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ORGANIC MICROPOLLUTANTS ANALYSIS AND THEIR METROLOGICAL TRACEABILITY TO SI UNITS

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CHAPTER 1

INTRODUCTION

The PhD activity described in the present work concerned the determination of organic micropollutants in various matrices (food, environmental samples), at trace level, with the aim of correctly evaluating the measurement uncertainty and establishing metrological traceability for the results of these measurements.

The metrological traceability of organic micropollutants measurements is a relevant issue due to the potential adverse effects that these substances can act on human health and on natural ecosystems. Many organic micropollutants have been classified as Persistent Organic Pollutants (POPs) by the United Nations Environment Programme (UNEP), in the framework of the Stockholm Convention (2001) [1] and 12 classes of POPs have been considered until now.

Nowadays, food safety is a field of fundamental importance for the consumers, for the food industry and for the economy in general. For these reasons, it is necessary to have reliable methods and instrumentation to perform accurate and efficient quality controls, in order to prevent adverse effects on the consumer health. In this framework, the contribution of metrology is fundamental, as it provides the means to obtain accurate, traceable measurement results, which can be compared even if determined in different conditions, places and times.

During the PhD, the research project was articulated in two complementary parts: the first devoted to the development of analytical capabilities for the determination of different micropollutants and the second to traceability issues, i.e. to the development of correct metrological traceability chains to the units of the International System of Units (SI). The PhD activity has been carried out at INRiM Thermodynamic division under the supervision of Dr. Michela Sega.

In particular, the research activity was addressed to the study of some food matrices, in order to extract and analyse pollutants that could be possibly present in them. The attention was focused on two different matrices, green tea powder and milk, and on two organic molecules:

- endosulfan, an organochlorine pesticide banned in 2011 due to its toxicity, analysed in green tea;
- melamine, a molecule commonly used in the plastic industry which became famous for its fraudulent use as adulterant of the protein content of milk in 2008.

Ad hoc methods were developed for the extraction of these molecules from food samples and for their quantification by means of two different analytical techniques, i.e. gas-chromatography coupled with mass spectrometry (GC-MS), for the analysis of endosulfan, and Surface Enhanced Raman Scattering (SERS) spectroscopy for the analysis of melamine.

For the development of these methods a metrological approach was adopted and correct metrological traceability chains to the SI units were established. In order to achieve this goal, we considered all the steps which constitute an analytical method (extraction of the analytes from the sample, preparation of the sample for the analysis, method validation and quantification of the analytes), facing several problems typical of the chemical analysis, as complexity of real food samples, matrix effects (sample matrix interferences during the quantification step) and lack of primary methods applicable to routine measurements.

The activity concerning the Organochlorine Pesticides (OCPs) started for the participation in 2012 in an international comparison carried out in the framework of the *Comité Consultatif pour la quantité de matière* (CCQM), namely the "Pilot Study CCQM-P136 Mid-Polarity Analytes in Food Matrix: Mid-Polarity Pesticides in Tea". It concerned the determination of the mass fractions (between 100-1000 μg/kg) of two pesticides, β-endosulfan and its metabolite endosulfan sulphate in a food matrix, i.e. green tea powder.

Endosulfan is a broad-spectrum insecticide, widely used in agricultural practices, which was internationally banned as it is a strong neurotoxic agent, both on insects and on mammals, including humans. In addition endosulfan is an endocrine disruptor (agent which can "mime" the activity of some hormones) and many studies have documented its reproductive and developmental toxicity. Endosulfan may also bioaccumulate in the food chain, displaying high toxicity [2]. The CCQM-P136 pilot study (and the parallel Key Comparison CCQM-K95), co-organised by the Government Laboratory of Hong Kong (GLHK - Hong Kong) and the National Institute of Metrology (NIM - China), required the development of a procedure which involved extraction, clean-up, analytical separation and selective detection of the analytes in a food matrix. The samples of green tea object of the comparison were prepared by GLHK starting from a batch of Chinese commercial tea

purchased from a local market. Two bottles of sample were sent to the participants, one for the method development and another for the analysis with the preferred method.
The comparison was designed to test the capabilities of the participant NMIs for determining mid-polarity analytes in a food matrix.
My activity consisted in:

- the set up the analytical procedure for the extraction of the pesticides from the matrix and the preparation of the samples for the quantification step (clean-up, interferences reduction, concentration);
- the set up of the best analytical conditions and the quantification by means of GC-MS;
- the evaluation of measurement uncertainty.

Metrological traceability was established using suitable Certified Reference Materials (CRMs), both for the calibration of the analytical instrumentation and for the evaluation of the recovery efficiency of the analytes from the matrix. For the calibration of the GC-MS a CRM produced by the National Institute of Standards and Technology (NIST - United States) was used, which consisted of an organic solution containing some OCPs, among which endosulfan and endosulfan sulphate.
The calibration solutions were prepared by gravimetric dilution of the CRM, in order to obtain different concentrations of the analytes in the range of interest. The calibration curves were obtained by means of an algorithm based on the Weighted Least Squares [3], which calculates a linear correction to be applied to the instrument readings of the calibration solutions. This correction can be applied to the instrumental readings of the unknown samples, thus obtaining the correct values. The algorithm takes care of different sources of uncertainty, the standard solutions uncertainty, the repeatability of the instrument, the lack of fit, the instrumental resolution.
The recovery efficiency was determined by spiking samples of commercial green tea with known amounts of the two pesticides, then processing and analysing these samples in the same way as the unknown samples of the comparison to take into account all the possible losses during the whole sample preparation process. The recovery efficiency was calculated from the ratio of the measured concentrations and the theoretical concentrations in the spiked samples.
In addition, the evaluation of the moisture content of the sample was performed in order to determine the mass fractions of the pesticides on a dry mass basis, as requested by the

protocol of the comparison. The moisture content of green tea samples was determined by weighing some aliquots of tea before and after heating them to constant weight, obtaining by difference the content of humidity in the samples. Finally, for the uncertainty evaluation, two approaches were followed: the classical GUM approach based on the law of propagation of uncertainty [4] and the Monte Carlo method [5]. The latter method well applies to asymmetric probability distributions of the measurands and therefore it can be useful for the treatment of analytical data at low level concentrations.

Another activity in the field of micropollutants analysis in food matrices concerned the determination of melamine in milk. Melamine is an organic molecule very rich in nitrogen atoms, widely used in the plastics industry field. Due to the presence of amine functional groups (NH_2) and nitrogen atoms in the molecular structure, the melamine can alter some classical methods for the evaluation of the protein content in foodstuffs.
Melamine was involved in some cases of sophistication of milk destined in particular to baby nutrition and of animal feed in 2008. In addition, the melamine can accidentally contaminate foodstuffs, passing from the plastic packaging to the food itself.
This molecule can have adverse effects on human health, in particular in children and may be potentially responsible of cancer and reproductive damages in case of chronic exposure.
I carried out this activity with Dr. Andrea Mario Giovannozzi of INRiM Thermodynamic division and in this way I could get acquainted with a new analytical technique, the Raman spectroscopy. This spectroscopic technique is based on the measurement of inelastic scattering between the photons produced by a laser radiation and the atoms (or molecules) of a substance. It is very useful for the analysis of gaseous, liquid and solid (crystalline or amorphous) samples, providing information on molecular composition, chemical bonds, crystalline phases and structures.
A particular feature of this technique is the so-called SERS effect which increases the analytical performances of Raman spectroscopy. Indeed, when the sample is put in contact with a metallic irregular surface or constituted of metallic nanoparticles (gold or silver), a considerable intensity enhancement (by a factor of 10^6-10^{10}) of the Raman signal can be observed.
The use of this technique is becoming more and more popular in the scientific community, as it allows getting detailed information from complex matrices like biological samples (cells, tissues) or inorganic materials (plastic matrices, polymers), assuring high accuracy

and sensitivity. It can be used in various analytical fields: electrochemistry, bio-sensing, environmental analysis and, in general, all the analytical chemistry fields.
The experimental activity consisted in the set up of an extraction procedure of melamine from milk and its analysis by means of Raman spectroscopy, performed after mixing the samples with gold nanoparticles. Their synthesis is carried out in order to obtain particles having a diameter of 40 nm and a negatively charged surface, which allows their interaction with the melamine molecules (positively charged at acidic pH). These agglomerates are responsible for the amplification of the Raman signal. The instrument used is a Raman spectrophotometer which works at a laser wavelength of 780 nm. The melamine has typical Raman signals which can be amplified by the SERS effect and can be used for its quantification in the analysed samples.
This procedure was validated by studying different parameters (linearity, repeatability, limit of detection, limit of quantification and recovery). The developed method allows the quantification of melamine in milk samples in accordance with the European law limits, fixed by the Codex Alimentarius Commission, due to its potential toxicity, to 1 mg/l for powder infant formula and 2.5 mg/l for other foods and animal feed [6]. The quantification range obtained for the Raman spectrophotometer used is between 0.57 and 5.0 mg/l of melamine in the matrix with a limit of detection (LOD) of 0.17 mg/l. The recovery efficiency of the method was calculated by preparing and analysing samples containing melamine concentrations of 1 mg/l and 3 mg/l. For the determination of the calibration curve of the Raman spectrophotometer and of the uncertainties of the related coefficients, an algorithm based on Total Weighted Least Squares [7] was used, which takes into account both the instrumental repeatability uncertainty and the uncertainty deriving from the calibration solutions used for the calibration of the measuring instrument.

