ			
bromopropylate	511	8.8	-11.5	No
azinphos--methyl	At LOQ			
lambda--cyhalotrin	138.4	7.9	-1.8	No
permethrin	391	9.6	-7.7	No
cypermethrin	550.1	6.3	-8.0	No
azoxystrobin	612.9	7.2	-12.0	No

Table 40: Results of the short–term stability study for the pesticides in the sterilized test material. Test temperature +4 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Sterilized test material				
phorate	Not detected			
propyzamide	244.1	6.7	-7.3	Yes
diazinon	41	10.9	-1.2	No
vinclozolin	200.3	12.6	-10.8	Yes
chlorpyrifos--methyl	At LOQ			
metalaxyl	490.8	10.21	-5.4	No
pirimiphos--methyl	192.2	11.2	-10.6	Yes
malathion	484	16.2	-31.4	Yes
chlorpyrifos	304.8	14.5	-20.5	Yes
parathion	298.8	10.3	-10.3	Yes
mecarbam	228.8	10.2	-12.8	Yes
procymidone	193.6	12.4	-12.3	Yes
endosulfan (a+b)	At LOQ			
triazophos	At LOQ			
iprodione	At LOQ			
bromopropylate	459.6	14.7	-39.0	Yes
azinphos--methyl	At LOQ			
lambda--cyhalotrin	At LOQ			
permethrin	391.8	14.4	-16.2	No
cypermethrin	515.1	14.5	-19.3	No
azoxystrobin	603.6	12	-21.2	Yes

Table 41: Results of the short–term stability study for the pesticides in the sterilized test material. Test temperature +18 °C, reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter /week]	Slope significant [95% level of confidence]
Sterilized test material				
metalaxyl	404.6	31.5	7.4	No

Table 42: Results of the short–term stability study for the pesticides in the sterilized test material. Test temperature +60 °C, reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter /week]	Slope significant [95% level of confidence]
Sterilized test material				
metalaxyl	479.9	22.2	7.3	No

The analysis of the experimental results of the short term stability study was done by:

- Evaluating the stability data by process (frozen, freeze-dried, sterilized sample batches)
- Evaluating the stability data of each analyte for each storage temperature and matrix type in order to find similarites and/or inconsistencies of the behaviour of the analytes for different storage temperatures/ type of matrices (wet and freeze dried matrices).

12.2 Short term stability of the frozen batch

As shown in Table 34, the content of the target analytes showed no significant changes when stored at -20 °C up to 4 weeks, except for bromopropylate, cypermethrin, endosulfan (a+b), mecarbam, and permethrin. For the 5 mentioned analytes, the reason for a positive significant slope was checked for inconsistencies in the analytical sequence/sample means or any other analytical reason. A positive analytical trend was found for all these analytes and this fact could justify a deficient analysis pointing out the GC-MS instability for the analysis of the mentioned compounds, although all samples were analysed in a short interval to avoid time trends. For those compounds a conclusion due to stability parameters cannot be done.

12.3 Short term stability of the freeze dried batch

At the same, in Tables 35 and 36 all target analytes were stable in the freeze dried matrix up to a time period of 4 weeks at -20 °C, and all, except phorate, lambda-cyhalotrin, permethrin and cypermethrin, were stable at +4 °C over the same time span. These compounds were found at the LOQ and therefore they were not taken into consideration for a stability analysis. No analytical trends were found for the stable compounds.

The study revealed that the stability at +18 °C was compromised for the majority of the target pesticides. Stability was observed for iprodione, metalaxyl, endosulfan (a+b), chlorpyrifos and azoxystrobin, and at +60 °C, azoxystrobin, metalaxyl and permethrin were stable.

Mecarbam, procymidone and iprodione showed a positive significant trend in the analytical sequence at +60 °C and therefore no conclusions about stability cand be made for these compounds at +60 °C.

12.4 Short term stability of the sterilized batch

As presented in Table 39, all analytes measured were in the quantifiable range of the analytical method were stable up to 4 weeks in the sterilized test material at -20 °C, with the exception of chlorpyrifos and diazinon.

Analytical/sample means inconsistencies were investigated for those significant slopes (chlorpyrifos, diazinon). As no drifts were found the significant slopes are due to the analyte instability.

Most of the analytes were not stable when stored at +4 °C. No analytical reason was found for these negative significant slopes, so instability is more likely to occur. Stability was observed for diazinon, metalaxyl, permethrin, and cypermethrin, at +4 °C during a storage period of 4 weeks.

The only analyte that showed stability in the sterilized matrix at + 18 °C and at + 60 °C was metalaxyl.

12.5 Comparison of stability issues between the processes (wet vs dried) and by storage temperature

Storage at -20 °C

Except chlorpyrifos and diazinon all other pesticides were stable during storage at -20 °C for up to fouir weekes, irrespective of the stabilising process used (frozen, freeze dried and sterilized matrix). Chlorpyrifos and diazinon were not stable in the sterilized matrix but proved to be stable in the frozen matrix. This could be due to specific interactions with the matrix.

Storage at +4 °C

Most of the pesticides (17 out of 21 pesticides) that were stable in the freeze dried matrix at +4 °C were not stable in the sterilized matrix. No inconsistencies such as a time drift in theanalytical sequence could be identified. Most problaby the heat treatment and the storage period of 4 weeks

in a wet matrix have contributed to degradation pathways that pesticides might undergone, even being stored at the same T (+4 °C). It suggests that the T is not the only factor behind the pesticide stability, but also its surrounding environment.

Storage at + 18 °C

At +18 °C inconsistencies of stability were found on iprodione, metalaxyl, endosulfan (a+b), chlorpyrifos and azoxystrobin. These were stable in the freeze dried matrix but not in the sterilized one up to 4 weeks of storage. Analysis of potential drifts showed no trends in analytical sequence/sample means found, so again a different behaviour due to the type of processed matrix (dried vs wet sterilized matrix) can explain such differences of behaviour under storage at the same temperature but in a different type of environment.

Storage at +60 °C

Formatiert: Nummerierung und Aufzählungszeichen

At +60 °C inconsistencies about the analytes stability were found, regarding storage at same T in different surrounding environments namely for permethrin and azoxystrobin, which tended to be stable in the dried matrix and not in the sterilized matrix.

12.6 Conclusions

Regarding the short term stability studies, the freeze dried matrix is the preferred matrix in order to achieve stability of all target pesticides at -20 °C. It was also found out that transport of the candidate reference material would be feasible even at +4 °C, if phorate, lambda-cyhalotrin, permethrin and cypermethrin were not of interest.

The short stability data for the sterilized samples showed that 13 analytes were stable at -20 °C up to 4 weeks. The sterilized matrix, although processed, is rather similar to a real carrot material in comparison to a freeze dried material, which has to be reconstituted before use.

Sixteen out of the 21 pesticides were stable in the frozen test material, at -20 °C up to 4 weeks.

13. Long term stability evaluation of the test materials (frozen, freeze-dried and sterilized carrots)

For the long-term stability study a period of 5 months and 4 time intervals were considered (0, 3, 4 and 5 month) following an isochronous study scheme.

At the end of the isochronous scheme (5 months), samples were stored at the reference temperature for a short period (1 week) and analysed in the laboratory as follows:

Day 1 Thawing of samples at +4 °C, overnight;

Day 2 Preparation of samples for extraction: weighing of sample intake and reconstitution in the case of freeze dried sample, followed by the addition of adequate amount of internal standard (at MRL level, falling near middle point of calibration curve). Samples were processed in random order. Samples were stored overnight at +4 °C.

Day 3 Run of the analytical method for all samples under repeatability conditions. Sample extracts stored at -20 °C.

Day 4, 5, 6 Injection following randomized sequence in GC-MS (a new GC column was used for the long term stability studies and the liner was changed for each batch of samples to avoid cross contamination of the inlet system between sample batches).

For all three type of materials, the average (3 sample replicates, 2 injections each) pesticide concentration expressed in ng/g dry matter was plotted against time of storage. Slopes of these regression lines were tested for significance using SoftCRM software.The outcome of the study is summarised in Tables 42-48.

Table 42: Results of the long-term stability study for the pesticides in the frozen test material. Test temperature -20 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Frozen test material				
phorate	432.7	3.9	3.4	No (2 outliers)
propyzamide	241.6	3.4	0.8	No
diazinon	96.4	11.7	1.9	No
vinclozolin	486.8	5.8	6.7	No
chlorpyrifos--methyl	390	7.9	6.0	No
metalaxyl	442.5	8.8	-0.02	No (1 outlier)
pirimiphos--methyl	451.3	6.0	3.6	No (1 outlier)
malathion	4507.9	5.9	-13.3	No (1 outlier)
chlorpyrifos	378.9	3.1	4.8	No (1 outlier)
parathion	454.9	5.6	4.0	No (1 outlier)
mecarbam	582.2	6.8	-1.6	No
procymidone	215.2	4.9	-1.3	No
endosulfan (a+b)	368.5	9.1	3.6	No
triazophos	207.9	8.5	-0.3	No
iprodione	137.1	17.4	3.3	No
bromopropylate	366.2	10.6	8.2	No
azinphos-methyl	421.6	9.4	-0.8	No
lambda--cyhalotrin	129.8	8.7	2.3	No
permethrin	323.6	10.6	6.6	No
cypermethrin	425.9	10.7	9.5	No
azoxystrobin	534.1	14.9	11.3	No

Table 43: Results of the long-term stability study for the pesticides in the freeze dried test material. Test temperature -20 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Freeze dried test material				
phorate	133,9	10.6	0.7	No
propyzamide	144.7	23.7	-4.5	No
diazinon	47.9	13.5	-0.9	No
vinclozolin	236.5	24.8	-10.1	No
chlorpyrifos--methyl	125.7	22.8	-3.7	No
metalaxyl	345.7	10.4	-7.5	No (month 3 below LOQ)
pirimiphos--methyl	289	19.0	-8.9	No (2 outliers)
malathion	2340.5	8.7	60.2	No
chlorpyrifos	278.1	8.3	0.12	No (month 3 below LOQ)
parathion	250.0	15.1	-9.04	No (3 outliers)
mecarbam	341.5	13.2	-15.5	Yes (3 outliers)
procymidone	138.7	9.5	-4.3	Yes (month 3 below LOQ)
endosulfan (a+b)	Below LOQ			
triazophos	108.9	23	-1.3	No (2 outliers)
iprodione	112.7	24	-5.8	Yes
bromopropylate	328.2	11.2	-1.4	No
azinphos-methyl	252.9	12.1	-6.9	No (month 3 below LOQ)
lambda-cyhalotrin	Below LOQ			
permethrin	324.5	4.2	-0.8	No
cypermethrin	Below LOQ			
azoxystrobin	271.1	33.2	-13.4	Yes

Table 44: Results of the long–term stability study for the pesticides in the freeze dried test material. Test temperature +4 °C, reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Freeze dried test material				
phorate	Below LOQ			
propyzamide	149.6	18.7	-7.7	Yes but not at 99% c.l.
diazinon	41.9	25.6	-3.6	Yes
vinclozolin	235.7	25.4	-17.9	Yes
chlorpyrifos--methyl	Below LOQ			
metalaxyl	336.6	10.2	-11.5	No
pirimiphos--methyl	210.4	8.9	-10.2	Yes but not at 99 % c.l.
malathion	2070.1	8.8	-48.1	Yes but not at 99% c.l.
chlorpyrifos	294.4	13.6	-2.5	No
parathion	241.4	17.8	-13.4	Yes
mecarbam	316.5	21.5	-22.7	Yes
procymidone	128.6	18.9	-6.9	Yes but not at 99% c.l.
endosulfan (a+b)	Below LOQ			
triazophos	121.5	11.85	-1.9	No
iprodione	104.4	20.32	-6.7	Yes
bromopropylate	320.9	7.3	1.2	No
azinphos-methyl	250.3	8.74	-8.4	Yes
lambda-cyhalotrin	Below LOQ			
permethrin	Below LOQ			
cypermethrin	Below LOQ			
azoxystrobin	281.6	21.9	-19.71	Yes

Table 45: Results of the long–term stability study for the pesticides in the freeze dried test material. Test temperature +18 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Freeze dried test material				
phorate	At LOQ			
propyzamide	152	12.5	-6.5	Yes
diazinon	42.47	20.0	-2.9	Yes
vinclozolin	246.7	16.4	-13.1	Yes
chlorpyrifos-methyl	At LOQ			
metalaxyl	353.5	8.0	-3.3	No
pirimiphos--methyl	At LOQ			
malathion	1881.7	19.1	-107.5	Yes
chlorpyrifos	At LOQ			
parathion	Al LOQ			
mecarbam	317.7	17.6	-21,6	Yes
procymidone	136.6	11.5	-4.0	Yes but not at 99% c.l.
endosulfan (a+b)	At LOQ			
triazophos	103.9	15.4	-4.3	Yes but not at 99% c.l.
iprodione	109.1	14.8	-4.6	Yes
bromopropylate	At LOQ			
azinphos-methyl	206.1	22.4	-19.7	Yes
lambda-cyhalotrin	At LOQ			
permethrin	At LOQ			
cypermethrin	At LOQ			
azoxystrobin	260.1	25.1	-21.4	Yes

Table 46: Results of the long-term stability study for the pesticides in the sterilized test material. Test temperature -20 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter/week]	Slope significant [95% level of confidence]
Sterilized test material				
phorate	Not detected			
propyzamide	188.6	15.1	-10.5	Yes
diazinon				
vinclozolin	170.9	15.4	-10.2	Yes
chlorpyrifos--methyl	At LOQ			
metalaxyl	465.1	10.2	-2.9	No
pirimiphos--methyl	171.1	19.1	-14.1	Yes
malathion	543.7	7.8	3.7	No
chlorpyrifos	258	16	-13.6	Yes
parathion	200.8	18.9	-16.2	Yes
mecarbam	201.2	15.6	-8.7	Yes
procymidone	130.3	22.0	-9.6	Yes
endosulfan (a+b)	At LOQ			
triazophos	At LOQ			
iprodione	At LOQ			
bromopropylate	293.9	19.3	-20	Yes
azinphos-methyl	At LOQ			
lambda-cyhalotrin	102.9	13.9	-4.3	Yes
permethrin	279.1	17.5	-18.1	Yes
cypermethrin	412.5	12.9	-10.9	No
azoxystrobin	552	8.0	-5.9	No

Table 47: Results of the long–term stability study for the pesticides in the sterilized test material. Test temperature +4 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter /week]	Slope significant [95% level of confidence]
Sterilized test material				
phorate	Not detected			
propyzamide	At LOQ			
diazinon	At LOQ			
vinclozolin	At LOQ			
chlorpyrifos--methyl	At LOQ			
metalaxyl	498.5	10.7	5.9	No
pirimiphos--methyl	At LOQ			
malathion	390.9	20.5	-39.2	Yes
chlorpyrifos	At LOQ			
parathion	At LOQ			
mecarbam	At LOQ			
procymidone	138.6	8.6	-5.2	Yes
endosulfan (a+b)	At LOQ			
triazophos	At LOQ			
iprodione	At LOQ			
bromopropylate	351.3	8.5	-2.3	No
azinphos-methyl	At LOQ			
lambda-cyhalotrin	109.05	9.5	0.2	No
permethrin	320.15	11.6	1.2	No
cypermethrin	473.6	15.8	-0.4	No
azoxystrobin	511.7	18.9	12.7	No

Table 48: Results of the long–term stability study for the pesticides in the sterilized test material. Test temperature +18 °C; reference temperature -70 °C.