Finally, during the third PhD year, I spent a training period in Paris (France) at the *Laboratoire National de Métrologie et d'Essais* (LNE), the French Metrological Institute. In this occasion, I worked at the set up of a new analytical procedure for the determination of some Polycyclic Aromatic Hydrocarbons (PAHs) in the particular matter suspended in water used for human consumption. The PAHs are part of the 33 priority water pollutants, as stated in the European Water Framework Directive [8].
With this activity I could use my experience concerning this class of organic pollutants, obtained thanks to the research previously carried out at INRiM for my Master Degree in Chemistry.

The work was done in the framework of the activities of the chemical metrology group of LNE. In particular my activity concerned the set up of an analytical method for the extraction and the analysis of some PAHs in the particular matter suspended in water by means of an extraction technique called accelerated solvent extraction (ASE) and gas-chromatography coupled with isotopic dilution mass spectrometry (GC-IDMS). This quantification technique is more and more used in the chemical metrology field and is classified among the primary ratio methods of measurement. The activity that I carried out at LNE is part of the European project EMRP ENV08 "Traceable measurements for monitoring critical pollutants under the European Water Framework Directive (WFD) 2000/60/EC". An overview of the activity carried out at LNE is reported in Appendix A.

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CHAPTER 2

METROLOGICAL TRACEABILITY OF CHEMICAL MEASUREMENTS

2.1 Introduction

Metrology is defined as the "science of measurement and its application". It includes all theoretical and practical aspects of measurements, whatever are the measurement uncertainty and field of application. [1].

Its objectives are the definition of the measurement units, the realisation of the related standards, their replication and traceability, the technologies and methods of measurement.

The need of defining a system of units is the basis of the metrology concept. In order to establish a system of units, such as the International System of Units (the SI), it is necessary first to establish a system of quantities, including a set of equations defining the relations between those quantities.

Indeed, the equations between the quantities determine the equations relating the units.

It is also convenient to choose definitions for a small number of units called *base units*, and then to define units for all other quantities as products of powers of the base units, that we call *derived units*. It is important that the definition of each base unit is made with particular care, since they provide the foundation for the entire system of units [2].

This need was satisfied in 1960 with the introduction of the SI (from the French *Système International d'Unités*), established and defined by the General Conference on Weights and Measures, the CGPM. The base quantities used in the SI are length, mass, time, electric current, thermodynamic temperature, amount of substance and luminous intensity and these base quantities are by convention assumed to be independent in the actual configuration and definition of the SI.

The measurements carried out in accordance with the SI are based on the comparison of the measured quantities in unknown samples with the same quantities in reference samples. Indeed, each measurement process is based on the comparison with a measurement standard and on the definition of the related units.

A measurement procedure needs a suitable metrological traceability, which can be achieved by establishing unbroken traceability chains for each quantity, having accessible standard (national or international) at their top.
In order to be reliable, the measurement results have to be expressed together with their measurement uncertainty (defined in par. 3.1.3): this parameter gives an estimation of the quality of each link in the traceability chains to the SI, starting from the value of a measurand in an unknown sample, usually referring to a certified value of the same measurand in a reference standard.
The accuracy of a measurement result is quantified by means of its uncertainty, which has to be evaluated taking into account the contributions deriving from the possible sources. In order to compare two measurements, the results need to be expressed in the same units, with their uncertainty, evaluated and expressed following coherent criteria and their comparability is shown by the overlapping of the uncertainty bands of the measurement results.

2.2 Metrological traceability to the SI of measurements of amount of substance in chemistry

The metrological traceability is defined as the "property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty" [1].
A metrological traceability chain is a sequence of measurement standards and calibrations that is used to relate a measurement result to a reference and thus establishing metrological traceability of a measurement result. The purpose of establishing traceability is to ensure that measurements at the end of a traceability chain can be stated with quantified uncertainties in SI units so that they are accurate, comparable with measurements made by other methods and in other domains, and stable in the long term.
A direct way of establishing traceability to the SI in measurements of amount of substance, or of any other quantity, requires that the measurements are made using a primary method of measurement, which is correctly applied and stated with an evaluated uncertainty.
There may be other indirect ways of establishing traceability to the SI, beyond those covered by primary methods, which may include, among others:

1) combinations of methods that are not established as primary, but have combined uncertainties where the evaluation requires the incorporation of the links to national or international measurement standards of each SI unit involved;
2) comparison with reference materials of the same or similar substance, or with a mixture of substances, which are themselves linked to the SI through a chain of other comparisons, culminating in a measurement using a primary method; the uncertainty components due to the matrix effects must be evaluated;
3) comparison with other standards which realise or represent an accurate chemical composition (e.g. standard gas mixture generator, standard UV spectrometer for ozone determination) which themselves are linked to the SI [3].

A primary method of measurement is defined as "a method having the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be written down in terms of SI units, and whose results are, therefore, accepted without reference to a standard of the quantity being measured" [3].

Primary methods are classified as direct and ratio methods. The former measure an unknown value without reference to a standard of the same quantity, the latter allow measuring the value of a ratio between an unknown value and a standard of the same quantity. Among the direct primary methods there are coulometry, gravimetry, titrimetry, determination of freezing-point depression while among the ratio primary methods there are those based on isotope dilution mass spectrometry.

Even if primary methods allow reaching the smaller uncertainties possible, these methods cannot be applied easily to routine analysis.

When primary methods are not applicable, metrological traceability in chemical measurements can be obtained by means of the following approaches:

- instrument calibration with traceable reference standards;
- use of a pure certified substance;
- use of a certified reference material in a matrix;
- use of a well defined and accepted procedure.

Metrology in chemistry has its own features, which distinguish it from classical metrology: due to the lack of primary methods applicable in routine measurements, metrological traceability of measurement results can be achieved by using in a proper way suitable certified reference materials (CRMs). Indeed, the use of CRMs, which have been

characterised by means of highly reliable methods, as primary ones, can assure a direct relation to a reference, thus assuring metrological traceability of measurement results.

The application of metrological concepts to the determination of organic pollutants in different matrices is a challenge. Indeed, it deals with an enormous amount of different compounds, which are present at various concentrations, often at trace level, and can interact with the matrix constituents. For such kinds of measurements, the achievement of metrological traceability is undoubtedly a non-trivial aspect and one of the tasks to be carried out is the evaluation of measurement uncertainty taking into consideration all relevant contributions. The developing of an analytical method under a metrological approach has to be done step by step and the quantification is only the last and the less problematic one both in terms of metrological traceability and of contribution to uncertainty, as it is necessary to establish a traceability chain comprising all the steps which constitute the analytical procedure (sampling, sample preparation, quantitative analysis, measurement uncertainty evaluation). It is necessary to calculate the contribution to uncertainty in each step of the analytical method, in order to evaluate the combined uncertainty of the final result.

In figure 2.1 an example of traceability chain for chemical measurements is shown [4].

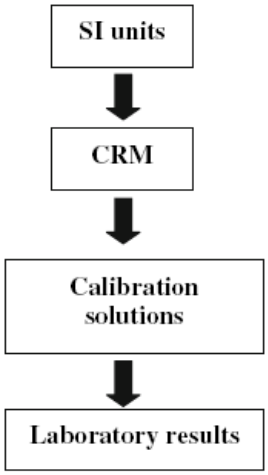

Figure 2.1 – Block diagram of a traceability chain for chemical measurements [4].

It is important to be able to compare with confidence the results obtained by different laboratories or by the same laboratory at different times. This is achieved by ensuring that all laboratories are using the same measurement scale, or reference points. This is possible,

in many cases, by establishing a chain of calibrations leading to primary national or international standards.

In routine measurement, the consistency of measurements between one laboratory (or time) and another is greatly aided by establishing traceability for all relevant intermediate measurements, used to obtain or control a measurement result.

The agreement between laboratories is limited, in part, by uncertainties incurred in each laboratory traceability chain. Traceability is accordingly intimately linked to uncertainty and it provides the means of placing all related measurements on a consistent measurement scale, while uncertainty characterises the "strength" of the links in the chain and the agreement to be expected between laboratories making similar measurements.

In general, the uncertainty on a result which is traceable to a particular reference will be the uncertainty on that reference together with the uncertainty on making the measurement relative to that reference.

2.3 Principles of metrological traceability

2.3.1 Introduction

Any measurement can be thought of as one or more determinations combined to give a result under specified conditions. For example, analysis of a soil sample for contaminants, typically involves the quantitative determination of the mass of soil taken and the concentration of analyte in the measured volume of solution containing an extract from the sample. All these parameters are qualified to some extent by the condition of measurement. Mass is determined by weighing, volume is typically taken as "volume at 20 °C" and extraction conditions are defined in terms of time, solvent and temperature. The mass, concentration and perhaps volume will of course vary from one measurement to the next (as different sized samples are taken) and they represent the measured values of the "variables" in the calculation of the final result. The extraction and other conditions are usually held close to their nominal value and are not expected to change, as they are fixed conditions and are not generally included in the calculation. For a given measurement method, if the fixed conditions change, so will the value of the result. It follows that both the fixed conditions required for the measurement and the other measured values obtained and put in the calculation of the result, affect the analytical result. These measured values are the "influence quantities" for the measurement. If two scientists want to get the same

readings for a measurement, the simplest method could be the use of the same measuring instrument, but this becomes unworkable very quickly. It is traceability to common reference standards which allows laboratories to obtain the same set of fixed conditions required for measurements and generates consistent measurements in different laboratories. Very similar principles apply when looking at the measured variables included in the calculation of the result, but the situation is more complex since the values are not supposed to be fixed, but consistent in some way. This consistency is achieved by using the same calibration standards for successive measurements.

The essential activities in establishing traceability are described in the EURACHEM/CITAC Guide "Traceability in chemical measurement" [5]:

1) Specifying the measurand, scope of measurements and the required uncertainty;

2) Choosing a suitable method of estimating the value that is, a measurement procedure with associated calculation (an equation) and measurement conditions;

3) Demonstrating, through validation, that the calculation and measurement conditions include all the influence quantities that significantly affect the result, or the value assigned to a standard;

4) Identifying the relative importance of each influence quantity;

5) Choosing and applying appropriate reference standards;

6) Evaluating the uncertainty.

This list does not necessarily imply an order or priority among the activities.

1) A meaningful measurement requires an unambiguous specification of the measurand and close attention needs to be paid to some specific issues, which are the identity of the analyte, the implied measurement conditions, the recovery correction and the specification in terms of a method. Indeed, chemical measurement most commonly quantifies particular molecular or elemental species. It will clearly be necessary to take extra care if different forms of a material occur and if the difference is important. In addition, it is important to understand exactly what conditions apply, as these form part of the formal definition of the measurand. It is fundamental to state clearly whether the quantity of interest is an amount of substance recovered from a substrate, or whether it is the total amount believed to be present. It is necessary an additional measurement, to calculate the recovery correction. Finally, it is often convenient to consider the required

performance of the measurement method and the most important concern is the measurement uncertainty required.

2) The choice of the method involves a range of factors including, for example, regulatory requirements for particular methods, costumer requirements, cost, experience of different methods, availability of equipments, and criticality of decisions.

Method development typically produces a standard operating procedure, incorporating a set of instructions for carrying out a measurement, a set of measurement conditions defining the values of parameters that must be held stable and an equation from which the result is calculated using the values of the measured parameters. This equation is expected to generate consistent results provided that specified conditions are correctly set and stable. The results will be consistent if the values of all these parameters are traceable to stable references. This expectation, however, is based on some assumptions, as linearity of the response, freedom from overall bias, absence of other significant effects. Method validation is the mechanism used to test these crucial assumptions, by reviewing the measurement model and making experimental tests.

3) Method validation should provide a reasonable test of measurement equation and conditions. Validation demonstrates that this equation and set of conditions is sufficiently complete for the purpose in hand. Establishing traceability ensures that the values of these measured quantities and the values of specified conditions are related to appropriate measurement standards. Traceable calibration against other reference values is essential for the critical quantities in the measurement.

Validation within a single laboratory will include a) assessment of selectivity and specificity, in order to ensure that the method responds to the particular species of interest and not to other similar species; b) a certified reference material check, which demonstrates that the method is not significantly biased by comparison with independently obtained traceable values; c) precision studies over a wide time interval and set of conditions as reasonably possible; d) additional studies on specific and likely sources of bias, including spiking and recovery studies, interferences and cross-reactivity studies; e) linearity check, detection capability, ruggedness studies, comparisons between analysts, different laboratories, or the results of independent methods.

Where an effect is discovered, the method needs to be modified and subjected to further development and validation. Such a modification can be the elimination of the effect, the reduction of the variation caused by the effect by adding or reducing a control range, or the

correction for the effect, by including it in the calculation of the result. The last two have the effect of introducing another measurement into the method, that is, another factor requiring traceability.

4) The importance of different influence quantities is a crucial point in deciding the appropriate degree of control or calibration. The importance of different influence quantities is dictated by their quantitative effect on measurement results. A second issue is the possible effect on the result given by the uncertainties or possible gross errors involved. Effects from physical quantities such as time, mass and volume are typically well controlled and easily measured compared to many chemical effects, particularly at trace levels.

Step 5) will be discussed in par. 2.4 and par. 2.5 while step 6) will be described in details in chapter 3.

2.3.2 Calibration

Calibration is defined as: "an operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication" [1].

A comparison between two measurement standards may be considered as a calibration, if the comparison is used to check and, if necessary, correct the quantity value and measurement uncertainty attributed to one of the measurement standards.

A calibration may be expressed by a statement, calibration function, calibration diagram, curve or table and should not be confused with adjustment of a measuring system.

The "calibration hierarchy" is the sequence of calibrations from a reference to the final measuring system, where the outcome of each calibration depends on the outcome of the previous calibration.

Metrological traceability requires an established calibration hierarchy and the measurement uncertainty necessarily increases along the sequence of calibrations.

In particular, the instrument measurement uncertainty is the uncertainty component which originates from the measurement instrument or the measurement system used. Its value is obtained by calibration of the measurement instrument or system, except for primary

standards. In addition, information concerning instrument uncertainty may be given in the specification of the instrument.

The calibration of a measurement instrument, allows quantifying the results of the measurement of a given quantity, with reference to a suitable reference standards and to make the results traceable and comparable with the results of measurements carried out in other laboratories, or to study other metrological properties, e.g. the effect of influence quantities on the instrument response.

For complex systems of measurement, the calibration may present various problems and, in these cases, it is necessary to consider carefully the type of analysis to perform, taking into account the characteristics of the sample and of the substances to determine, their concentration and the number of analyses to carry out.

Calibration is the fundamental process in establishing traceability and it is through calibration that traceability to appropriate reference standards is actually achieved in practice. Calibration can be, and usually is, applied to parts of a measurement system. Instruments such GC or Inductively-coupled Plasma tend to vary much more than balances or thermometers, and are typically calibrated more frequently, often in the same run as a set of test items. For this purpose, it is possible to use a pure chemical as the calibration material, and it may be added to a matrix similar to the samples expected in order to reduce the matrix effects. In this case, the reference standard values will appear in the calculation of the result and it is therefore clear that the result is traceable to these reference values.

In some cases, calibration standards are taken through the complete measurement process; for example, a matrix reference material may be analysed at the same time as the test samples and used to correct the results, or a known amount of material (a “spike”) may be used to estimate and correct for the actual analyte recovery during a run. If these procedures are employed, either the reference material value or the amount of spike added must appear in the calculation for the result, perhaps via an intermediate recovery factor and the results are accordingly traceable to the value used. Another situation, rare in practice, may be that during method development and validation, it is decided that a fixed correction should be applied to all future measurements, based on observations of a particular reference material which is not used for regular, day to day calibration.

After having chosen the analytical method to use and having analysed with this method a series of reference standards at known concentrations, it is necessary to build a calibration curve, usually plotting in a graph the instrument responses versus the different increasing concentrations of the analytes in the reference standards (i.e. the calibration standards),

which must comprise all the range of the concentrations of interest. The calibration curve is defined as the "expression of the relation between indication and corresponding measured quantity value" [1].
As the instrument signal depends both on the presence of interferences in the sample and on the type of matrix, it is advisable to choose reference standards having a composition as similar as possible to that of the analysed samples.

2.4 Choice of the appropriate reference standard

The choice of a reference can be made for:

- physical measurements made during analytical work;
- confirmation of identity;
- calibration with certified reference materials (CRMs);
- calibration with other materials;
- calibration using reference data;
- method development, validation and verification.

A large range of physical measurements is common in analytical work, but suitable calibration of physical equipment and availability of standards is rarely a major problem in analytical measurement. Equipment and reference standards for mass, length, volume, temperature, time and for electrical measurement normally provide calibration uncertainties well below any level of significance compared to the uncertainties found in analytical measurement.
The identity of materials needs to be confirmed by reference to an authentic sample or reference data. Certified pure materials will often serve for identity confirmation, where available. Comparison with reference data, for example in the form of spectroscopic data, is normally acceptable evidence of identity. In this case, it is important to ensure that the reference data are obtained under closely similar conditions to those used in the laboratory and are traceable to appropriate references (for example wavelength standards) so that direct comparison is possible.
Calibration can be carried out with pure materials or other types of reference materials. In many cases, the measurand is an amount of a chemically distinct substance, an element or single molecular species. Calibration with materials of well-established purity is accordingly a valid means of establishing traceability. Establishing purity relies primarily

on appropriate techniques for preparing and purifying a material, followed by the effort to detect significant impurities. A wide range of other materials and formulations is available for calibration including mixed element calibration solutions, alloys and pharmaceutical reference materials.