Pesticide	Average [ng/g dry matter]	RSD [%]	Slope [ng/g dry matter /week]	Slope significant [95% level of confidence]
Sterilized test material				
metalaxyl	404.6	31.5	7.4	No

13.1 Discussion and conclusions

The long term stability data evaluation was done by means of comparing:

- The analyte stability for each storage temperature and matrix type in order to find similarities and/or inconsistencies of the analyte behaviour for different storage temperature/matrix type combinations (wet and freeze dried matrices)
- Consistency with short term stability results.

A general strategy was followed for the analysis of the raw data of the three batches of samples, which included in chronological order, the following parameters:

- ❖ Pesticide name
- ❖ Content (ng/g dry matter) during screening measurements of the processed materials
- ❖ Limit of quantification (LOQ)
- ❖ Analysis of sample/analytical trends during analysis (if yes no conclusions about stability can be made)
- ❖ Analysis of outliers
- ❖ CV (%) target criteria for each time point average results (between 2-15 % maximum), which included the analysis of 2 consecutive injections of the same sample in GC-MS.

- ❖ Comparison of average value of data set of long-term stability with short-term stability average data set
- ❖ Conclusions about long-term stability parameter

13.2 Frozen batch long-term stability analysis

As seen in Table 42 and following the analytical strategy described above, all target pesticides were stable in the frozen carrot matrix kept at -20 °C over a period of 5 months.

As far as consistency with short-term stability studies is concerned, trends observed for bromopropylate, cypermethrin, endosulfan (a+b), mecarbam and permethrin in the short-term studies were not confirmed by the findings of the long-term stability studies (LTS). Therefore the trends observed in STS are analytical trends.

13.3 Freeze dried batch long-term stability analysis

All target analytes except mecarbam, procymidone, endosulfan (a+b), lambda-cyhalotrin cypermethrin, iprodione and azoxystrobin were stable in the freeze dried matrix at -20 °C over a period of 5 months (Table 43). At +4 °C and for the same time span, most of the pesticides stability was compromised and only metalaxyl, chlorpyrifos, triazophos and bromopropylate appeared to be stable. The only pesticide that was stable at +60 °C over a 5 month period was metalaxyl. For some analytes measurements of month 3 had systematically a negative bias and for some e.g. metalaxyl, chlorpyrifos and procymidone, concentration values were below the LOQ (these were treated as outliers). This should not drastically influence the conclusions since the outliers were in the middle part of the regression function and points at the edge would influence the results and the derived conclusions to a greater extent. If all of pesticides appeared to be stable in the freeze dried matrix over a period of 4 weeks, this assumption is no longer seen during long term stability studies. Seven out of the 21 appeared to be instable over a longer storage period. All significant slopes

were tested for any potential trends in analytical sequence/sample means but no correlation was found. The stability tables are designed in a way that makes possible to divide the compounds in groups of physico-chemical similarities (increasing Rt in the GC column) and the corresponding labelled ISTD used for their quantification are printed in bold. The mentioned problematic 7 pesticides belong to the late elucting compounds and it was found out that the consecutive analysis of two injections of the same sample presented a 20 % difference, thus introducing great variability in the results.

13.4 Sterilized batch long-term stability analysis

In the sterilized matrix only metalaxyl, malathion, cypermethrin and azoxystrobin were stable at -20 °C (Tables 46-48). At +4 °C metalaxyl, bromopropylate, lambda-cyhalotrin, permethrin, cypermethrin and azoxystrobin out of 21 analysed pesticides were the only that remained stable. Again, at higher T (+60 °C) metalaxyl was the only pesticide appearing to be stable in the whole list of target analytes.

It is clearly seen that during storage of the samples for a longer period of time (5 months), irrespective of the temperature, pesticides do not remain stable in the sterilized carrot matrix.

13.5 Comparison of stability issues between the processes (wet vs dried), by storage temperature

Storage at -20 °C

There are a number of pesticides (7 analytes) that do not appear to be stable in the freeze-dried matrix but stable in the frozen matrix. However, as it has been mentioned before, GC-MS measurements of consecutive injections of the same sample seemed to be out of control, at least for the late elucting compounds in the freeze dried matrix.

Storage at +4 °C

Metalaxy and bromopropylate appear to have the same behaviour in the two types of matrices (wet vs. dry) at +4 °C, but no explanation was found for the irregularity concerning chlorpyrifos and triazophos, which were stable in the dry but not on the wet matrix. On the contrary, lambda-cyhalotrin, permethrin, cypermethrin and azoxystrobin were stable in the wet but not on the dried matrix at +4 °C over a time span of 5 months.

Storage at +18 °C

Only metalaxy seems to be fairly stable in wet and dry matrices at higher temperatures (+18 ° C).

13.6 Conclusions

This section aims at showing how the generated stability data fits into the existing knowledge. Mainly reference will be made to the Pesticide Manual Compendium [57], which is the only available source which contains stability data of a wide list of pesticides in use.

Chlorpyrifos-methyl and diazinon are both high volatile pesticides. Chlorpyrifos-methyl is relatively stable in neutral media but it is hydrolysed under both acidic (pH 4-6) and more readily under alkaline (pH 8-10) conditions. Diazinon is readilyt hydrolysed at +20 °C. In fact, during short term stability studies of the freeze dried material at +18 °C and + 60 °C the average concentrations of these pesticides dropped substancially when T increased.

In the sterilized material the highly volatile chlorpyrifos methyl was found at the LOQ and diazinon appeared to degrade at +18 °C. This was confirmed later on in the long-term stability study.

Parathion is known to rapidly hydrolyse at pH 5-6 and +25 °C, and that on heating it isomerizes to the O-S-diethyl isomer. During short term stability of the three batches of samples, it was not stable above +4 °C. Chlorpyrifos is described as a pesticide whose rate of hydrolysis in water increases with pH

and T. It proved to be stable at +18 °C, but not at +60 °C in the freeze dried test material during the short term stability studies. In the sterilized material the heat applied during the processing (+120 °C, 15 min) seems to have contributed to its degradation and in this material it was mostly found at the LOQ. Bromopropylate is a fairly stable pesticide in neutral or slightly acid media (carrot/potato based baby food matrix pH=5.2). Looking at the short term and long term stability data, bromopropylate appears to be stable in all batches at low temperatures (-20 °C) and even at +4 °C in the freeze dried material. Data revealed that it is also fairly stable to heat treatment (+120 °C, 15 min) of the sterilized batch. The same did not happen when this pesticide was exposed to temperatures higher than + 4 °C for prolonged times.

With regard to metalaxyl, the pesticide manual data indicates that metalaxyl is stable at T < +300 °C. Indeed the analysis of short and long term stability data indicated that this is the only compound in the target list of pesticides of the present study that revealed to be stable in all batches of samples exposed to storage temperature of +60 °C.

Stability data in the pesticide manual refer only to the neat compounds and describe stability only in relation to T and pH, while in the present study other elements may be of influence (e.g. the different components of a real food matrix), which of course are difficult to predict empirically. In any case, the comparisons made above suggest that the stability of pesticides contained in a matrix do fit in the existing knowledge.

13.7 Uncertainty budget

From the perspective of the Guide to the Expression of Uncertainty in Measurement (GUM) [59], uncertainty of stability is a part of the total uncertainty of a CRM. In fact, uncertainty of stability refers to two distinctly different uncertainty components-possible degradation during short-term storage (transport to the user; μ_{sts}) and possible degradation during long-term storage (μ_{lts}).

$$\mu_{CRM} = \sqrt[2]{\mu_{char}^2 + \mu_{bb}^2 + \mu_{sts}^2 + \mu_{lts}^2} \qquad (1)$$

In eq. (1) μ_{char} and μ_{bb} refer to the uncertainties in batch characterization and between-unit variation, respectively. All components have to be converted into relative uncertainties to enable addition of the individual uncertainties. In reality, the uncertainties associated with degradation do not necessarily reflect apparent degradation but even in the absence of degradation they reflect the uncertainties associated with the measurements used to determine degradation.

As discussed before t-tests are used to test significance of the slope in a stability study. The assumption of linear degradation is justified because possible degradation must be small if the material is to be a CRM, and a small degradation can be described approximately by a linear function. Materials for which significant trends are observed will usually be insuitable for certification.

In Tables 49, 50 and 51 the combined uncertainty (μ^{*}_{bb} or $S_{bb,}$ μ_{sts}, μ_{lts}) of three bacthes of test materials only for conditions/materials whose slopes of the stability study were not significant is presented.

Combined uncertainty values ranged from 3.8 % to 12.2 % (and a high value of 35 % for azoxystrobin), 5 % to 16.7 %, and 10 % to 17 %, respectively for the frozen, freeze dried and sterilized bacth of test materials spiked with pesticides.

Table 49: Combined uncertainty budget for the target pesticides in the frozen test material at -20 °C.

Pesticide	Standard Uncertainty [%]			Combined Uncertainty [%]	Expanded Uncertainty [%]
	$(U_1)^1$	$(U_2)^2$	$(U_3)^3$	(U_c)	$(U=2*U_c)$
	Frozen test material				
phorate	2.3	3.0	2.2	4.4	8.7
propyzamide	2.0	5.1	2.1	5.8	11.7
diazinon	1.5	3.1	5.6	6.6	13.2
vinclozolin	1.4	3.4	4.4	5.8	11.5
chlorpyrifos--methyl	2.0	3.9	4.2	6.1	12.2
metalaxyl	2.3	4.7	5.0	7.2	14.5
pirimiphos--methyl	1.5	2.4	3.9	4.8	9.5
malathion	1.3	1.9	3.6	4.3	8.5
chlorpyrifos	3.5	4.1	3.7	6.6	13.1
parathion	1.4	2.4	2.7	3.9	7.7
mecarbam	1.1	2.1	3.3	4.1	8.1
procymidone	1.0	2.6	2.5	3.8	7.6
endosulfan (a+b)	2.5	4.4	5.0	7.1	14.3
triazophos	2.5	11.4	3.7	12.2	24.4
iprodione	3.0	6.4	6.4	9.5	19.1
bromopropylate	2.3	4.5	5.4	7.4	14.7
azinphos--methyl	3.5	4.5	4.0	7.0	14.0
lambda--cyhalotrin	3.0	6.6	5.4	9.1	18.2
permethrin	2.9	4.7	6.5	8.5	17.0
cypermethrin	2.9	8.6	7.7	11.9	23.8
azoxystrobin	33.0	10.7	6.3	35.3	70.5

1- $\mu^*_{bb\ or}\ S_{bb}$

2- μ_{STS}

3 - μ_{LTS}

Table 50: Combined uncertainty budget for the target pesticides in the freeze dried test material at -20 °C.

Pesticide	Standard Uncertainty [%]			Combined Uncertainty [%]	Expanded Uncertainty [%]
	$(U_1)^1$	$(U_2)^2$	$(U_3)^3$	(U_c)	$(U=2*U_c)$
	Frozen test material				
phorate	2.5	3.1	11.8	12.5	25.0
propyzamide	3.1	3.6	12.3	13.2	26.4
diazinon	1.7	4.1	4.4	6.2	12.4
vinclozolin	1.8	5.2	10.1	11.5	23.0
chlorpyrifos--methyl	1.4	2.9	7.9	8.5	17.1
metalaxyl	6.3	11.5	6.6	14.7	29.4
pirimiphos--methyl	1.0	3.3	7.3	8.0	16.1
malathion	3.9	3.8	9.5	10.9	21.8
chlorpyrifos	5.4	3.8	3.1	7.2	14.5
parathion	2.2	6.1	6.6	9.2	18.4
mecarbam	1.5	6.8			
procymidone	1.4	7.7			
endosulfan (a+b)	1.8	5.3			
triazophos	2.7	12.9	10.2	16.7	33.3
iprodione	3.2	26.8			
bromopropylate	1.5	5.3	5.9	8.1	16.2
azinphos--methyl	5.3	5.1	7.0	10.2	20.3
lambda--cyhalotrin	1.4	3.3			
permethrin	1.6	4.1	2.4	5.0	10.0
cypermethrin	1.8	4.6			
azoxystrobin	3.0	7.4			

1- $\mu^*_{bb\ or}\ S_{bb}$

2- μ_{STS}

3 - μ_{LTS}

Table 51: Combined uncertainty budget for the target pesticides in the sterilized test material at -20 °C.