In some situations, reference data are used either to support calibration using a well characterised material, or as calibration factors. Examples might be the use of reference spectroscopic data to calibrate wavelength scales (as in infrared spectroscopy) or the use of reference absorbance data to establish concentrations directly from absorbance measurements. It is important to ensure that the reference data apply under the conditions used in the measurement and the reference data are traceable to appropriate references.

Reference materials, particularly matrix reference materials, play an important role in method development, validation and verification. It is important that the material should not only provide traceable reference values, but should also be relevant to the application.

A reference material is defined as a "material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in a measurement or in examination of nominal properties" [1].

Matrix effects and other factors such as concentration range can be more important than the uncertainty of the certified value. The factors to consider include: measurand and measurand range, matrix match and potential interferences, sample size, homogeneity and stability of the samples, measurement uncertainty, characterisation and certification procedures.

2.5 Calibration with Certified Reference Materials (CRMs)

A certified reference material (CRM) is formally defined as a "reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceabilities, using valid procedures" [1].

CRMs assure measurement traceability and allow assessing, by comparison, the coherence of results obtained by different laboratories; a disagreement between the certified value and the measured value causes errors in the measurement method. CRMs are also used to check non certified RM, to be used as working standards.

CRMs certification is a task of different institutions, as National Metrology Institutes (NMIs), calibration centres and accredited laboratories, or the producers themselves.

CRMs are essential instruments in all the fields of analytical chemistry: all chemical measurement results depend upon and are ultimately traceable to the values of measurement standards of various types, such as those of mass, volume and the amount of a particular chemical species. If results obtained by different laboratories are to be comparable, it is essential that all results are based on reliable measurement standards, whose values are linked to a stated reference. The accuracy of measurement results must be adequate to the level of uncertainty required and the reliability of the results largely depends on the availability of CRMs. It is a requirement of standards such as ISO/IEC 17025 [6] that test results should be traceable, preferably to national or international standards.

To clarify the concept of establishing traceability by means of suitable CRMs, consider two laboratories, A and B, carrying out measurements of samples of broadly the same type (fig. 2.2). Each calibrates their equipment using a reference standard with a known nominal concentration (x_1 and x_2 respectively). They calculate their respective results y_1 and y_2 from a calibration equation including the respective values of x. The result y is a function of the reference value x and, where there is such a relationship, the calculated value can always be claimed to be traceable to the reference value. Here, y_1 is traceable to x_1 and y_2 to x_2. In this first case, there is no basis for comparing the two results and it is not possible to write a mathematical equation that would show, for example, y_1 in terms of y_2.

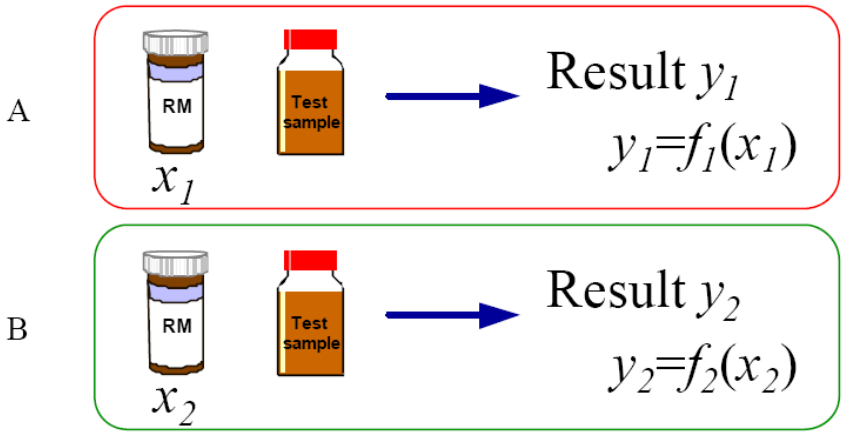

Figure 2.2: two laboratories measure the same test sample using independent working standards (x_1 and x_2). Here there is non common reference and thus the results are not comparable [5].

If, however, the two reference standards are both calibrated against some common reference (x_0) a comparison become meaningful (fig. 2.3). Both results are now derived from the same value and will have the same units of measurement, and in this way direct comparison of the values y_1 and y_2 is possible. Traceability does not make the results

identical, but permits meaningful comparison by ensuring consistency of measurement units.

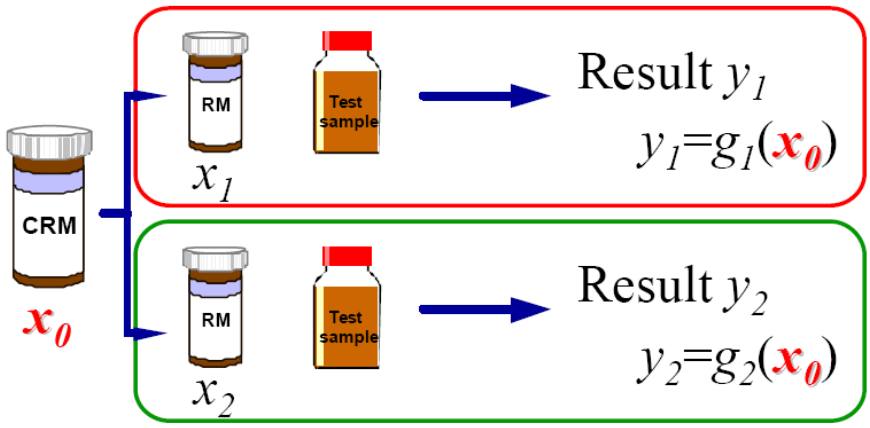

Figure 2.3: a relationship between results from lab. A (y_1) and lab. B (y_2) can now be established because they are traceable to common reference (x_0). [5]

The analysis of persistent organic pollutants (POPs) in environmental, food or feed samples is complex and typically involves extraction, clean up, further fractionation, and a final determination of the contaminants. Every stage of the analysis has critical parameters that should be optimised in order to reduce the uncertainty of the final result. Policy makers rely on data produced by various laboratories (e.g. when monitoring the compliance of products or carrying out risk assessment.

The ISO/IEC 17025 [6] standard requires that accredited laboratories use validation methods, demonstrate traceability of calibrations, and apply an appropriate quality control programme. Proficiency testing (PT) schemes are important tools to compare a laboratory performance with external laboratories. Nowadays, a number of international PT schemes are available for a wide range of contaminants in food and environmental matrices. CRMs are valuable tools to validate the trueness of analytical methods. Many European projects have been devoted to the production of CRMs for the analysis of POPs [7].

In 2004, the ERM (*European Reference Material*) range of reference materials was launched. It was the result of collaboration between three major reference material producers, the Institute for Reference Materials and Measurements (IRMM) in Belgium, the Laboratory of the Government Chemist (LGC) in the UK and the Bundesanstalt für Materialforschung und –prüfung (BAM) in Germany. A further added value comes from the fact that the three institutes are also NMIs or Designated Institutes (DIs).

This consortium promotes the cooperation between laboratories, research institutes and reference materials producers. The partners are committed to using the most advanced principles for the production of CRMs; the certified values have clearly defined and stated traceability and are internationally recognised through participation of the partners in key comparisons organised by the Bureau International des Poids et Mesures (BIPM), being the three partners NMIs or DIs.

After a feasibility study has been carried out for a "candidate" CRM, the production of the material can be sub-divided into four stages:

- production of the material;
- homogeneity study;
- stability study;
- certification study.

The production of materials is carried out according to ISO Guide 35 [8] and BCR guidelines (Community Bureau of Reference – European Union) [9, 10].

The National Institute for Standards and Technology (NIST), the US NMI, has developed CRMs for determination of organic contaminants in environmental matrices since the 1971, when the former National Bureau of Standards (NBS) issued the first natural matrix standard reference material (SRM) for environmental measurements. Assignment of the certified concentrations for this natural-matrix SRM was based on the approach of combining results from two or more independent and reliable analytical methods. During the 1970s, eleven additional natural-matrix SRMs for trace-element content were developed, but in the late 1970s, the newly created Organic Analytical Research Division at NBS began to address the need to develop SRMs for determination of individual trace level (μg/g or ng/g) organic compounds in complex matrices. The first natural-matrix SRM with certified concentrations for organic environmental contaminants was issued by NBS. The use of multiple independent methods for the certification of selected organic constituents required that all the steps in the measurement process (extraction, isolation, separation, and detection) be as chemically independent as possible. The development of natural-matrix SRMs at NIST for the determination of organic environmental contaminants has focused primarily on persistent organic pollutants (POPs), including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and chlorinated pesticides.

Two types of CRM are can be used for food and environmental analysis:

1. solutions containing several compounds;
2. matrix materials.

The solution CRMs are useful for validating and calibrating the chromatographic separation and detection step of the measurement process (e.g. the retention time and detector response). The natural-matrix CRMs, which are similar to the actual samples analysed, are used to evaluate and validate the complete analytical procedure, including solvent extraction, cleanup of the extract, isolation/enrichment of the compounds of interest, and the final chromatographic separation, detection and quantification.

The typical modes used for certification of chemical composition of CRMs are:

1. measurements using a primary method, i.e. a method of high precision for which all sources of bias have been rigorously investigated;
2. measurements using two or more independent and reliable methods;
3. measurements from several laboratories participating in a multi-laboratory comparison exercise, e.g. round-robin or inter-laboratory studies.

The requirement of using two or more analytical techniques is based on the assumption that agreement of the results from independent methods minimises the possibility of biases within analytical methods [11].

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CHAPTER 3

UNCERTAINTY EVALUATION

3.1 Evaluation of measurement uncertainty

3.1.1 Definition of measurement

"The objective of a measurement is to determine the value of the measurand, that is, the value of a particular quantity to be measured. A measurement therefore begins with an appropriate specification of the measurand, the method of measurement and the measurement procedure." [1].

In most cases, a measurand Y is not measured directly, but is determined from N other quantities $X_1, X_2, \ldots, X_N$, through a functional relationship f, called model equation:

$$Y = f(X_1, X_2, ..., X_N) \tag{3.1}$$

The result of a measurement is only an approximation or estimate of the value of the measurand Y, denoted with y, and thus is complete only when accompanied by a statement of the uncertainty of the estimate.

The input quantities $X_1, X_2, \ldots, X_N$ upon which the output quantity Y depends, may themselves be viewed as measurands and may depend on other quantities, including corrections and correction factors for systematic effects, thereby leading to a complicated functional relationship f. Further, f may be determined experimentally or exist only as an algorithm that must be evaluated numerically. The function f is to be interpreted as that function which contains every quantity, including all corrections and correction factors that can contribute a significant component of uncertainty to the measurement result.

The set of input quantities $X_1, X_2, \ldots, X_N$ may be categorised as:

- quantities whose values and uncertainties are directly determined in the current measurement. These values and uncertainties may be obtained from, for example, a single observation, repeated observations, or judgements based on experience, and

may involve the determination of corrections to instrument readings and corrections for influence quantities, such as ambient temperature, barometric pressure, and humidity;

- quantities whose values and uncertainties are brought into the measurement from external sources, such as quantities associated with calibrated measurement standards, certified reference materials, and reference data obtained from handbooks.

An estimate y of the measurand Y is obtained from equation (3.1), using input estimates x_1, x_2, …, x_N for the values of the N quantities X_1, X_2, …, X_N. Thus the output estimates y, which is the result of the measurement, is given by:

$$y = f(x_1, x_2, ..., x_n) \tag{3.2}$$

In many cases, the result of a measurement is determined on the basis of series of observations obtained under repeatability conditions, which implies the agreement between the results of successive measurements of the same measurand, carried out under the same measurement conditions (same measurement procedure, observer, instrument, location, day in which the measurement are made).

Variations in repeated observations are assumed to arise because influence quantities that can affect the measurement result are not held completely constant. The mathematical model of the measurement that transforms the set of repeated observations into the measurement result is of critical importance because, in addition to observations, it generally includes various influence quantities that are inexactly known. This lack of knowledge contributes to the uncertainty of the measurement result, as do the variations of the repeated observations and any uncertainty associated with the mathematical model itself [1].

3.1.2 Errors and measurement uncertainty

In general, a measurement has imperfections that give rise to an error in the measurement result. Traditionally, an error is viewed as having two components, namely a random component and a systematic component.

Random errors presumably arise from unpredictable or stochastic temporal and spatial variations of influence quantities. The effects of such variations, hereafter termed random effects, give rise to variations in repeated observations of the measurand. Although it is not possible to compensate for the random errors of a measurement result they can usually be reduced by increasing the number of observations. The experimental standard deviation of the arithmetic mean or average o f a series of observations is not the random error of the mean, but is instead a measure of the uncertainty of the mean due to some random effects. The exact value of the random error in the mean arising from these effects cannot be known.

The systematic error is defined as a component of error which, in the course of a number of analyses of the same measurand, remains constant or varies in a predictable way. It is independent of the number of measurements made and cannot therefore be reduced by increasing the number of analyses under constant measurement conditions. [2]

Systematic errors cannot be eliminated, but they can often be reduced. If a systematic error arises from a recognised effect of an influence quantity on a measurement result, hereafter termed a systematic effect, the effect can be quantified and, if it is significant in size relative to the required accuracy of the measurement, a correction or correction factor can be applied to compensate for the effect [1].

When a measurement is carried out, it is therefore necessary to evaluate all the possible sources of error. Even if all the error components, known or presumed, have been evaluated and the relative corrections have been done, an uncertainty on the result still remains, that is a “doubt” if the result represents the effective value of the measured quantity.

The result of a measurement after correction for recognised systematic effect is still only an estimate of the value of the measurand because of the uncertainty arising from random effects and from imperfect correction of the result for systematic effects.

More precisely, the measurement uncertainty can be defined as “non negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used” [2] and may be a standard deviation or the half-width of an interval having a stated level of confidence [1].

The evaluation of the overall uncertainty has to be carried out combining the uncertainty contributions expressed as standard deviations. Therefore it is necessary to define a mathematical model of the measurement process, in which all the factors that influence the result are expressed.

The uncertainty of a measurement result is usually evaluated using a mathematical model of the measurement and the law of propagation of uncertainty. A measurement can be modelled mathematically to the degree imposed by the required accuracy of the measurement.

Uncertainty components are grouped into two categories based on their method of evaluation, "A" and "B" [1]. These categories apply to uncertainty and are not substitutes of the words "random" and "systematic": the uncertainty of a correction for a known systematic effect may in some cases be obtained by a Type A evaluation while in other cases by a Type B evaluation, as may the uncertainty characterising a random effect.

The purpose of the Type A and Type B classification is to indicate the two different ways of evaluating uncertainty components but this classification is not meant to indicate that there is any difference in the nature of the components resulting from the two types of evaluation. Both types of evaluation are based on probability distributions and the uncertainty components resulting from either type are quantified by variances or standard deviations.

Type A evaluation of uncertainty is a method of evaluation by the statistical analysis of series of observations. The information come from the same experiment or measurement under examination and are based on a series of observations of X_i.

In the type B evaluation of uncertainty, information for the evaluation come from external sources. Type A evaluations of standard uncertainty components are founded of frequency distributions while Type B evaluations are founded on *a priori* distributions.

The estimated variance u^2 characterising an uncertainty component from a Type A evaluation is calculated from a series of repeated observations and is the statistically estimated variance s^2. The estimated standard deviation u is thus $u = s$ and is sometimes called a Type A standard uncertainty. For an uncertainty component obtained from a Type B evaluation, the estimated variance u^2 is evaluated using available knowledge and the estimated standard deviation u is sometimes called a Type B standard uncertainty.

Thus a Type A standard uncertainty is obtained from a probability density function derived from an observed frequency distribution, while a Type B standard uncertainty is obtained from an assumed probability density function based on the degree of belief that an event will occur (often called subjective probability).

For an estimate x_i of an input quantity X_i that has not been obtained from repeated observations, the associated estimated variance $u^2(x_i)$ or the standard uncertainty $u(x_i)$ is

evaluated by scientific judgement based on all of the available information on the possible variability of X_i.

The pool of information may include previous measurement data, experience with or general knowledge of the behaviour and properties of relevant materials and instruments, manufacturer specifications, data provided in calibration or other certificates, uncertainties assigned to reference data taken from handbooks. For convenience, $u^2(x_i)$ and $u(x_i)$ evaluated in this way are sometimes called a Type B variance and a Type B standard uncertainty, respectively.

A measured experimental data is the result of the observation of a random variable, which has its own probability distribution. The most common is the Gaussian distribution or normal error distribution. The equation describing the Gaussian curve is:

$$y = \frac{1}{\sigma \cdot \sqrt{2\pi}} \cdot e^{-(x-\mu)^2/2\sigma^2} \tag{3.3}$$

in which μ represents the mean of the population and σ the standard deviation for an infinite series of data, which measures the dispersion of the data around the mean value. Since it is impossible to carry out an infinite number of measurements, μ and σ cannot be determined. In fact, it is only possible to obtain estimates by means of the value of the arithmetic mean $\bar{x}$ and of the experimental standard deviation s. The less s, the more data will gather around the mean value.