Pesticide	Standard Uncertainty [ng/g dry matter]			Combined Uncertainty [ng/g dry matter]	Expanded Uncertainty [ng/g dry matter]
	(U1)1	(U2)2	(U3)3	(Uc)	(U=2*Uc)
	Sterilized test material				
phorate					
propyzamide					
diazinon					
vinclozolin					
chlorpyrifos--methyl					
metalaxyl	4.1	5.8	15.5	17.0	34.1
pirimiphos--methyl					
malathion	10.7	4.6	7.7	14.0	28.0
chlorpyrifos					
parathion					
mecarbam					
procymidone					
endosulfan (a+b)					
triazophos					
iprodione					
bromopropylate					
azinphos-methyl					
lambda--cyhalotrin					
permethrin					
cypermethrin	7.9	0.0	6.4	10.2	20.4
azoxystrobin	11.2	5.7	11.2	16.8	33.6

1- $\mu^*_{bb\ or}\ S_{bb}$

2- μ_{STS}

3 - μ_{LTS}

14. Discussion

14.1 Optimization of the Analytical method for determination of 21 EU priority pesticides in carrots baby food

The whole study provided an advance in scientific knowledge with what has been reported in the literature up to date.

With regard to the analytical method, the in-house validated parameters, the in-house validation programme delivered method performance characteristics (recovery, precision, etc.) that were fully equivalent to reports from interlaboratory studies using the QuEChERS method for determination of pesticides in fruit/vegetable matrices (Table 52). The method was robust enough to be applied to new types of matrices (processed and no processed carrots, spinach and orange baby food) without loss of performance.

Table 52: Results of interlaboratory tests using the QuEChERS analytical method and similar type of matrices/techniques for the quantification of the 21 EU priority analytes of the present study [60].

Pesticide	GC	Matrix type	Spiking level (mg/kg) Min-max	Recoveries			Number of laboratories
				Rec (%)	RSD (%)	Number of results	
azinphos--methyl	GC	High water content	0,010-0,2	95	**18**	92	4
azoxystrobin	GC	High water content	0,010-1	96	11	50	4
bromopropylate	GC	High water content/dry	0,1-1,0	103/90	11/11	77/2	6/3
chlorpyriphos	GC	High water content/dry	0,025-0,1	103/106	8/12	80/2	8/3
chlorpyriphos--methyl	GC	High water content/dry	0,01-1	102/122	11/12	85/5	6/2
cypermethrin	GC	High water content/dry	0,1-1	100/112	**16**/_	64/1	4/1
diazinon	GC	High water content/dry	0,01-1,0	101/89	9/13	92/2	6/3
endosulfan (α+β)	GC	High water content/dry	0,1-1,0	96/98	**17**/_	92/1	6/1
iprodione	GC	High water content/dry	0,01-0,5	99/98	**18**/_	64/1	5/1
lambda-cyhalotrin	GC	High water content	0,025/0,25	100	7	64	7
malathion	GC	High water content/dry	0,01-1	101/92	13/_	93/1	5/1
mecarbam	GC	High water content/dry	0,01-1	102/98	13/_	76/1	6/1
metalaxyl	GC	High water content	0,025/25	104/103	10/5	47/50	5
parathion	GC	High water content/dry	0,01-1	102/100	10/_	89/1	5/1
permethrin	GC	High water content/dry	0,01-1	98/119	13/10	82/2	5/1
phorate	GC	High water content/dry	0,01-1	91/90	13/_	65/1	5/1
pirimiphos-methyl	GC	High water content/dry	0,01-1	103/116	10/15	96/2	6/1
procymidone	GC	High water content	0,025	103	6	35	7
propyzamide	GC	High water content	0,025/0,25	105/105	6/5	60/60	6
triazophos	GC	High water content/dry	0,05-1,0	99/98	12/8	46/12	3/1
vinclozolin	GC	High water content/dry	0,01-1	101/108	11/13	113/2	6/1

In the present work method validation results for each pesticide in different types of matrices tested (processed and non processed) were described along with an uncertainty budget (at 95 % confidence level). The parameters presented in Table 52 can serve as a comparison, since spiking levels (mg/kg) were in the same range and similar techniques were employed. In both instances, azinphos-methyl, cypermethrin, endosulfan (α+β) and iprodione were considered as “difficult” analytes. In the carrots matrix their peak shapes were the main adverse factor at low detection limits.

Obviously, all of the presented work refers to the use of spiked samples and not incurred samples, but the overall objective of the study was to work towards a system that can be very well characterized and which will act primiraly as a “reference system” for other measurement activities (e.g. method validation, by comparing results with the certified value).

Also, one must consider that pesticides are not comparable to veterinary drugs which tend to be bound to a various degree to the matrix. Pesticides tend to be adsorbed at the surfaces of fruits/vegetables when applied during agricultural practices. This is confirmed by the results of proficiency tests with samples that contained incurred polar and nonpolar residues, where shaking has been an acceptable technique compared with blending based methods [15] using the general QuEChERS approach.

14.2 The use of IDMS in the quantification of pesticides in food matrices

Experiments showed that calibration in solvent was possible for accurate measurement of a sample in matrix, when a labelled isotope analog of the native pesticide was used as an internal standard. This is possible because IDMS is largely unaffected by matrix suppression or enhancement, as only isotope ratios have to be measured. Therefore both isotopes will be affected in the same way. It enabled high accuracy and small measurement uncertainties, when applied properly [55]. However it has disvantages because among others it is expensive, and it is a destructive method.

14.3 New processed matrices and the effects on pesticides survival

Processes involving heat can increase volatilization, hydrolysis or other chemical/degradation reactions and thus reduce residue levels. On the contrary drying processes may result in higher concentrations of residues due to loss of moisture. The sterilization process and the set up conditions can vary. The details of time, T, degree of moisture loss and wether the system is closed or open are important to the quantitative effects of residue levels. Several reviews have appeared over the last 10 years [56] on the effects of processing on pesticides residues. The emphasis has been mainly on the organochlorine insecticides. Also the persistence and distribution of residues of post harvest fruits and vegetables has been the subject of a recent thorough review [56]. In the present work it was necessary to investigate the effect of storage (freezing) or processing (sterilization and freeze drying) with an intent to rationalize this information in the context of the thesis work which included specific conditions, matrices and compounds.

14.4 Water content determinations

Although in literature/legislation MRL´s are given in mg/kg (wet/frozen correspondent fruit/vegetable), in the present study and in order to achieve comparability between the three tested processes results were given in ng/g dry matter. Therefore water content determinations were of crucial importance in order to provide such type of measurement result.

For samples with low water content (1 % water (m/m) -8 % water (m/m), an AOTF-NIR technique described in detail by Kestens et al. [58], which provides online measurements by being attached to the capping machine of the freeze dried samples, was used.

For confirmation purposes Karl fisher titration (KFT) operated under ISO 17025 was employed, and the results compared.

For samples with high water content (frozen and sterilized materials) two methods were used in those measurements, namely KFT and oven drying.
Although oven drying is not selective for water and KFT is, it demonstrated to be more precise for samples with high water content.

After conducting homogeneity/stability studies, frozen and freeze dried materials were elected as the best option for the end-purpose. Therefore and based in the above discussion, a strategy based on elemental content (Ca, Mg, and P) of the frozen/freeze dried matrices, was developed to contribute for the measurements accuracy and eliminate the high water measurement uncertainties in the frozen samples by KFT. This methodology is dicussed in detail in Annex 7.

14.5 Homogeneity and stability studies

The whole study provided an advancement of the scientific knowledge in comparison to what has been reported in the literature up to date.

With regard to the analytical method applied, there are not many RM for pesticides in fruit/vegetable matrices available besides the natural matrix (pureed tomato) CRM containing residue concentrations of pesticide at Australian MRL level prepared by the National Measurement Institute of

Australia (NMIA) [41], in which two identical substances (chlorpyrifos and permethrin) were included and others with similar size, polarity, and vulnerability to heat processing (parathion methyl and α and β endosulfan), were used in both studies, serving as a comparison model for experimental results. The NMIA sample was stabilized by means of heat sterilization in sealed cans.

In this case, the reported uncertainty of homogeneity of the NMIA CRM can serve as a comparison, althought a different analytical method using IDMS calibration was employed and the matrix in question was acidic (which has no relevance if one compares analytes that are not acid or based sensitive). Here it is of interest to compare NMIA findings with the sterilized carrot matrix, since this is the common stabilization process.

The reported uncertainty contribution of inhomogeneity for chlorpyrifos and permetrin were 9.9 % and 3.2 % respectively, and below 15 % for the other NMIA studied pesticides, while in the carrot matrix it was 15.4 % and 6.2 % for the same compounds. In fact, inhomogeneity contributions of pesticides in the sterilized carrot matrix also gave very high values, up to 15 %.

The NMIA report mentioned that the concentration of all pesticides measured during homogeneity testing were in all cases lower than the spiking level and this could be due to the heat sterilization process itself. This is similar to what was found in the sterilized spiked carrots, for which heat treatment did contribute to degradation of the majority of pesticides.

For the NMIA samples short periods of refrigerated or ambient temperatures are acceptable during transport, which is not in accordance with findings of this study where most pesticides are only stable when frozen, even during a 4 week period.

Results of stability showed that storage in a freezer is required for the long term stability of the NMIA sample for all pesticides except parathion-methyl, which presented a high homogeneity uncertainty contribution and instability due to heat. This is somewhat dissimilar to the sterilized carrots sample for which long term stability could not be achieved even during freezer storage for the majority of the studied pesticides.

Homogeneity/stability results obtained for the three processed matrices of the present study and their uncertainty contributions are a major contribution

for the decision making certification process of those pesticides in the carrots matrix. An overview of this data is summarized and discussed here.

Long term stability was done for a period of 5 months for the three tested matrices, so it would be indicative of pesticides behaviour in different type of processed matrices. This time span was sufficient to prove that only a few pesticides (four) remained stable in the sterilized matrix. The heat treatment did not contribute positively to samples stability, which would eliminate this type of stabilization technique for the use of the carrots matrix as a RM.

As far as the freeze dried matrices are considered, the purpose of using a freeze dried matrix, which would require a reconstitution step, is mostly due to the fact that it would avoid the use of large quantities of dry ice to ship a frozen sample. Instead cool bags (keeping the sample bellow 0 °C) can be used for shipping samples to the end consumer.

All studied pesticides remained stable for a period of 5 months in the carrots matrix with an average combined uncertainty contribution of 8.2 % and 10.1 % in the frozen and freeze dried matrix respectively, to the exception of some late elucting compounds in the freeze dried matrix. For those substances subsequent injections in the GC instrument of the samples revealed to be out of control, probably due to adsorption mechanisms or formation of new active sites that could influence at least these pesticides analysis. This needs further experimental confirmation.

With these findings it is concluded that freezing and freeze drying are acceptable stabilization techniques that meet the purpose of the whole study.

The scientific advance consisted in using differently processed matrices and a wider list of target pesticides for the preparation of a RM, which has never been studied for homogeneity and stability parameters in any other natural matrix as far as literature searches provide.

The homogeneity/stability uncertainty contributions of the pesticides in the processed matrices by means of freezing, sterilization and freeze drying provide valuable information for the certification process of a candidate RM and this was the main goal of the study.

15. Outlook and future work

The present study revealed the potential of the chosen analytical method for detection and quantification of the low MRL values of pesticides in food commmodities as demanded by European food legislation. The application of the method for various types of food commodities and analytes, including its suitability for concurrent analysis in both GC and LC instrumentation, makes it a very promising technique.

This method could be used to assign values to a candidate reference material. As the results show, the analysis of the LTS of the freeze dried batch were somehow out of control, at least for the late elucting compounds and more investgations need to be done in order to confirm and to complete the results on the LTS study of the freeze dried batch of sample. Consequently, the analytical technique should be improved in order to obtain better overall accuracy for the large set of samples resulting from isochronous stability studies. One possibility would be to implement a technique that speeds up the analysis time so that sample through-put increases; by doing so more replicates could be run, improving the robustness of the precision estimates. Maštovská and Lehotay [61] have described several practical approaches to fast chromatography for pesticide residue analysis, which are either based on (1) short, microbore capillary GC columns, (2) fast temperature programming, (3) low-pressure GC-MS, (4) supersonic molecular beam for MS at high GC carrier gas flow, and (5) pressure-tunable GC-GC. Another possibility of improving the precision of the GC-MS results would be the use of special GC inlet devices allowing the removal of nonvolatile matrix components that would normally contaminate the inlet after every injection [62, 63]. Another advantage is the possibility to switch from GC to LC and improve sensitivity of more polar compounds [64, 65] by using large sample input devices.

16. Summary

In the present study, a new concept towards reference materials for pesticide analysis in a food matrix has been investigated. The proper monitoring of this class of compounds requires the use of CRMs to ensure worldwide comparability of pesticide data.

The developed concept is based on commercially available baby food spiked with a range of pesticides. The matrix was stabilised by either freezing, freeze-drying or sterilization. The freeze-dried matrix has to be reconstituted before actual use.

The basic requirements related to the development of reference materials, namely homogeneity, stability and matrix properties were investigated.

Homogeneity and stability studies of the candidate RMs were carried out, i.e. a number of jars containing pesticides spiked into frozen, sterilized and freeze dried carrot matrix were kept for different periods at different temperatures, in order to detect possible instability.

The homogeneity data was assessed using one way ANOVA, which allows the separation of heterogeneity and method repeatability influences. The experimental set up of the study ensured that the errors resulting from measurement, sampling and sample treatment were similar for all samples, only the degree of homogeneity could vary from sample to sample.

Method repeatability was better then 10 % for the majority of compounds and between bottle variation could not be detected for many compounds in the three tested materials, therefore u^{*}_{bb} was adopted as potential inhomogeneity contribution.

For the majority of compounds in the three tested materials a small heterogeneity contribution could be detected, with values bellow 5%. Azoxystrobin in the frozen and sterilized batch, metalaxyl in the freeze dried batch and cypermethrin, azoxystrobin, chlorpyrifos, lambda-cyhalotrin, malathion and permethrin in the sterilized batch presented a significant degree of inhomogeneity.