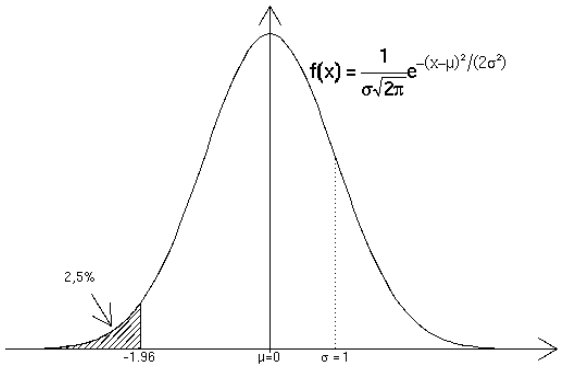

Figure 3.1 – Error Gaussian curve centred on the value $\mu = 0$ [4].

The Gaussian curve, shown in fig. 3.2, represents a probability density function symmetric in respect to $x = \mu$ (the value μ is assumed equal to zero for the sake of simplicity).

This indicates that the most probable value for x corresponds to $x = \mu$. The probability that the measurement result x falls in a given interval of the curve is proportional to the area

underneath the probability distribution of the interval. As the sum of the probabilities off all the measurements must be unit, also the area includes under the entire curve from $x = -\infty$ a $x = +\infty$ must be unit. The term $1/\sigma \cdot \sqrt{2\pi}$ in equation (3.3) is defined normalisation factor and it guarantees that the area underneath the curve is unit.

The standard deviation σ measures the amplitude of the error normal curve: the more is the value of σ, the more the curve will be broad.

In a Gaussian curve, the 68.27% of the area is comprised in the interval $\mu \pm 1\sigma$, i.e. more than the two out of three of the measures will be included among in one standard deviation of the mean.

The 95.45% of the area is comprised in $\mu \pm 2\sigma$, while the 99.73% corresponds to $\mu \pm 3\sigma$. These probability percentage values are also known as intervals of confidence. [5]. In order to obtain the expanded uncertainty $U(y)$ it is necessary to choose a suitable coverage factor k which has to be multiplied for the combined standard uncertainty. In experimental conditions, if the probability distribution is approximately normal and the number of effective degrees of freedom is sufficiently high (at least $\nu_{eff} = 6$), a value of $k = 2$ gives a level of confidence of 95,45 %, while for $k = 3$ the level of confidence is equal to 99,73 % (see tab. 3.1).

To obtain the value of a coverage factor k_p that produces an interval corresponding to a specified level of confidence p requires detailed knowledge of the probability distribution characterised by the measurement result and its combined standard uncertainty.

For quantity z described by a normal distribution with expectation μ_z and standard deviation σ, the value of k_p, that produces an interval $\mu_z \pm k_p \cdot \sigma$ that encompasses the fraction p of the distribution, and thus has a coverage probability or level of confidence p, can be readily calculated [1].

If the estimate x_i is taken from a manufacturer specification, calibration, certificate, handbook or other source, and its quoted uncertainty is stated to be a particular multiple of a standard deviation, the standard uncertainty $u(x_i)$ is simply the quoted value divided by the multiplier and the estimated variance $u^2(x_i)$ is the square of that quotient. The quoted uncertainty of x_i is not necessarily given as a multiple of a standard deviation and it may define an interval having a 90, 95 or 99 % level of confidence. Unless otherwise indicated, it may be assumed that a normal distribution was used to calculate the quoted uncertainty,

and recover the standard uncertainty of x_i by dividing the quoted uncertainty by an appropriate factor for the normal distribution.

In some cases, it may be possible to estimate only bounds (upper and lower limits) for X_i, in particular to state that "the probability that the value of X_i lies within the interval a_- to a_+ for all practical purposes is equal to one and the probability that X_i lies outside this interval is essentially zero". If there is no specific knowledge about the possible values of X_i within the interval, one can only assume that it is equally probable for X_i to lie anywhere within it. This is a uniform or rectangular distribution of possible values. Then x_i, the expected value of X_i, is the midpoint of the interval $x_i = (a_-+a_+)/2$, with associated variance (eq. 3.4):

$$u^2(x_i) = \frac{(a_+ - a_-)^2}{12} \tag{3.4}$$

If the difference between the bounds a_+-a_- is denoted by $2a$, the equation 3.4 becomes:

$$u^2(x_i) = \frac{a^2}{3} \tag{3.5}$$

Because there is no specific knowledge about the possible values of X_i within its estimated bounds a_- to a_+, one may only assume that it is equally probable for X_i to take any value within those bounds, with zero probability of being outside them. Such step function discontinuities in a probability distribution are often unphysical and, in many cases, it is more realistic to expect that values near the bounds are less likely than those near the mid point. It is then reasonable to replace the symmetric rectangular distribution with a symmetric trapezoidal distribution having equal sloping sides (an isosceles trapezoid), a base of width a_+-a_- = $2a$ and a top of width 2aβ, where $0 \le \beta \le$. As $\beta \rightarrow 1$, this trapezoidal distribution approaches the rectangular distribution, while for β = 0, it is a triangular distribution. Assuming such a trapezoidal distribution for X_i, the expectation of X_i is $x_i = \frac{(a_- + a_+)}{2}$ and its associated variance is (eq. 3.6)

$$u^2(x_i) = \frac{a^2(1+\beta^2)}{6} \tag{3.6}$$

which becomes for the triangular distribution, $\beta = 0$,

$$u^2(x_i) = \frac{a^2}{6} \tag{3.7}$$

In fig. 3.2, the estimation of the value of an input quantity X_i is represented, and the evaluation of the uncertainty of the estimate from an *a priori* distribution of possible values of X_i, or probability distribution of X_i, based on all the available information. For both cases shown, the input quantity is assumed to be a temperature t.

In fig. 2 a), a case is illustrated in which it is assumed that little information is available about the input quantity t and that it is only possible to suppose that t is described by a symmetric, rectangular *a priori* probability distribution. For the case illustrated in fig. 2 b), it is assumed that the available information concerning t is less limited and that t can be described by a symmetric, triangular, *a priori* probability distribution of the same lower bound [1].

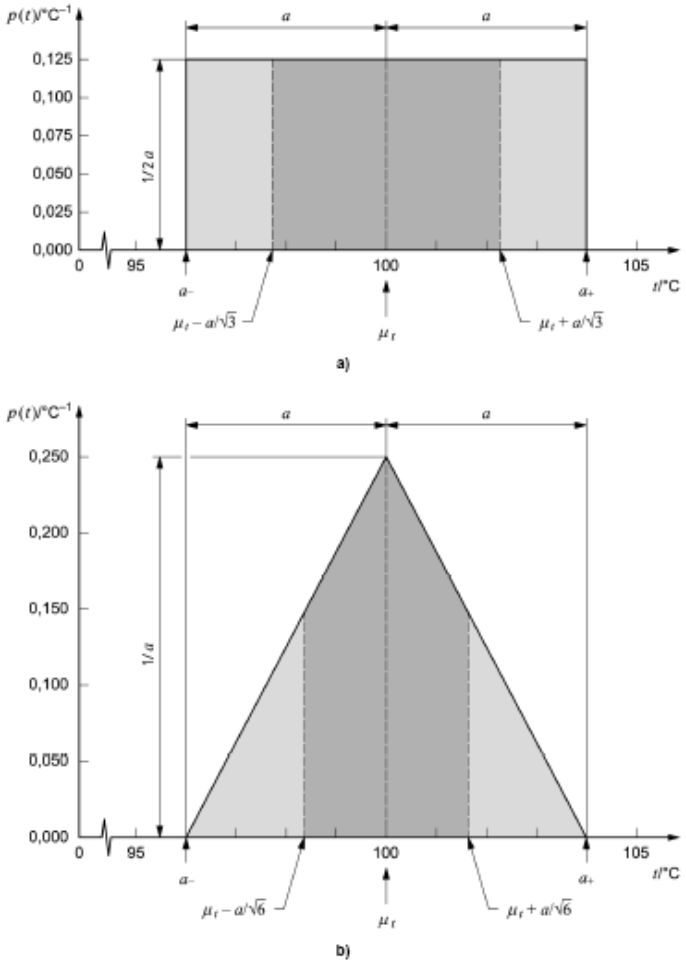

Figure 3.2: Graphical illustration of evaluating the standard uncertainty of an input quantity from an a priori distribution [1].

3.1.3 Expression of the uncertainty

The International regulation concerning the evaluation of uncertainty is expressed in the "Guide to Expression of Uncertainty in Measurement", also known with the acronim GUM and first published in 1995. In 2008, the Joint Committee for Guidance in Metrology (JCGM) issued a revised version of the GUM, "JCGM 100:2008 – GUM 2005 with minor corrections". The Guide was prepared by a joint working group consisting of experts nominated by the BIPM, the *Bureau International des Poids et Mesure*, IEC, the International Electrotechnical Commission, ISO, the International Organization for Standardization, and OILM, the *Organisation Internationale de Métrologie Légale*.

The following seven organisations supported the development of the Guide, which is published in their names:

- BIPM (*Bureau International des Poids et Mesure*)
- IEC (International Electrotechnical Commission)
- IFCC (International Federation for Clinical Chemistry and Laboratory Medicine)
- ISO (the International Organization for Standardization)
- IUPAC (International Union of Pure and Applied Chemistry)
- IUPAP (International Union of Pure and Applied Physics)
- OILM (*Organisation Internationale de Métrologie Légale*).

In 2005 the ILAC (International Laboratory Accreditation Cooperation) officially joined the seven founding international organisations.

The GUM gives general rules to evaluate and express measurement uncertainty and underlines the need of the knowledge and the comprehension of the measurement process in order to evaluate uncertainty. The GUM was introduced in Europe in 19999 as the experimental regulation ENV 13005:1999, from which the Italian regulation UNI CEI ENV 13005:2000 [1].

Concerning the chemical measurement field, it is necessary to cite the EURACHEM/CITAC Guide "Quantifying Uncertainty in Analytical Measurement" [3], in which the GUM principles are applied to chemical measurements and many practical examples are given.

In [2] the following definitions of measurement uncertainty are reported:

- Standard uncertainty $u(x)$: measurement uncertainty expressed as a standard deviation. It may also be written as $s(x)$;

- Combined standard uncertainty $u_c(y)$: standard measurement uncertainty that is obtained using the individual standard measurement uncertainties associated with the input quantities in a measurement model;
- Expanded uncertainty $U(y)$: product of a combined standard measurement uncertainty and a factor larger than the number one;
- Coverage factor k: number larger than one by which a combined standard measurement uncertainty is multiplied to obtain an expanded measurement uncertainty.

The expanded uncertainty is defined as:

$$U(y) = k \cdot u_c(y) \tag{3.8}$$

The standard uncertainty of y or combined standard uncertainty $u_c(y)$ is expressed as the positive square root of a sum of terms, the terms being the variances or covariances of other quantities (the input quantities) weighted according to how the measurement result varies with changes in these quantities. This sum is defined as combined variance $u_c^2(y)$ and derives from the law of propagation of uncertainty:

$$u_c^2(y) = \sum_{i=1}^{N} \left(\frac{\partial f}{\partial x_i} \right)^2 \cdot u^2(x_i) \tag{3.9}$$

where f is the function given in eq (3.1) and each $u(x_i)$ is a standard uncertainty (Type A or B). The partial derivatives $\partial f / \partial x_i$ are equal to $\partial f / \partial X_i$ evaluated at $X_i = x_i$. These derivatives, often called sensitivity coefficients, describe how the output estimate y varies with changes in the values of the input estimates $x_1, x_2, \ldots x_N$. The equation (3.9) is based on a first-order Taylor series approximation of $Y = f(X_1, X_2, ..., X_N)$.

The change in y produced by a small change Δx_i in input estimate x_i is given by $(\Delta y_i) = (\partial f / \partial x_i)(\Delta x_i)$. If this change is generated by the standard uncertainty of the estimate x_i, the corresponding variation in y is $(\partial f / \partial x_i)u(x_i)$. The combined variance $u_c^2(y)$ can therefore be viewed as a sum of terms, each of which represents the estimated variance associated with the output estimate y generated by the estimated variance associated with each input estimate x_i. This suggests writing the equation (3.9) as:

$$u_C{}^2(y) = \sum_{i=1}^{N} \left[c_i u(x_i) \right]^2 \equiv \sum_{i=1}^{N} u_i^2(y) \tag{3.10a}$$

where:

$$c_i \equiv \partial f / \partial x_i, \quad u_i(y) \equiv | c_i | u(x_i) \tag{3.10b}$$

Equation (3.9) is valid only if the input quantities X_i are independent or uncorrelated. If some of the X_i are significantly correlated, the correlations must be taken into account. When the input quantities are correlated, the appropriate expression for the combined variance $u_c^2(y)$ associated with the result of a measurement is:

$$u_c^2(y) = \sum_{i=1}^{N}\sum_{j=1}^{N}\frac{\partial f}{\partial x_i}\frac{\partial f}{\partial x_j}u(x_i,x_j) = \sum_{i=1}^{N}\left(\frac{\partial f}{\partial x_i}\right)^2 u^2(x_i) + 2\sum_{i=1}^{N-1}\sum_{j=i+1}^{N}\frac{\partial f}{\partial x_i}\frac{\partial f}{\partial x_j}u(x_i,x_j) \qquad (3.11)$$

where x_i and x_j are the estimates of X_i and X_j and $u(x_i, x_j) = u(x_j, x_i)$ is the estimated covariance associated with x_i and x_j. The degree of correlation between x_i and x_j is characterised by the estimated correlation coefficient:

$$r(x_i,x_j) = \frac{u(x_i,x_j)}{u(x_i)u(x_j)} \qquad (3.12)$$

where $r(x_i, x_j) = r(x_j, x_i)$ and $-1 \leq r(x_i, x_j) \leq +1$. If the estimates x_i and x_j are independent, $r(x_i, x_j) = 0$, and a change in one does not imply an expected change in the other. In terms of correlation coefficients, which are more readily interpreted than covariances, the covariance term of eq. (3.11) may be written as :

$$2\sum_{i=1}^{N-1}\sum_{j=i+1}^{N}\frac{\partial f}{\partial x_i}\frac{\partial f}{\partial x_j}u(x_i)(x_j)r(x_i,x_j) \qquad (3.13)$$

Equation (3.11) then becomes:

$$u_C^{\ 2}(y) = \sum_{i=1}^{N}c_i^{\ 2}u^2(x_i) + 2\sum_{i=1}^{N-1}\sum_{j=i+1}^{N}c_ic_ju(x_i)u(x_j)r(x_i,x_j) \qquad (3.14)$$

In order to obtain the expanded uncertainty $U(y)$ it is necessary to choose a suitable coverage factor k_p which has to be multiplied for the combined standard uncertainty. As defined in par. 3.1.2 in experimental conditions, if the probability distribution is approximately normal and the number of effective degrees of freedom is sufficiently high (at least $\nu_{eff} = 6$), a value of $k = 2$ gives a level of confidence of 95,45 %, while for $k = 3$ the level of confidence is equal to 99,73 % (see tab. 3.1).

Level of confidence (%)	Coverage factor k_p
68,27	1
90	1,645
95	1,960
95,45	2
99	2,576
99,73	3

Table 3.1: Value of the coverage factor k_p that produce san interval having level of confidence p assuming a normal distribution [1].

3.2 Evaluation of measurement uncertainty in chemical measurement

According to the EURACHEM/CITAC Guide [3], the process of evaluating measurement uncertainty consists of four steps:

1) Specification of the measurand;
2) Identifying uncertainty sources;
3) Quantifying uncertainty components;
4) Calculating the combined uncertainty.

In the first step of the process, it is necessary to define the measurand and to formulate a quantitative expression which relates the measurand value to the input quantities on which it depends, including possible corrections for the systematic effects.

In order to evaluate uncertainty as much accurately as possible, it is necessary to take into account all the possible sources, including all the parameters which appear in the measurand definition.

However, even parameters which do not explicitly appear in the chosen mathematical model can be potential uncertainty sources and have effects on the measurement. There are many uncertainty sources, that are not necessary independent from each other.

In an analytical method, typical sources of uncertainty are: sampling, storage conditions, instrument effects, reagent purity, stoichiometry of the chemical reactions, measurement

conditions, sample effects, computational effects, blank correction, operator effects and random effects.

When the sampling forms part of a specified measurement procedure, it contributes significantly to the uncertainty of the entire analytical process. For this reason, it is necessary to minimise the risks of contamination and losses of the analytes, during the sampling and the storage, to reduce the random variations between different samples.

The duration of the storage and the conditions during storage should be considered as uncertainty sources. Variations in space and time of the analytes in the samples should not be underrated, in order to avoid any bias in the sampling procedure.

After this stage, the samples can be pre-treated before the actual analysis, among which extraction from the matrix, precipitation, pre-concentration, and each step contributes to the final uncertainty.

In addition, problems can arise if the analytes are contained in a substrate, or *matrix*, which can influence the response of the instrument used, or if we have to deal with a class of substances not singularly defined (e.g extractable fats). In other cases, the substance can be present at trace level or in different chemical forms (speciation). In this last case, the analyte can assume many similar chemical forms and the form of interest must be clearly specified, as the way to determine it.

Instrument effects may include, for example, the limits of accuracy on the calibration of an analytical balance, a temperature controller that may maintain a mean temperature which differs (within specification) from its indicated set-point, an auto-analyser that could be subject to carry-over effects.

The purity of the substances used for the preparation of calibration solutions is usually stated by manufacturers as being *not less than* a specified level as the parent materials have been assayed. Indeed, some uncertainty related to the assaying procedure remains and any assumptions about the degree of purity will introduce an element of uncertainty.

Where an analytical process is assumed to follow a particular reaction stoichiometry, it may be necessary to allow for differences from the expected stoichiometry, or for incomplete reaction or side reactions.