A GC-IDMS method was developed that contributed to the accurate determination of pesticides in carrot matrix.

Based on the elemental content (Ca, Mg ,P) of the frozen/freeze dried matrices, a method was developed in order to eliminate the concentration of pesticide (given in ng/g dry matter) in the frozen test material as it based on a high uncertainty water content analysis. (Annex 7), that contributes to reduce the overall uncertainty.

Stability of a natural matrix candidate RM refers to two components: the stability of the matrix itself and stability of the target analytes. However, these factors are correlated and the study set up chosen did not allow to assess the two factors separately. In the available literature there are no stability data of pesticide, only the pesticide manual compendium [57] has stability data of pesticides, taking into account the influence of pH, temperature, light and moisture.

In any case the overall objective is to assess stability issues that might arise during storage of the candidate RM, which along with the t/T that might affect the pesticide concentration. These are the conditions that the test materials (matrix and analytes) might undergo before they arrive to a customer laboratory.It means that the set up of the stability study did not allow to separate analyte and matrix stability but they are effectively studied together.

The conditions that might influence that stability property (analyte+matrix) during transport and storage of the material and that can be tested during the study are the temperature and time of transport/storage, so this are the “changing” parameters “behind” the set up of the study (time and temperature).

The sterilized test material seems to be the less suitable surrounding environment to keep pesticides stable during long term storage.

The frozen material is similar to a routine carrot sample, but it has the disadvantage of the need of being shipped on dry ice (e.g. high quantities of dry ice are necessary for a shipment of 48 h), and this can possibly be avoided because the results of freeze dried sample demonstrated that it can be shipped at higher temperature (e.g. +4 °C), for majority of pesticides under study.

Depending on the target maximum combined uncertainty, decisions have to be made in relation to the choice of both the type of processed matrix and pesticides of interest to be certified. It is important to note that the processed

blank and its correspondent CRM must be provided to the end costumer, as matrix effects might influence the result and the proper calibration of the samples (in this case the CRM itself). Matrix enhancement effects are different in a processed and non processed matrix.

17. Annexes

Annex 1

Experimental/statistical protocol for homogeneity and stability studies of the candidate Reference Materials

This Annex include a detailed plan of spiking procedures, bottling, and homogeneity/stability studies necessary for carrying out the feasibility study of producing of three candidate RMs (frozen, freeze dried and sterilized carrots matrices spiked with pesticides at the specific MRL level).

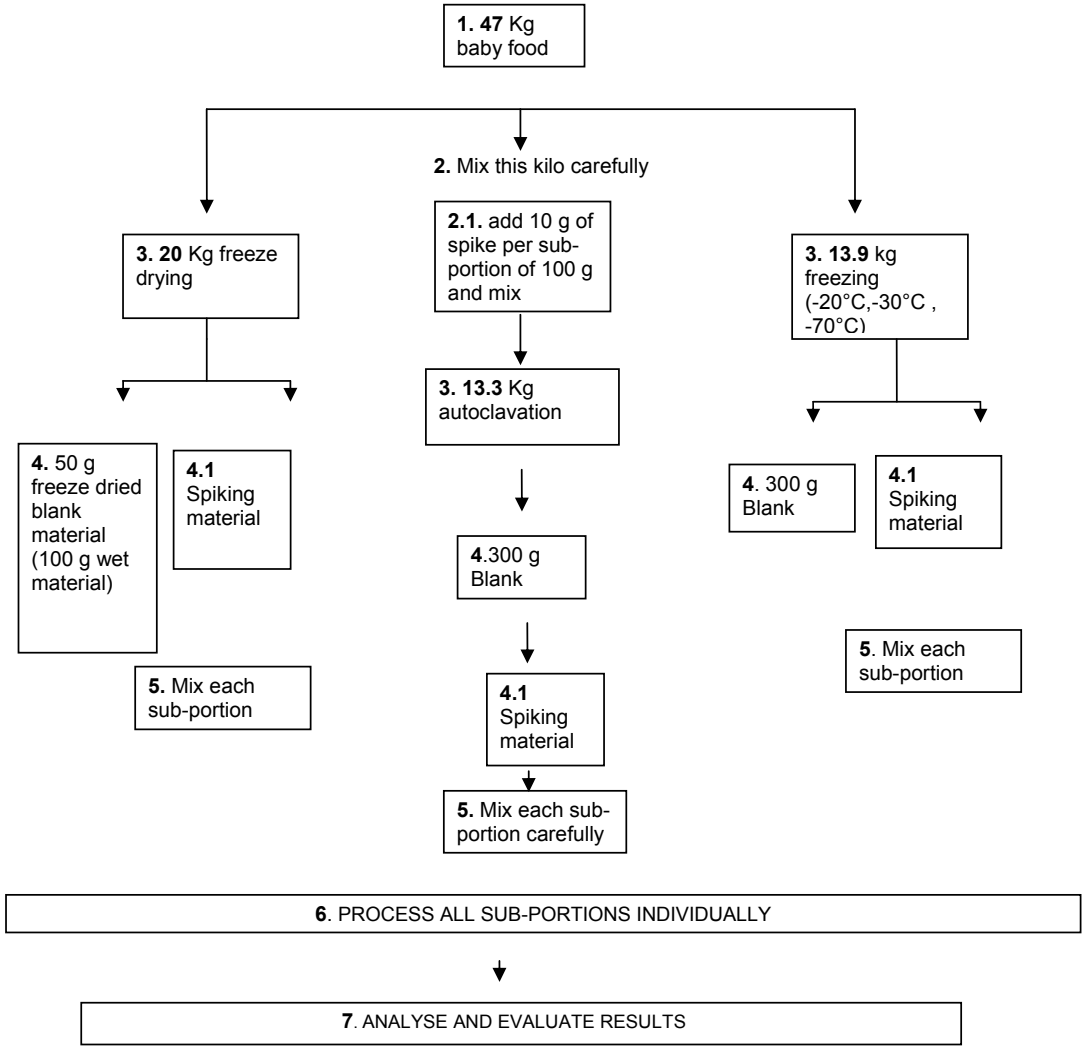
The extent to which pesticide residues are removed by processing depends on a variety of factors, such as the chemical properties of a pesticide, the nature of the food commodity, the processing step and the length of time the compound has been in contact with the food.

The work described here, is intended to determine the effect of different processing operations (freeze, freeze drying, and sterilization) on the pesticides residues in fruits and vegetables and for that, commercially purchased baby food carrots with potatoes is intended to simulate the homogenized correspondent main fruit/ vegetable (carrots).

A. Experimental

Figure 1 shows the flow chart followed by a description of each step undertaken at the processing plant at RM unit of the JRC-IRMM (Joint Research Center-Institute for Reference Materials and Measurements)

Figure 1. Flow chart of food processing steps



A.1 Flow chart description

The raw material (baby food) is stored at room temperature (+18 °C).

A.1.1- Weigh 47 Kg of baby food to be tested.

A.1.2- Mix carefully this 47 Kg of baby food and measure:

- Water content
- pH

A.1.3-SPIKING: The mix at MRL is to be prepared in acetonitrile (acetonitrile is soluble in water) and should be diluted appropriately to ensure that the spiking level is done by weighting always 10 mL (approx. 10 g) of mix per 100 g of blank material, to ensure homogenization of the spiking process . Pesticide concentration should be around 10 % (mass of spiking/ weight of baby food).

This ensures that the homogenisation process is the same for the total amount of baby food to be used in the three different processes (freeze, freeze-drying and autoclavation) and it is done at the same time.

A.1.4-Allow a stabilization period of 30 min.

A.1.5-Weigh separately 20,10 and 10 Kg of well homogenized and spiked material to be used in the processing step, respectively for freeze drying, sterilization and freezing (-70 °C, -30 °C and -20 °C). The quantities shown in the flow chart (4 and 4.1) applies to the temperatures that will be used in the stability testing. Freeze dryer minimum batch size is 1 Kg.

A.1.6-CALIBRATION: Weight 240 g for a matrix blank, the matrix blank will serve to construct the matrix-matched calibration curve, using 10 g sample for each extraction and 3 replicates of each calibration point (0.25 MRL, 0.5 MRL, MRL, 1.5 MRL, 2 MRL) taking into account possible wastes (15%) (10 g sample * 5 points * 3 replicates = 150 g blank material). For freeze dry blank material, the same replicates applies, sample intake is approx 2 g (2 g * 5 points* 3 replicates = 30 g freeze dry material).

A.1.7-Weigh 20, 13 and 13 Kg well of homogenized and spiked material (corresponding to each processing treatment) and account for possible wastes (15 %).

A.1.8-Mix (homogenize well) each subsample individually (CALIBRATION and SPIKING).

A.1.9-Allow a stabilization period of 1 hour at + 4 °C and in the dark (cover with aluminium foil if necessary)

A.2-Bottling

A.2.1- All material (spiked and blank) is filled in glass jars with metal screw cap. Jars are filled with 70 g wet material and 13 g dry material.

A.2.2- Process individually each portion (e.g. 1 Kg per each freeze dryer tray, but all processed at the same time.

A.2.3- After processing and for sample analysis, a reconstitution step for freeze dried samples is necessary (to be able to use the QuEChERS method and water content adjusted to 85 - 90 % (m/m)).
The target water content of the freeze drying processed samples is 3 %.
Prepare matrix-matched calibration curve and analyse samples with validated QuEChERS method. Give results in ng pesticide/g dry matter for all 3 processes (freeze, freeze drying and sterilization)
Store samples at -70 °C (freezer) if not readily analysed, and in dark (cover with aluminium foil if necessary).

A.3-Conclusions

The determination of the recoveries using a calibration curve obtained for each process, will show how the extractability of different pesticides is affected by each treatment, type of matrix (coextractives) and pH.

B. Plan of the homogeneity study according to Reference Material Unit Procedure (RM PR/ 0004 RM PR70017)

B.1-Between–Unit

- Average method repeatability for the target analytes is 5 % at a sample intake 10

- Targeted maximum contribution of inhomogeneity is 2 %.
- Number of units to be assessed is 10 (e.g. N=1000 units batch$^{1/3}$=10 units
- It is assumed that the method repeatability of 5 % cannot be decreased.
- Number of replicates [48]:

$$u_{bb}^{*} = \frac{RSD_{method}}{\sqrt{n}} \sqrt[4]{\frac{2}{N(n-1)}}$$

Where:

N= number of units to be assessed

n= number of replicates per unit

u^{*}_{bb}= envisaged uncertainty of homogeneity (between- bottle)

RSD_{method}= RSD method repeatability

With RSD method = 5%, N=10 units, several values for n are obtained

n	U*bb
2	2.37
3	1.62
4	1.3

Two replicates per sample unit are enough to detect (hidden) inhomogeneity above 2 %, given a total of 2 replicates * 10 units = 20 measurements, for each process (freeze, freeze drying and sterilization).

Safety factor=2, Total Units=20 per type of process.

B.2 Within-Unit Homogeneity

Six replicates per unit should be analysed, to check if method repeatability is the same as given in method validation and should permit to determine the minimum sample intake value.

C. Plan of stability studies according to Reference Material Unit Procedure (RM PR/0009)

C.1 Background information

Stability testing is of the highest importance as CRM may be sensitive to degradation by several factors (pH, T, light, etc.). All studies must be carried out using highly repeatable and reproducible methods.

C.2 Plan of short-term stability studies

Duration 1 month (exceeding a normal time allowed for transportation)
Temperature [-20 °C, + 4 °C, + 18 °C, + 60 °C]
Number of time points: 3 time points, **not including T ref**, t= 0, 1, 2, 4 weeks
Number of replicates and units: 2 units per each time point, **3 replicates per unit**
Measurement method: GC-MS

Analytes to be determined: 21 pesticides

Sample intake -10g

1 unit = 70 g test product

2 units per each time point,3 time points
= 30 units

Safety factor = 2, Total Units to be produced= 60

Units at Reference temperature: 3 unit T_{ref} (freezer 1) and 3 units T_{ref} (freezer 2). Total 66 units

C.3 Planning vs evaluation of long-term stability studies

Duration: 5 months

Temperatures [-20 °C, + 4 °C, + 18 °C] 3 time points not including T ref [-70 °C]

Number of time points: 3 time points not including T ref, t= 0, 3, 4, 5 months

Measurement method: GC-MS

If 3 replicates per time point are measured

The targeted uncertainty due to long term stability should be related to the targeted shelf –life:

Decided: The uncertainty of spiked carrots baby food for a shelf life of 5 months should be less than 3%.

$$u_{lts\,[\%]} = \frac{RSD_{method\,[\%]}}{\sqrt{n\sum(Xi-\overline{X})^2}} * X_{shelf}$$

X_i -time points

$\overline{X}$ -average time points

X_{shelf}-envisaged shelf life

Time points = 0, 3, 4, 5 months

Calculations:

$\overline{X}$ = 3

$\Sigma (X_i - \overline{X})^2$ = 15

μ_{lts} (%) = 5% / (SQRT (3*48.8))*9 months = **3,73 %**

So, the number of replicates must be larger to achieve a lower u_{lts} for a shelf life of 5 months.

Calculation of number of replicates per time point (n):

$$n = \left(\frac{RSD_{method[\%]} * X_{shelf}}{u_{lts[\%]}} \right)^2 * \frac{1}{\sum (X_i - \overline{X})^2}$$

$\Sigma (Xi-X)^2 = 15$

N = (5 % *5 months /3 %)* 1/15

n= 4,6 replicates= 5 replicates per time point

Units: 2 units per each time point.

Note: it does not matter for the study if the several replicates per time, come from two or more units, generally the more heterogeneous a material is, the more different units per time point shall be used

Sample intake: 10 g

Total number of units: 2 units * 3 time points = 6 units /per each temperature

18 Units total

And 5 replicates per time point

Safety factor = 2, Total Units= 36 units to be produced

Plus 3 unit T_{ref} (freezer 1) and 3 units T_{ref} (freezer 2)

Total= 42 Units per each process

Annex 2

Table 1: Calibration in solvent given in ng/g solvent (toluene)

Pesticide	MRL Level	MRL ratio										
	ug/kg	ug/g MIX MRL	0.25 MRL1	0.25 MRL 2	0.5 MRL1	0.5 MRL 2	MRL1	MRL 2	1.5 MRL1	1.5 MRL 2	2 MRL 1	2 MRL 2
azinphos-methyl	50	1.0	14.2	14.2	24.6	24.6	48.2	49.6	75.6	72.9	100.0	102.7
azoxystrobin	50	1.1	14.4	14.4	24.9	24.9	48.8	50.1	76.5	73.8	101.3	103.9
bromopropylate	50	1.1	14.3	14.3	24.8	24.7	48.4	49.8	76.0	73.3	100.5	103.2
chlorpyriphos	50	1.1	14.7	14.7	25.5	25.5	49.9	51.3	78.3	75.5	103.7	106.4
chlorpyriphos-methyl	50	1.0	14.2	14.3	24.7	24.6	48.3	49.7	75.8	73.1	100.3	102.9
cypermethrin	50	1.0	14.2	14.2	24.6	24.5	48.1	49.4	75.4	72.7	99.8	102.4
diazinon	10	0.2	3.1	3.1	5.3	5.3	10.4	10.6	16.2	15.7	21.5	22.1
endosulfan a+b	50	1.1	14.3	14.3	24.8	24.7	48.4	49.8	76.0	73.3	100.6	103.2
iprodione	20	0.4	5.8	5.8	10.1	10.0	19.7	20.2	30.9	29.8	40.8	41.9
lambda-cyhalotrin	20	0.4	5.8	5.8	10.0	10.0	19.6	20.1	30.7	29.6	40.7	41.7
malathion	500	10.5	142.6	142.7	247.2	246.5	483.4	497.0	758.3	731.2	1003.5	1029.7
mecarbam	50	1.1	14.3	14.3	24.8	24.7	48.5	49.9	76.1	73.4	100.7	103.4
metalaxyl	50	1.1	14.5	14.5	25.1	25.0	49.0	50.4	76.9	74.2	101.8	104.5
parathion	50	1.0	14.2	14.2	24.6	24.5	48.0	49.4	75.4	72.7	99.7	102.3
permethrin	50	1.1	14.3	14.4	24.9	24.8	48.7	50.0	76.3	73.6	101.0	103.6
phorate	50	1.1	14.4	14.4	24.9	24.8	48.7	50.1	76.4	73.7	101.1	103.7
pirimiphos-methyl	50	1.0	13.9	13.9	24.1	24.0	47.1	48.5	73.9	71.3	97.9	100.4
procymidone	20	0.4	5.7	5.7	9.9	9.8	19.3	19.8	30.3	29.2	40.1	41.1
propyzamide	20	0.5	6.2	6.2	10.7	10.6	20.9	21.5	32.7	31.6	43.3	44.5
triazophos	20	0.4	5.8	5.8	10.0	10.0	19.6	20.1	30.7	29.6	40.6	41.6
vinclozolin	50	1.1	14.3	14.3	24.8	24.7	48.5	49.8	76.0	73.3	100.6	103.2

Table 2: Preparation of solvent based standards

	0.25 MRL1	0.25 MRL 2	0.5 MRL1	0.5 MRL 2	MRL1	MRL 2	1.5 MRL1	1.5 MRL 2	2 MRL 1	2 MRL 2
MIX MRL(g)	0.07	0.07	0.12	0.12	0.23	0.24	0.36	0.36	0.48	0.49
MIX labelled (ISTD) (g)	0.06	0.07	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.07
TPP wsol (g)	0.31	0.31	0.32	0.31	0.31	0.31	0.32	0.32	0.32	0.31
toluene (g)	4.57	4.56	4.55	4.52	4.41	4.40	4.27	4.39	4.18	4.16
TOTAL (g)	5.01	5.01	5.06	5.02	5.02	5.01	5.03	5.14	5.05	5.04

e.g.

C each compound at their respective MRL level (mix labelled) = 4870 ng/g

C TPP ws = 2500 ng/g

Table 3: Calibration in matrix given in ng/g blank extract (ng/g sample) covering a concentration range from 0.25 MRL to 2 MRL of each pesticide in carrot/potato matrix

Pesticide	MRL level (µg/Kg)	mix MRL ug/g	0.25 MRL 1	0.25 MRL 2	0.5 MRL 1	0.5 MRL 2	MRL 1	MRL 2	1.5 MRL1	1.5 MRL 2	2 MRL 1	2MRL2
Azinphos-methyl	50.0	1.3	17.5	16.6	30.8	29.8	57.8	57.1	241.4	98.8	110.7	106.9
Azoxystrobin	50.0	1.3	17.5	16.6	30.9	29.8	57.9	57.2	241.9	99.0	110.9	107.2
Bromopropylate	50.0	1.3	17.3	16.5	30.5	29.5	57.3	56.7	239.4	98.0	109.8	106.1
Chlorpyriphos	50.0	1.3	17.4	16.6	30.7	29.7	57.7	57.0	240.9	98.6	110.5	106.7
Chlorpyriphos-methyl	50.0	1.3	16.6	15.7	29.2	28.3	54.8	54.2	228.9	93.7	105.0	101.4
Cypermethrin	50.0	1.3	17.5	16.7	30.9	29.9	58.0	57.3	242.3	99.2	111.1	107.4
Diazinon	10.0	0.3	3.8	3.6	6.7	6.5	12.6	12.5	52.7	21.6	24.2	23.3
Endosulfan a+b	50.0	1.4	17.7	16.8	31.1	30.1	58.4	57.8	244.1	99.9	111.9	108.1
Iprodione	20.0	0.5	7.1	6.7	12.5	12.1	23.4	23.2	97.9	40.1	44.9	43.4
Lambda-cyhalotrin	20.0	0.6	7.7	7.3	13.5	13.1	25.4	25.1	106.0	43.4	48.6	47.0
Malathion	500.0	12.9	168.5	160.0	296.8	287.1	557.1	550.6	2326.9	952.7	1067.3	1030.9
Mecarbam	50.0	1.3	17.3	16.5	30.6	29.6	57.4	56.7	239.5	98.1	109.9	106.1
Metalaxyl	50.0	1.3	17.6	16.7	30.9	29.9	58.0	57.4	242.4	99.2	111.2	107.4
Parathion	50.0	1.4	17.8	16.9	31.3	30.3	58.7	58.0	245.2	100.4	112.5	108.6
Permethrin	50.0	1.3	17.3	16.5	30.6	29.6	57.4	56.7	239.6	98.1	109.9	106.1
Phorate	50.0	1.3	17.4	16.5	30.7	29.7	57.6	56.9	240.6	98.5	110.3	106.6
Pirimiphos-methyl	50.0	1.4	18.0	17.1	31.7	30.7	59.5	58.8	248.6	101.8	114.0	110.2
Procymidone	20.0	0.5	7.1	6.7	12.4	12.0	23.3	23.1	97.5	39.9	44.7	43.2
Propyzamide	20.0	0.6	7.3	7.0	12.9	12.5	24.3	24.0	101.4	41.5	46.5	44.9
Triazophos	20.0	0.5	7.1	6.7	12.4	12.0	23.4	23.1	97.6	39.9	44.8	43.2
Vinclozolin	20.0	1.2	15.8	15.0	27.8	26.9	52.1	51.5	217.7	89.1	99.8	96.4

Table 4: Preparation of Matrix-matched standards

	0.25 MRL 1	0.25 MRL 2	0.5 MRL 1	0.5 MRL 2	MRL 1	MRL 2	1.5 MRL1	1.5 MRL 2	2 MRL 1	2MRL2
MIX MRL	0.04	0.04	0.07	0.07	0.13	0.13	0.54	0.22	0.25	0.24
MIX labelled	0.31	0.30	0.32	0.30	0.31	0.31	0.32	0.32	0.31	0.32
blank extract (1g/mL) (g)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
TPP ws (2490 ng/g)	0.31	0.29	0.28	0.3	0.3	0.28	0.31	0.3	0.3	0.32

Table 5: Example of calculations of ISTDs in the calibration standards

		MIX Labelled/ TPP ws	0.25 MRL 1	0.25 MRL 2	0.5 MRL 1	0.5 MRL 2	MRL 1	MRL 2	1.5 MRL1	1.5 MRL 2	2 MRL 1	2MRL2
C labelled parathion in Matrix-matched standards	ng/g	447.3	46.2	45.4	47.9	45.1	46.4	46.7	47.7	47.7	45.9	47.8
C labelled phorate in Matrix-matched standards	ng/g	460.0 460	503.1 50.3	487.7 48.7	514.3 51.4	483.8 48.3	498.1 49.8	501.0 50.1	511.9 51.2	511.9 51.2	493.2 49.3	513.1 51.3
C labelled pirimiphos-methyl in Matrix-matched standards	ng/g	405.2	42.5	41.2	43.4	40.8	42.0	42.3	43.2	43.2	41.6	43.3
C TPP WS in the Matrix-Matched standards	ng/g	2490	257.3	240.7	232.4	249.0	249.0	232.4	257.3	249.0	249.0	265.6

Annex 3

Table 6: Information about the pesticides under study on the 2002-2005 EU monitoring programme

Pesticide	use	mg/kg	Class	MW (g/mol)	Formula	Vp (mPa)	Water sol. (mg/L)25 C	Pkow	Analysis	Rt in GC-MS	Masses
		lowest MRL									
acephate	I	0.02	OP	183.2	$C_4H_{10}NO_3PS$	0.227	8.18E+05	-0.85	GC and **LC**	6.4	136, 94
aldicarb	I	0.05	oxime carbamate	190.3	$C_7H_{14}N_2O_2S$	4.6	6030	1.13	LC		
azinphos-methyl	I	0.05	Organothiophosphate	317.3	$C_{10}H_{12}N_3O_3PS_2$	0.213	20.9	2.75	GC	18.2	132,160
azoxystrobin	F	0.05	stobilurin	403.4	$C_{22}H_{17}N_3O_5$	1.1x10-7	6	2.5	**LC** and GC	22.3	344,345
benomyl	F	0.10	Benzimidazole carbamate	290.3	$C_{14}H_{18}N_4O_3$	Negligible	3.6	2.12	LC		
carbendazim	F		Carbamate	191.2	$C_9H_9N_3O_2$	Negligible	29	1.52	LC		
thiophanate -methyl	F		Carbamate	342.4	$C_{12}H_{14}N_4O_4S_2$	0.01	26.6	1.4	LC		
bromopropylate	A	0.05	Bridget diphenyl	428.1	$C_{17}H_{16}Br_2O_3$	0.011	0.1	5.4	GC	17.6	341,343
captan	F	0.05	Phthalimide	300.6	$C_9H_8Cl_3NO_2S$	0.012	5.1	2.8	GC	13.5	79,149
chlorothalonil	F	0.01	OC	265.9	$C_8Cl_4N_2$	0.076	0.81	2.92	GC	9.9	266,264,268
chlorpyrifos	I	0.05	OP	350.6	$C_9H_{11}Cl_3NO_3PS$	2.7	1.4	4.7	**GC** and LC	11.9	197,258,314
chlorpyrifos-methyl	I	0.05	OP	322.5	$C_7H_7Cl_2O_4P$	3	2.6	4.24	**GC** and LC	10.7	286,290
cypermethrin	I	0.05	Pyrethroid	416.3	$C_{22}H_{19}Cl_{12}NO_3$	Negligible	0.004	6.6	GC	19836.0	163,181,209
deltamethrin	I	0.01	Pyrethroid	505.2	$C_{22}H_{19}Br_2NO_3$	0.002	0.002	6.2	GC	21.9	181,253

diazinon	I	0.01	OP	304.4	$C_{12}H_{21}N_2O_3PS$	11.9	40	3.81	GC	9.5	137,179, 304
dichlofluanid	F	0.10	Phenylsulfamide	333.2	$C_9H_{11}Cl_2FN_2O_2S_2$	0.015	1.3	3.7	GC	11.6	167,224
dicofol	A	0.02	Bridget diphenyl	370.5	$C_{14}H_9Cl_5O$	0.053	0.8	5.02	GC	17.7	139,251
dimethoate	I	0.02	OP	229.3	$C_5H_{12}NO_3PS_2$	1,133	25000	0.78	GC	8.9	125,229
endosulfan (α+β)	I	0.05	OC	406.9	$C_9H_6Cl_60_3S$	0.023	0.325	3.83	GC	15.7	339,341
folpet	F	0.02	Phthalimide	296.6	$C_9H_4Cl_3NO_2S$	0.021	0.8	2.85	GC	13.3	260,262
imazalil	F	0.02	Imidazole	297.2	$C_{14}H_{14}Cl_2N_2O$	0.158	180	3.82	LC		
kresoxim-methyl		0.05							GC	15.8	131,206
iprodione	F	0.02	Imidazole	330.2	$C_{13}H_{13}Cl_2N_3O_3$	Negligible	13.9	3	GC	15.7	131,206
lambda-cyhalothrin	I	0.02	Pyrethroid	449.9	$C_{23}H_{19}ClF_3NO_3$	Negligible	0.000853	7	GC	18.4	181,197
malathion	I	0.05	OP	330.4	$C_{10}H_{19}O_6PS_2$	0.0451	143	2.36	GC	11.6	158,173
maneb	F	0.05	dithiocarbamate	295.4	$C_4H_6MnN_2S_4$	0.01	6	0.62			
mancozeb	F	0.05	dithiocarbamate	541.0	$C_8H_{12}MnN_4S_8Zn$	Negligible	6.2	1.33			
metiram	F	0.05	dithiocarbamate	504.1	$C_8H_{16}N_5S_8Zn$	0.01	1.45E+04	0.3			
propineb	F	0.05	dithiocarbamate	357.1	$C_5H_{10}N_2S_4Zn_2$	0.02	987	2.06			
zineb	F	0.05	dithiocarbamate	275.7	$C_4H_6N_2S_4Zn$	0.01	10	1.3			
mecarbam	I	0.05	Organothiophosphate	329.4	$C_{10}H_2ONO_5PS_2$	0.431	1000	2.29	GC	13.9	159,296,32 9
methamidophos	I	0.01	OP	141.1	$C_2H_8NO_2PS$	4.7	1.00E+06	-0.8	GC and **LC**	5.3	94,141
metalaxyl	F	0.05	anilide	279.3	$C_{15}H_{21}NO_4$	0.749	8400	1.65	GC	11.0	206,249
methidathion	I	0.02	Organotiophosphate	302.3	$C_6H_{11}N_2O_4PS_3$	0.449	187	2.2	GC	11.6	206,249

methiocarb	I		Carbamate	225.3	$C_{11}H_{15}NO_2S$	0.036	27	2.92	GC	12.0	168,153
methomyl	I	0.02	oxime carbamate	162.2	$C_5H_{10}N_2O_2S$	0.72	5.80E+04	0.6	LC		
omethoate	I	0.02	Organothiophosphate	213.2	$C_5H_{12}NO_4PS$	3,306	1.00E+06	-0.74	GC	8.0	110,156
oxydemeton-methyl	I	0.02	Organothiophosphate	246.3	$C_6H_{15}O_4PS_2$	0.0038	1.00E+06	-0.74	GC	4.8	142,168
parathion	I	0.05	OP	291.3	$C_{10}H_{14}NO_5PS$	0.891	11	3.83	GC	12.0	291.109.97
permethrin	I	0.05	Pyrethroid	391.3	$C_{21}H_{20}Cl2O_3$	0.0015	0.006	6.1	GC	19.0	163,183
phorate	I	0.05	OP	260.4	$C_7H_{17}O_2PS_3$	85	50	3.56	GC	8.9	260.75
pirimiphos-methyl	I	0.05	OP	305.3	$C_{11}H_{20}N_3O_3PS$	2	8.6	4.2	GC	11.4	290,305
procymidone	F	0.02	dicarboximide	284.1	$C_{13}H_{11}Cl_2NO_2$	18	4.5	3.14	**GC** and LC	14.1	283,285
propyzamide	H	0.02	amide	256.1	$C_{12}H_{11}Cl_2NO$	0.058	15	3.43		9.4	173,175
thiabendazole	F	0.05	benzimidazole	201.3	$C_{10}H_7N_3S$	0.00046	30	2.39	**LC**		
tolyfluanid	F		N-trihalomethylthio	347.3	$C_{10}H_{13}Cl_2FN_2O_2S_2$	0.2	0.9	3.9	GC and **LC**	13.0	238,240
triazophos	I	0.02	Organothiophosphate	313.3	$C_{12}H_{16}N_3O_3PS$	0.387	39	3.34	GC	16.9	161,162
vinclozolin		0.05	dicarboximide	286.1	C12H9Cl2NO3	0.016	2.6	3.1	GC	10.7	214,212

A= Acaricide I = Insecticide F= Fungicide H= Herbicide OC= Organochlorine OP = Organophosphate

The MRLs presented here are the minimum of the EU-MRLs set for each analyte/ matrix combinations (47 analytes in 6 matrices, resulting from the EU 2002-2005 monitoring programme)

In bold: preferred methodology

Annex 4

Table 7: LOD/LOQ determinations in an Apple/pear based blank extract (signal to noise ratio > 10 => Quantification (+); signal to noise ratio> 4 => Detection (+); at LOQ when signal to noise ratio = 10)

IN APPLE/PEAR BASED BABY-FOOD

Pesticide	end ratio MRL							
	ug/kg	Rt in GC in matrix	LC or GC	1/2 MRL	1/4 MRL	1/5 MRL	1/6 MRL	1/10 MRL
phorate	50	8.45	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
propyzamide	20	9.34	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
diazinon	10	9.51	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
vinclozolin	50	10.64	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
chlorpyriphos-methyl	50	10.67	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
metalaxyl	50	10.97	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
pirimiphos-methyl	50	11.39	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
malathion	*500*	11.63	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
chlorpyrifos	50	11.92	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
parathion	50	11.93	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
mecarbam	50	13.11	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ at LOQ
procymidone	20	13.34	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
methidathion	20	13.57	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
endosulfan a	10	13.91	GC detected but LC pref	Poor peak shape	Poor peak shape	Poor peak shape	Poor peak shape	No detected
endosulfan b	*50*	*15.63*	*GC*	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ at LOQ
triazophos	20	16.34	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ at LOQ
iprodione	20	17.43	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
bromopropylate	50	17.58	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
azinphos-methyl	50	18.10	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
lambda-cyhalotrin	20	18.38	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
permethrin	50	18.97,19.08	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)
cypermethrin	50	19.80,19.89,19.98	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ at LOQ
azoxystrobin	50	22.28	GC	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)	Detect (+)/ Quantif (+)

Annex 5

Table 8: Ion ratios for each target pesticide (%)

Pestiocide	Tgt, Q1, Q2	Tgt	Q1	Q2
azinphos-methyl	160,132	100	86	
azoxystrobin	344,345	100	34.1	
bromopropylate	341,343	100	45.5	
chlorpiriphos	197,314,258	100	82.8	49.1
chlorpiriphos-methyl	286,290	100	19.3	
cypermethrin (α+β+c)	181,163,209	100	145.1/165.3/148.3	129.8/136.4/138.9
diazinon	304,137,179	100	852.1	577.1
endosulfan (α+β)	339,341	100	69.10/72.8	
iprodione	314,316	100	64.1	
lambda-cyhalotrin	181,197	100	73.1	
malathion	173,158	100	42.7	
mecarbam	159,329,296	100	44.5	28.2
metalaxyl	206,249,279	100	50.6	19.7
parathion	291,109,97	100	103.3	131.9
permethrin (1+2)	183,163	100	64.6/56.6	
phorate	260,75	100	460.8	
pirimiphos-methyl	290,305	100	64.7	
procymidone	283,285	100	65.5	
propyzamide	173,175	100	61.7	
triazophos	161,162	100	66.5	
vinclozolin	212,214	100	196.4	
labelled phorate (ISTD)	264,125,235	100	140.8	54.9
labelled malathion (ISTD)	183,132	100	65.5	
labelled parathion (ISTD)	301,115,99	100	65.6	78.2
labelled cypermethrin (ISTD) mix of isomers	187,163,207	100	113.6/136.2/137.8	49.3/106.1/30.4
TPP (ISTD)	325,326,233	100	123,9	20.5

Annex 6: Uncertainty Budget

The uncertainty was calculated using the top-down approach taking into account the uncertainty of the preparation of the standards, the repeatability, the intermediate precision, the calibration curve and the recovery (MRL level/ total number of days)

$$U = k * \sqrt{u_{(Cst)}{}^2 + u^2{}_{(Calib)} + \frac{u_r{}^2}{n_1} + \frac{u_{ip}{}^2}{n_2} + \left(\frac{CV}{\sqrt{n_3}}\right)^2}$$

k	coverage factor (k=2)	
U Calib	Uncertainty of calibration curve	
U Cst	uncertainty of the standards used	
u r	uncertainty of repeatability	
n1	total number of measurements	
u ip	uncertainty of intermediate precision	
n2	total number of days measured	
CV	Average of coefficient of variation of the results on 5 days (2 levels)	30= 6 replicates *5 days
n3	number of independent samples	30= 6 replicates *5 days
n2	total number of days measured	5 days
CV	Average of coefficient of variation of the results on 5 days (2 levels)	
n3	number of independent samples	27

Table 9: Uncertainty budget calculations (1) Uncertainty of the Preparation of the standards

Pesticide	**Neat solid**	ME 235 P-OCE				**indiv. stock sol**	ME 235 P-OCE				**Indiv.Stock dilution**	ME235 P-OCE				ME 235P-OCE						**MIX MRL level**	ME235 P-OCE				
	intercept	slope	(mg)	u (mg)	**u (%)**	intercept	slope	(g)	u (g)	**u (%)**	intercept	slope	(g)	u (g)	**u (%)**	intercept	slope	(g)	(g)	u (g)	**u (%)**	intercept	slope	(g)	u (g)	**u (%)**	Ucstd
azinphos-methyl			0.056	0.0001	**0.21**			25.014	0.000	**0.002**			0.4068	0.0001	**0.031**				22.363	0.0004	**0.002**			2.4302	0.0002	**0.0060**	0.2904
azoxystrobin			0.057	0.0001	**0.21**			25.1153	0.0005	**0.002**			0.40104	0.0001	**0.032**				20.5314	0.0004	**0.002**			2.2715	0.0002	**0.0070**	0.3608
bromopropylate			0.050	0.0001	**0.24**			25.0237	0.0005	**0.002**			0.41722	0.0001	**0.031**				20.0442	0.0004	**0.002**			2.3780	0.0002	**0.0070**	0.3774
chlorpyriphos			0.076	0.0001	**0.16**			29.8226	0.0005	**0.002**			0.4048	0.0001	**0.031**				20.1905	0.0004	**0.002**			2.0100	0.0002	**0.0070**	0.3316
chlorpyriphos-methyl			0.049	0.0001	**0.24**			25.1207	0.0005	**0.002**			0.5394	0.0001	**0.024**				23.3167	0.0005	**0.002**			2.1742	0.0002	**0.0070**	0.3795
cypermethrin			0.056	0.0001	**0.21**			25.8586	0.0005	**0.002**			0.48	0.0001	**0.027**				21.1916	0.0004	**0.002**			2.0163	0.0002	**0.0070**	0.3622
diazinon			0.062	0.0001	**0.19**			25.4362	0.0005	**0.002**			0.4291	0.0001	**0.030**				19.9655	0.0004	**0.002**			0.4082	0.0001	**0.0310**	0.3522
endosulfan a+b			0.055	0.0001	**0.22**			25.0749	0.0005	**0.002**			0.41252	0.0001	**0.031**				20.1117	0.0004	**0.002**			2.2193	0.0002	**0.0070**	0.3650
Iprodione	0.000121	0.0000142	0.056	0.0012	**0.21**	0.000121	0.0000142	24.8216	0.0005	**0.002**	0.000121	0.0000142	0.3618	0.0001	**0.035**	0.000121	0.0000142		19.8812	0.0004	**0.002**	0.000121	0.0000142	0.9876	0.0001	**0.0140**	0.3631
lambda-cyhalotrin			0.052	0.0001	**0.23**			25.1532	0.0005	**0.002**			0.40597	0.0001	**0.031**				20.0322	0.0004	**0.002**			0.9627	0.0001	**0.0140**	0.6239
malathion			0.057	0.0001	**0.21**			25.2411	0.0005	**0.002**			0.57663	0.0001	**0.022**				30.18414	0.0006	**0.002**			23.0210	0.0004	**0.0020**	0.3600
mecarbam			0.188	0.0001	**0.06**			47.8946	0.0008	**0.002**			0.2102	0.0001	**0.059**				20.0337	0.0004	**0.002**			2.4329	0.0002	**0.0060**	0.3020
metalaxyl			0.048	0.0001	**0.25**			24.9522	0.0005	**0.002**			0.4311	0.0001	**0.030**				20.0092	0.0004	**0.002**			2.4371	0.0002	**0.0060**	0.3851
parathion			0.06	0.0001	**0.20**			25.426	0.0005	**0.002**			0.42395	0.0001	**0.030**				20.1814	0.0004	**0.002**			2.0030	0.0002	**0.0070**	0.6131
permethrin			0.049	0.0001	**0.24**			24.2431	0.0005	**0.002**			0.3845	0.0001	**0.033**				19.9277	0.0004	**0.002**			2.5803	0.0002	**0.0060**	0.6297
phorate			0.054	0.0001	**0.22**			25.078	0.0005	**0.002**			0.40081	0.0001	**0.032**				20.0825	0.0004	**0.002**			2.3295	0.0002	**0.0070**	0.3675
pirimiphos-methyl			0.059	0.0001	**0.20**			25.0714	0.0005	**0.002**			0.40634	0.0001	**0.031**				20.0688	0.0004	**0.002**			2.0241	0.0002	**0.0070**	0.3555
procymidone			0.049	0.0001	**0.24**			27.2695	0.0005	**0.002**			0.4669	0.0001	**0.027**				21.0068	0.0004	**0.002**			0.9811	0.0001	**0.0140**	0.6112
propyzamide			0.052	0.0001	**0.24**			25.3483	0.0005	**0.002**			0.40561	0.0001	**0.031**				21.4856	0.0004	**0.002**			1.1101	0.0001	**0.0120**	0.6239
triazophos			0.053	0.0001	**0.22**			25.0202	0.0005	**0.002**			0.43256	0.0001	**0.029**				20.2791	0.0004	**0.002**			0.9238	0.0001	**0.0150**	0.3699
vinclozolin			0.0484	0.00012	**0.252**			25.1243	0.0005	**0.002**			0.3704	0.0001	**0.034**				19.4892	0.0004	**0.002**			2.7370	0.0002	**0.0060**	0.2544

2) Uncertainty of the purity

Pesticide	P	u (P)		**u (P) (%)**
azinphos-methyl	0.9650	0.0048	0.0028	**0.2887**
azoxystrobin	0.9990	0.0050	0.0029	**0.2887**
bromopropylate	0.9920	0.0050	0.0029	**0.2887**
chlorpyriphos	0.9920	0.0050	0.0029	**0.2887**
chlorpyriphos-methyl	0.9990	0.0050	0.0029	**0.2887**
cypermethrin	0.9670	0.0097	0.0056	**0.5774**
diazinon	0.9990	0.0050	0.0029	**0.2887**
endosulfan a+b	0.9750	0.0049	0.0028	**0.2887**
iprodione	0.9990	0.0050	0.0029	**0.2887**
lambda-cyhalotrin	0.9850	0.0049	0.0028	**0.2887**
malathion	0.9730	0.0097	0.0056	**0.5774**
mecarbam	0.9890	0.0049	0.0029	**0.2887**
metalaxyl	0.9850	0.0049	0.0028	**0.2887**
parathion	0.9880	0.0049	0.0029	**0.2887**
permethrin	0.9450	0.0095	0.0055	**0.5774**
phorate	0.9450	0.0095	0.0055	**0.5774**
pirimiphos-methyl	0.9990	0.0050	0.0029	**0.2887**
procymidone	0.9950	0.0050	0.0029	**0.2887**
propyzamide	98.1000	**not stated**	0.5485	**0.5591**
triazophos	0.7100	0.0071	0.0041	**0.5774**
vinclozolin	0.9960	0.0050	0.0029	**0.2887**

3) Final Calculations

Pesticide	Ucstd	Ucalib	Ur	Uip	CV (average 5 days) for 2 conc levels	SQRT(n3)	U (%)
azinphos-methyl	0.29	0.016	2	14.04	7.37		12.91
azoxystrobin	0.36	0.006	3.33	5.83	3.12		5.54
bromopropylate	0.38	0.004	4.98	2.61	3.38		3.32
chlorpyriphos	0.33	0.047	8.76	0.49	1.40		3.34
chlorpyriphos-methyl	0.38	0.088	42.69	0.51	1.51		15.63
cypermethrin	0.36	0.042	4.6	3.72	2.88		3.96
diazinon	0.35	0.038	2.2	2.69	1.33		2.68
endosulfan a+b	0.36	0.017	3.11	3.91	1.77		3.81
iprodione	0.36	0.011	5.19	3.84	3.92		4.27
lambda-cyhalotrin	0.62	0.005	4.77	1.56	3.39		2.87
malathion	0.36	0.583	2.16	3.45	2.12		3.56
mecarbam	0.30	0.028	3.27	4.18	1.26	5.19	4.00
metalaxyl	0.39	0.089	6.08	8.21	2.76		7.78
parathion	0.61	0.036	2.59	1.31	1.33		2.01
permethrin	0.63	0.016	5.02	1.25	2.61		2.69
phorate	0.37	0.031	1.93	0.77	1.29		1.33
pirimiphos-methyl	0.36	0.056	2.77	4	2.30		3.89
procymidone	0.61	0.078	2.22	2.82	1.51		2.98
propyzamide	0.62	0.038	2.51	2.09	2.13		2.56
triazophos	0.37	0.107	4.13	8.94	3.03		8.26
vinclozolin	0.25	0.012	2.66	3.39	1.96		3.31

Annex 7

The accuracy of measurement results of pesticide content (ng/g dry matter) in spiked carrots samples and the use of a “normalizer” based on inorganic elemental content (Ca, Mg, P).

Summary

The water content measurement in the frozen batch of carrots baby food spiked with pesticides present a high uncertainty value (approx. 12 %). Therefore a correction factor based on the elemental inorganic content of frozen vs freeze dried batches of samples was applied and calculations as follows:

1) The average elemental content of Ca, Mg and P, in the frozen and freeze dried samples was determined. This elements were chosen because they are found at relatively high levels in raw carrots. Samples were measured by a method based on that described in RM WI0247 (Trace Elements in Food matrices). Each element was measured by ICP-OES using the instrument manufacturer´s recommended emission. In each case, at least one alternative emission line was measured to confirm that the analytical line was free from interferences. No correction was made for calibration linearity or instrumental drift, as the influence of these parameters on results was found to be less that of the repeatability of ICP-OES measurements.
2) Measurements of wet samples were not corrected for water content.
3) All measurements were corrected for recovery. For each element, the recovery was estimated by making two measurements on each of three certified reference materials with similar matrices to the samples (BCR 100, beech leaves, NIST SRM-8438,

wheat flour, NIST SRM-8418, wheat gluten). In the case that the recovery of an element lay outside of acceptable criteria (as defined in RM PR 0025), sample measurements were corrected by the mean observed bias.

4) Uncertainty was estimated on the measurements by combination of uncertainties associated to the following parameters: sample weights, dilution of digest, dilution of extract, dilution of standards, ICP-OES measurement repeatability, blank level, Trueness. For wet samples, uncertainty associated to the repeatabilities of the water determinations were also included.
5) The calculations for water content correction, dilutions and sample intake masses were made in the ICP-OES (validated) software. The corrections for recovery and the uncertainty estimations were made in Excel software.

Table 1: Results for the elemental content (mg/kg) measured in the Frozen carrot/potato matrix spiked with pesticides.

Frozen carrot spiked with pesticides (test material)	
Elemental content	Average Result (±expanded uncertainty)/Unit
Ca	1.37±9 mg/kg
Mg	98.9±6 mg/kg
P	2.37±13 mg/kg

Table 2: Results for the elemental content (g/kg and mg/kg) measured in the Freeze-dried carrot/potato matrix spiked with pesticides

Freeze-dried carrot spiked with pesticides (test material)	
Elemental content	Average Result (±expanded uncertainty)/Unit
Ca	16.8±0.11 g/kg
Mg	935.8±57 mg/kg
P	21.5±0.12 g/kg

Calculation of the correction factor (CF) using the following mathematical expressions:

$$CF(\%) = \frac{Ca_{frozen}}{Ca_{freezedried}} 100 \qquad (1)$$

$$CF(\%) = \frac{Mg_{frozen}}{Mg_{freezedried}} 100 \qquad (2)$$

$$CF(\%) = \frac{P_{frozen}}{P_{freezedried}} 100 \qquad (3)$$

Where:

CF-correction factor
Ca_{frozen}-calcium content in the carrots frozen test material
$Ca_{freeze\text{-}dried}$-calcium content in the freeze dried test material
Mg_{frozen}-magnesium content in the carrots frozen test material
$Mg_{freeze\text{-}dried}$-magnesium content in the freeze dried test material
P_{frozen}-phosphrous content in the carrots frozen test material
$P_{freeze\text{-}dried}$-phosphrous content in the freeze dried test material

The mathemathical result of equations (1), (2), and (3), will enable the calculation of an average value of the correction factor ($CF_{average}$).

$$CF_{average} = 10\ \% \qquad (4)$$

In fact it was previously demonstrated, that the average dry matter of the frozen test material is about 10 % of the average dry content of the freeze dried material.

The same way it will be possible to express pesticide concentrations of the frozen test material (ng/gdry matter) as a function of the concentrations of pesticide the freeze dried material (4), (these based on the water content determinations associated with a low uncertainty water content determination (max.3 %)) using the correction factor and therefore eliminate the pesticide concentrations of the frozen material as a function of the water content which tend to be linked to a high measurement uncertainty (12 %).

$$C^{Frozen}{}_{pesticide} = CF_{average} * C^{Freezedried}{}_{pesticide} \quad \text{(ng/g dry matter)} \qquad (5)$$

Conclusion: the accuracy of the content of pesticide in the frozen material (ng/g dry matter) was improved, since the high uncertainty water content measurements of the frozen test material was replaced by a correction factor derived from frozen/freeze dried elemental content measurements associated with a lower uncertainty level (5-6 %).

18. Appendices

Appendix 1

The simplified IDMS approach equations 1 and 2, derives from the following calibration approach using peak ratios and mass ratios by plotting the peak ratio PR cal mix (A pest cal mix/ A ISTD cal mix) of each calibration level against the dimensionless mass ratiop m pest cal mix/ mISTD cal mix (C $_{pest}$ m $_{pest}$ $^{cal\ mix}$)/ (C $_{ISTD}$ $^{cal\ mix}$ m $_{ISTD}$ $^{cal\ mix}$) of the standard solution. From the corresponding calibration graph obtained:

$$PR^{calmix} = a_{cal}\ x \frac{m_{pest}^{calmix}}{m_{ISTD}^{calmix}} + b_{cal} \qquad (1)$$

Each expected mass ratio m $_{std}$ $^{cal\ mix}$/ m $_{ISTD}$ $^{cal\ mix}$ can be calculated as follows:

$$\frac{m_{pest}^{calmix}}{m_{ISTD}^{calmix}} = \frac{PR^{calmix} - b_{cal}}{a_{cal}} \qquad (2)$$

The slope can be calculated as follows:

$$a_{cal} = \frac{PR^{calmix} - b_{cal}}{\frac{m_{pest}^{\ calmix}}{m_{ISTD}^{\ calmix}}} \qquad (3)$$

The mass ratio $m_{pest}^{sample}/ m_{ISTD}^{sample}$ in the final extract depends on the mass fraction w_R of the pesticide in the test portion m_a and the mass of the internal standard m_{ISTD}^{sample} (C_{ISTD} x m_{ISTD}^{Sample}) added to the test portion.

$$\frac{m_{pest}^{sample}}{m_{ISTD}^{sample}} = \frac{W_R x m_a}{C_{ISTD}\ x m_{ISTD}^{sample}} \qquad (4)$$

When the peak ratio PR^{sample} ($A_{pest}^{sample}/A_{ISTD}^{sample}$) obtained from final extract is identical to the peak ratio $PR^{cal\ mix}$ obtained from calibration mixture, the mass ratios, $m_{pest}^{sample}/ m_{ISTD}^{sample}$ and $m_{pest}^{cal\ mix}/m_{ISTD}^{cal\ mix}$ are identical. From equation 3 and 4 follows:

$$W_R = \frac{PR^{sample} - b_{cal}}{a_{cal}} x \frac{m_{ISTD}^{sample}}{m_a}\left(\frac{mg}{kg}\right) \qquad (5)$$

Or under equation (6):

$$W_R = \frac{PR^{sample} - b_{cal}}{\frac{PR^{calmix} - b_{cal}}{\frac{m_{pest}^{calmix}}{m_{pest}^{calmix}}}} * \frac{m_{ISTD}^{sample}}{m_a}\left(\frac{mg}{kg}\right) \qquad (6)$$

These equations can be simplified to equation (2) using equation 1 for the calibration graph of the IDMS simplified approach.

Variables used:

Mass of pesticide in calibration mixture ……..	$m_{pest}^{cal\ mix}$	[µg]
Mass of pesticide in final extract ……..	m_{pest}^{sample}	[µg]
Mass of internal standard in calibration mixture……	$m_{ISTD}^{cal\ mix}$	[µg]
Mass of internal standard added to test portion……	m_{ISTD}^{sample}	[µg]
Concentration of pesticide in pesticide mixture……	C_{pest}	[µg/g]
Concentration of pesticide in calibration mixture……..	$C_{pest}^{cal\ mix}$	[µg/g]
Concentration of the ISTD in ISTD-solution added to test portion……	C_{ISTD}	[µg/g]
Concentration of the ISTD in ISTD-solution used for calibration mixture…….	$C_{ISTD}^{cal\ mix}$	[µg/g]
Mass of pesticide mixture used for preparation of calibration mixture…….	$m_{pest}^{cal\ mix}$	[µg]
Mass of ISTD used for preparation of calibration mixture……..	$m_{ISTD}^{cal\ mix}$	[µg]
Mass of ISTD added to test portion……...	m_{ISTD}^{sample}	[µg]
Mass of test portion……....	m_a	[g]
Mass fraction of pesticide in the sample……..	W_R	[µg/g=mg/kg]
Peak area of pesticide obtained from calibration mixture……..	A_{pest}^{sample}	(counts)
Peak area of ISTD obtained from calibration mixture……	$A_{ISTD}^{cal\ mix}$	(counts)
Peak area of pesticide obtained from the final extract……	A_{pest}^{sample}	(counts)
Peak area of ISTD obtained from the final extract……..	A_{ISTD}^{sample}	(counts)
Peak ratio obtained form from calibration mixture…….....	$PR^{cal\ mix}$	(dimensionless)
Peak ratio obtained from final extract……..	PR^{sample}	(dimensionless)

Slope of calibration graph……a_{cal} (dimensionless)

Bias of calibration graph……..b_{cal} (dimensionless)

Appendix 2

$$U = k * \left(u_{(cst)}{}^2 + u^2{}_{(calib)} + \frac{u_r{}^2}{n_1} + \frac{u_{ip}{}^2}{n_2} + \left(\frac{CV}{\sqrt{n_3}} \right)^2 \right)^{1/2} \quad (1)$$

Result measurement:

$$x_{sample} \pm U(k=2) = MRLconc \pm U * MRLconc$$

Where:

u	expanded uncertainty;
k	coverage factor (k=2)
$u(c_{st})$	uncertainty of standards used
u(cal)	uncertainty of calibartion
u_r	uncertainty of repeatability
n_1	total number of measurements
u_{ip}	uncertainty of intermediate precision
n_2	total number of days
$u_{rec} = CV/\sqrt{n_3}$	
CV	coefficient of variation for the results of recovery
n_3	total number of independent samples used in the recovery experiments

Appendix 3

The statistical approach used for the estimation of the significance of matrix effects in carrots baby food is an adapted version proposed by Egea Gonzalez et al., [66].

The experiments (calibration in blank matrix and in solvent) were repeated every month during a period of three months, with an in house validated method (QuEChERS). During this time the usual maintenance operations were made and consequently minor changes in the chromatographic conditions occurred.

Initially each replicate of calibration in solvent and calibration in matrix was treated separately using Validata software [74], data was fitted to straight lines according to Mandel test for linearity. The residual standard deviations of the first and second order calibration functions are examined for significant (99%) differences. If such a difference exists, the working range should be reduced as far as necessary to receive a linear calibration function (otherwise the information values of analyzed samples must be evaluated using a non-linear calibration function). According to this information, when necessary the working range initially from ¼ MRL to 2 MRL has been reduced.

In a first step, the slopes and intercepts were compared with a 2 sided t-test at 95% level of confidence using the following formula to compare two regression coefficients [67]:

$$t_{calc} = \frac{|b_1 - b_2|}{\sqrt{\frac{\left(S^2{}_{y1*x1}(n1-2) + S^2{}_{y2.x2}(n2-2)\right)}{n1+n2-4} * \left[\frac{1}{Q_{x1}} + \frac{1}{Q_{x2}}\right]}} \qquad df = n_1 + n_2 - 4 \quad (1)$$

Nullhypothese: $b_1 = b_2$ b1, Alternative hypothese $b_1 \neq b_2$

n_1 = number of replicate measurements calibration curve 1

n_2 = number of replicate measurements calibration curve 2

$S^2_{y1.x1}$ and $S^2_{y2.x2}$–residual variance

Q_{x1} and Q_{x2} = $\sum\left(X - \overline{X}\right)$

When residual variances are not constant (variances are tested (F-test) for significant differences at (99%) using validata software), the number of degrees of freedom must be substituted by the following equation, where:

$$c = \frac{\frac{S^{2}_{y1.x1}}{Q_{x1}}}{\frac{S^{2}_{y1.x1}}{Q_{x1}} + \frac{S^{2}_{y2.x2}}{Q_{x2}}} \qquad df = \frac{1}{\frac{c^2}{n_1 - 2} + \frac{(1-c)^2}{n_2 - 2}}$$

If the calculated t value (t_{calc}) was less then the tabulated t value (t_{tab}) considering a 95 % confidence, the slopes of the replicates did not differ. The same procedure was applied to the intercepts in order to check if replicates are coincident or parallel. t_{calc} was also less then $t_{tab,}$ so it was concluded that neither solvent nor matrix calibration changed during the period of time , each batch was analysed.

Under this finding, a unique calibration curve was then recalculated for both calibration in solvent and calibration in matrix, using the 3 replicates at each concentration level of each anayte tested. A narrower working range was used in the statistical study in the cases the linearity test failed with the above working range.

Again t test statistics were applied to the new calibration curves, for both slopes and intercepts, independently to the data obtained in each monthly experiment. The same conclusions were obtained in all cases.

19. References

1 http://www.fao.org/index_fr.htm.

2 Pollution issues - Pesticides, http://www.pollutionissues.com/Na-Ph/Pesticides.htmL

3 Pesticide Action Network North America, http://www.panna.org/.

4 Duke, S.O., Natural pesticides from plants.Janick, J. and Simon, J.E. eds., p. 511-517, Portland,1997.

5 Linde, C.D., Physico-chemical properties and environmental fate of pesticides. Environmental Hazards Assesment Program, Environmental Protection Agency. Department of Pesticide Regulation. California. USA., 1994.

6 Schmidt, W.F., Bilboulian S., Rice C.P., Fettinger J.C., McConnell L.L., and Hapeman, C.J., Thermodynamic, spectroscopic, and computational evidence for the irreversible conversion of β- to α-endosulfan. Journal of Agricultural and Food Chemistry, 2001. **49**: p. 5372-5376.

7 Glenn, M.S. and Sharpf, W.G. ACS Symp. Ser.1977. **42** (116).

8 http://europa.eu.int/comm/food/plant/protection/index_en.htm.

9 Otles, S., Methods of analysis of Food Components and Additives. Chapter 12. Determination of pesticides Residues. Ege University, Department of Food Engineering. Izmir, Turkey. CRC Press. Taylor& Francis Editor. p.329-359.

10 USDA, www.nal.usda.gov/fnic/foodcomp/.

11 George Fong W., Seiber N.,Toth J., Pesticide Residues in food, Methods, Techniques and Regulamentations: Willey Interscience. NY, USA.

12 Mastovska K., Lehotay S.J.., Evaluation of common organic solvents for gas chromatographic analysis and stability of multiclass pesticides. Journal of Chromatography A, 2004. **1040**: p. 259-272.

13 Nemoto S., Sasaki K., Toyoda M., Bull, Studies on the stability of 89 pesticides in organic solvent. Natl. Inst. Health Sci, 1997. **115** (86).

14 Kocourek, V., Hajslova, J. ,Holadova, K. and Poustka, J., Stability of pesticides in plant extracts used as calibrants in the gas chromatographic analysis of pesticides. J . chromatography A, 1998. **800**: p. 297-304.

15 Anastassiades, M., Stajnbaher, D. and Schenk, F.J., Fast and Easy Multiresidue method Employing acetonitrile extraction / portioning and “Dispersive Solid- Phase Extraction” for the Determination of pesticide residues in produce. AOAC International, 2003. **86** (2): p. 412-431.

16 Erney, D.R., Gillespie, A.M., and Gilvydis, D.M., Explanation of the matrix-induced chromatographic response enhancement of organophosphorous pesticides during open tubular column gas chromatography with splitless or hot on column injection and flame photometric detection. J Chromatogr. A, 1993. **638**: p. 57-63.

17 Hajslova J. et al. Matrix effects in (ultra)trace analysis of pesticide residues in food and biotic matrices. J . chromatography A, 2000. **891**: p. 45-67.

18 Godula, M., Hajslova, J., Mastovska,K., and Kinvankova,J., Optimization and application of the PTV injector for the analysis of pesticde residues. J.Sep.Science, 2001. **24**: p. 355-366.

19 Zrostilova, J., Hajslova, J. Godula, M. and Mastovska, K., Performance of programmed temperature vaporized, pulsed splitless and on-column injection techniques in the analysis of pesticide residues in plant matrices. J. Chromatography A, 2001. **937**: p. 73-86.

20 European Commission, Quality control procedures for pesticide residue analysis - guidelines for residues monitoring in the European Union. 1997.

21 Anastassiades, M., Mastovska, K., Lehotay, S.J., Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides. Journal of Chromatography A, 2003. 1015(1-2): p. 163-184.

22 Godula, M., Hajslova, J., Mastovska, K., and Kinvankova,J., Optimization and application of the PTV injcetor for the analysis of pesticde residues. J.Sep.Science, 2001. **24**: p. 355-366.

23 Zrostilova, J., Hajslova, J. Godula,M. and Mastovska,K., Performance of programmed temperature vaporized, pulsed splitless and on-column injection techniques in the analysis of pesticide residues in plant matrices. J. Chromatography A, 2001. **937**: p. 73-86. 19.

24 Zrostilova, J., Hajslova, J. Godula, M. and Mastovska, K., Performance of programmed temperature vaporized, pulsed splitless and on-column

injection techniques in the analysis of pesticide residues in plant matrices. J . chromatography A, 2001. **937**: p. 73-86. 19.

25 Lehotay, S.J., Analysis of pesticides residues in mixed fruit and vegetables extracts by direct sample introduction/gas chromatography/tandem mass spectometry. J.AOAC Int., 2000. **83**: p. 680-697.

26 European Commission. Quality control procedures for pesticide residue analysis - guidelines for residues monitoring in the European Union. 1997.

27 Alba, A.R.F., Chromatographic – Mass Spectometric food analysis for trace determination of pesticide residues, ed. W. Wilsons. Vol. XLIII. 2005., Wilson & Wilsons.

28 Martinez Vidal, José L. and Garrido Frenich, A. Pesticide Protocols, C. Methods in Biotechnology. Chapter 17 Vol. 19. 2005. Totowa, NJ: Humana Press Inc.

29 Steger, H.F., The use of Matrix Reference Materials in Environmental Analytical Process. 1999, The Royal Society of Chemistry Special Publication No 238. Fajgelj, A. Parkany, M. Eds. Cambridge.

30 Development and use of Reference Materials and Quality Control Materials. Industrial Applications and Chemistry Section. International Atomic Energy Agency. 2003. Vienna, Austria.

31 Emons, H., Reference Materials - Insights and outlooks. Accred. Qual. Assur., 2007. **12**: p. 115-116.

32 International Organization of Standardization, ISO Guide 35, Reference materials - General and statistical principles for certification. 3rd edition. 2006.Geneva, Switzerland.

33 ISO guide 31. Reference materials - Contents of certificates and labels. ISO/REMCO, ed. ISO/REMCO. 2008, Geneva, Switzerland.

34 ERM, Policy for the statement of Metrological Traceability on Certificates of ERM Certified Reference Materials. 2008, http://www.iupac.org/reports/provisional/abstract07/Fajgelji_draft_2007-09-18.pdf.

35 De Bievre, P., Dybkaer R. Fajgelj A. and D.Brynn, Hibbert,. Metrological Traceability of Measurement Result. IUPAC Recommendations 2008.

36 Huntoon, R.D., Standard Reference materials and meaninful measurements, an overview. Vol. 408. 1975, Washington: Bureau of standards spect.

37 Ihnat, M., Biological reference materials for determination of elements, ed. Vol. 19. 1988, Amsterdam: Elsevier.

38 King.B., Traceability of chemical analysis. The Analyst, 1997. **122**: p. 197-204.

39 Uriano, G.A.G.C.C., The role of reference materials and reference methods in chemical analysis. CRC Crit. Rev Anal. Chem 1977(6): p. 361-41.

40 Armishaw R., J.M.M., P.J. Mclay, Development and certification of reference materials for residues of organochlorine and organophosphorus pesticides in beef fat ACSL CRM 1 and 2. Fresenius J. Anal. Chem., 1998. **360**: p. 630-636.

41 Armishaw P., M.R., A natural matrix (pureed tomato) candidate reference material containing residue concentrations of pesticide chemicals. Fresenius journal of analytical chemistry.2001. **370**(2-3): p. 291-296.

42 International Organization of Standardization., ISO, Quality Vocabulary (ISO/IEC standard 8402).1986, Geneva.

43 European Commission, Quality control procedures for pesticide residue analysis- guidelines for residues monitoring in the European Union. 1997.

44 Thompson M., Ellison Stephen L. Rand Wood R., Harmonized guidelines for single-laboratory validation of methods of analysis. Pure appl. Chem., 2002. **74**(5): p. 835-855.

45 Adrian M. H. Van der Veen, L., T.,Schimmel, H.,Lamberty, A.,Pauwels, J., Uncertainty calculations in the certification of reference materials. 4. Characterization and certification. Accred. Qual. Assur., 2001. **6**: p. 290-294.

46 Vesper, Hubert W., Reference Materials and Commutability. Clin. Biochem. Rev, 2007. **28**(4): p. 139-147.

47 King.B., Traceability of chemical analysis. The Analyst, 1997. **122**: p. 197-204.

48 Linsinger T.P.J., Pauwels J. and Lamberty A., Planning and combining of isochronous stability studies of CRMs. Accred. Qual. Assur., 2004. **9**: p. 464-472.

49 Poole, C.F., Matrix-induced response enhancement in pesticide residue analysis by gas chromatography. A review. Journal of Chromatography A, 2007. **1-2**(1158): p. 241-250.

50 Soboleva, E., Rathor,N.Mageto,A., and Ambrus,A., Estimation of significance of "matrix-induced" Chromatographic effects. Principles and practices of method validation. p.138-156. Royal society of Chemistry. 2000. Cambridge.

51 Loco, J.V., Elskens, M., Croux, C., Beernaert, H., Linearity of calibration curves: use and misuse of the correlation coefficient. Accred. Qual. Assur., 2002. **7**: p. 281-285.

52 Avramides, E.J., The long-term stability of pure standards and stock standard solutions of pesticides amenable to determination by gas chromatography. NAGREF Poster presentation., 2003.

53 Thompson,M. Ellison.L.R.; Fajgelj S., Willetts, P. and Wood, R., Harmonized guidelines for the use of recovery information in analytical measurement. Pure & Appl. Chem., 1999. **71**(2): p. 337-348.

54 Rohrer, Wegscheider and Neuboeck, Excel 97 Macro for method validation, Validata. © Rohrer & Wegscheider & Neuboeck Editor. 2005.

55 Colby, B.N., Rosecrance, A. E., Measurement parameter selection for quantitative isotope dilution gas chromatography/mass spectrometry. Anal. Chem., 1981. **53**: p. 1907-1911.

56 Holland, P.T., Effects of storage and processing on pesticide residues in plant products. Pure & Appl. Chem., 1994. **66**(2): p. 335 -356.

57 British Crop Protection Council. The Pesticide Manual: A World Compendium. Eighth edition. 1987, Suffolk.

58 Kestens, V., Charoud-Got, J., Bau, A., Bernreuther, A., Emteborg, H, Online measurement of water content in candidate reference materials by acousto-optical tuneable filter near-infrared spectrometry (AOTF-NIR) using pork meat calibrants controlled by Karl Fischer titration Food Chem, 2008. **106**(4): p. 1359-1365.

59 ISO, Guide for expression of uncertainty (GUM). Guide 98, 1995.

60 Lehotay, S. J., De Kok A., Hiemstra M., Van Bodegraven, P., Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid Chromatography and Mass spectrometric detection. Journal of AOAC International, 2005. **88** (2).

61 Lehotay, S.J., Maštovská, K., Practical approaches to fast gas chromatography-mass spectrometry. Journal of Chromatography A 2003, 1000, 153-180.

62 De Koning, S., Lach, G., Linkerhägner, M., Löscher, R., Tablack, P.H., Brinkman, U.A.Th. Trace-level determination of pesticides in food using difficult matrix introduction - Gas chromatography - Time-of-flight mass spectrometry. Journal of Chromatography A, 1008, 2003, 247-252.

63 Čajka, T., Maštovská, K., Lehotay, S.J., Hajšlová, J., Use of automated direct sample introduction with analyte protectants in the GC-MS analysis of pesticide residues. Journal of Separation Science 28, 2005, 1048-1060.

64 Alder, L., Greulich, K., Kempe, G., Vieth, B., Residue analysis of 500 high priority pesticides: Better via GC-MS or LC-MS/MS? Mass Spectrometry Reviews 25, 2006 838-865.

65 Fernández-Alba, A.R., García-Reyes, J.F., Large-scale multi-residue methods for pesticides and their degradation products in food by advanced LC-MS. TrAC - Trends in Analytical Chemistry 27, 2008, 973-990.

66 Egea Gonzalez, F.J. et al. Estimation and correction of matrix effects in gas chromatographic pesticide multiresidue analytical methods with a nitrogen-phosphorous detector. Analyst,127, 1038-1044,(2002).

67 Angewandte Statistik, Anwendung statistischer methoden. Sechste Auflage.Springer-Verlag, 1992.

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