Measurement conditions should be considered in evaluating measurement uncertainty: for example, temperature effects should be considered and corrected, e.g. when volumetric glassware is used at an ambient temperature different from that at which it was calibrated, any uncertainty in the temperature of liquid and glass should also be considered. Humidity

may be also very important when dealing with materials which are sensitive to possible changing in humidity.

The recovery of an analyte from a complex matrix, or an instrument response, may be affected by composition of the matrix. In addition, the stability of a sample/analyte may change during the analysis because of a changing thermal regime or photolytic effect.

When a spike is used to estimate recovery, the recovery of the analyte from the sample may differ from the recovery of the spike, introducing an uncertainty which needs to be evaluated.

Other factors which may influence the uncertainty of a measurement result are blank correction, operator and random effects. There will be an uncertainty on both the value and the appropriateness of the blank correction and this aspect is particularly important in trace analysis. Operator effects include, among others, the possibility of reading scales or meters consistently high or low, or the possibility of making a slightly different interpretation of the method.

When the component to be quantified has been identified and detected among the other components of a mixture or matrix, the actual separation can be carried out. If the separation is considered complete, the material can be quantified by weighing or, in the most common case, the instrument response is calibrated against reference standards, generally constituted by pure substances in suitable means [3].

In order to quantify the contribution of all the uncertainty sources two possible approaches may be followed:

a) evaluating the uncertainty related to each source and combining all the contributions to calculate the combined standard uncertainty (metrological approach);

b) determining directly the uncertainty by using data from previous studies on the performances of the method used (holistic approaches, e.g. Horwitz's approach).

In this framework, the importance of the measurements carried out on certified reference materials (CRMs) has been already highlighted, as CRMs allow characterising the complete measurement procedure with reference to traceable standards, and obtaining useful information concerning the combined effect of the various uncertainty sources. As not all the sources are significant for the whole evaluation of the uncertainty, it is preferable to carry out preliminary evaluations on each component, in order to identify which components have to be considered and which ones can be neglected. After having quantified all the contributions to the overall uncertainty, these contributions have to be

expressed as standard deviations and combined to calculate the combined standard uncertainty $u_c(y)$, from which the expanded uncertainty is obtained multiplying $u_c(y)$ for the appropriate coverage factor k.

3.3 Use of the Monte Carlo method for the evaluation of measurement uncertainty

3.3.1 Introduction ad scope

Supplement 1 to the GUM [5] provides a general numerical approach for carrying out the evaluation of measurement uncertainty in those cases in which the conditions for the GUM uncertainty framework are not fulfilled, or it is unclear whether they are fulfilled. The approach applies to arbitrary models with a single output quantity and a number n of input quantities which can be characterised by a specified probability density function (PDF). As in the GUM, Supplement 1 is primarily concerned with the evaluation of uncertainty for a well defined quantity, i.e. the measurand, which can be characterised by an essential unique value. Given the model relating the input quantities with the output quantity and the PDFs characterising the input quantities, there is a unique PDF for the output quantity representing the state of knowledge on it. Generally, it is very difficult to determine such PDF analytically. Supplement 1 can be used to provide a representation of the PDF for the output quantity from which:

a) an estimate of the output quantity,

b) the standard uncertainty associated with this estimate, and

c) a coverage interval for the quantity, corresponding to a specified coverage probability

can be obtained.

Therefore, the objective of the Monte Carlo approach is to determine a), b) and c) to a prescribed numerical tolerance, without linearizing the model ad prescribed in the GUM.

3.3.2 Basic principles

The main stages of uncertainty evaluation consist in formulation, propagation, and summarising:

a) Formulation: it consists in defining the output quantity Y and determining the input quantities $X_1, \ldots, X_N$ upon which Y depends; then developing a model relating Y and the

input quantities and assigning PDFs to the X_i, on the basis of available knowledge. It is possible to assign a joint PDF to those X_i that are not independent;

b) Propagation: propagate the PDFs of the X_i through the model to obtain the PDF for Y;

c) Summarising: it means to use the output PDF to obtain 1) the expectation of Y, taken as an estimate y of the quantity, 2) the standard deviation of Y, taken as the standard uncertainty $u(y)$ associated with y and 3) a coverage interval containing Y with a specified probability (the coverage probability).

The GUM uncertainty framework does not explicitly refer to the assignment of PDFs to the input quantities, however it is stated in [1] that Type A and Type B standard uncertainties are obtained from probability density functions (derived from an observed or an assumed frequency distribution) and that both approaches employ recognised interpretations of probability.

In Supplement 1, an efficient approach for determining a numerical approximation to the distribution function

$$G_Y(\eta) = \int_{-\infty}^{\eta} g_Y(z)\mathrm{d}z \tag{3.15}$$

for Y is considered. It is based on applying a Monte Carlo Method (MCM) as an implementation of the propagation of distributions.

In general, the propagation of distributions can be implemented in several ways:

a) analytical methods, i.e. methods that provide a mathematical representation of the PDF for Y;
b) uncertainty propagation based on replacing the model by a first-order Taylor series approximation [1], the law of propagation of uncertainty;
c) as b), except that contributions derived from high-order terms in the Taylor series approximation are included;
d) numerical methods that implement the propagation of distributions, specifically using MCM.

The propagation of the PDFs $g_{x_i}(\xi_i)$, $i = 1, \ldots, N$, for the input quantities X_i, through the model to provide the PDF $g_Y(\eta)$ for the output quantity Y is illustrated in fig. 3.3 for $N = 3$ independent X_i. Figure 3.3 may be compared to figure 3.4, representing the law of

propagation of uncertainty. $g_Y(\eta)$ is indicated as being asymmetric, as generally arises for non-linear models or asymmetric $g_{x_i}(\xi_i)$.

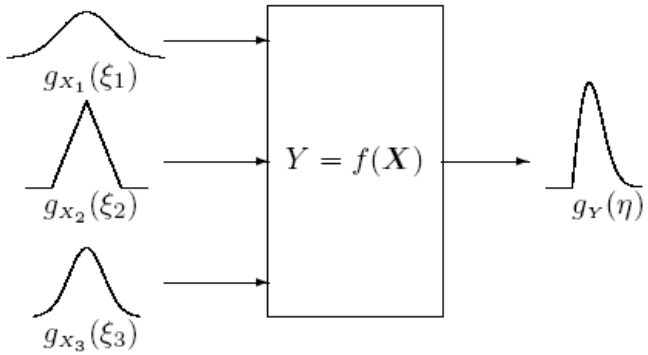

Figure 3.3: Illustration of the propagation of distributions for $N = 3$ independent input quantities[5].

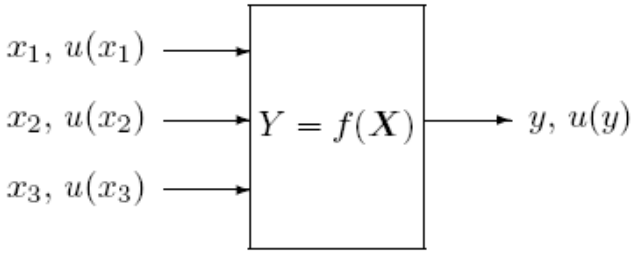

Figure 3.4: Illustration of the law of propagation of uncertainty for N = 3 independent input quantities [5].

3.3.3 Implementation of a Monte Carlo method

MCM provides a general approach to obtain an approximate numerical representation ***G***, of the distribution function $G_Y(\eta)$ for Y defined in eq. (3.15). The hearth of the approach is repeatedly sampling from the PDFs of the X_i, and evaluating the model in each case. Since $G_Y(\eta)$ encodes all the information known about Y, any property of Y such as expectation $E(Y)$, variance $V(Y)$ and coverage intervals can be approximated using ***G***. The quality of these calculated results improves with the number of the MC trials. Expectations and variances can be determined directly from the set of model values obtained. MCM can be stated as a step-by-step procedure:

1) select the number M of Monte Carlo trials to be made;

2) generate M vectors $\boldsymbol{x}_r$, by sampling from the assigned PDFs, as realisation of the set of N input quantities X_i;

3) for each vector, form the corresponding model value of Y, yielding M model values;

4) sort the M model values into a strictly increasing order, using the sorted values to provide **G**;

5) use ***G*** to form an estimate y of Y and the standard uncertainty $u(y)$ associated with y;

6) use ***G*** to form an appropriate coverage interval for Y, for a stipulated coverage probability p.

In an implementation of MCM, M vectors $\boldsymbol{x}_r$, $r = 1, \ldots, M$ are drawn from the PDFs $g_{x_i}(\xi_i)$ for the N input quantities X_i.

The model is evaluated for each of the M draws from the PDFs for the N input quantities. The model values are:

$$y_r = f(\boldsymbol{x}_r),\ r = 1, \ldots, M \tag{3.16}$$

The average $\tilde{y}$ and the standard deviation $u(\tilde{y})$ are taken respectively as an estimate y of Y and the standard uncertainty $u(y)$ associated with y.

The propagation of distributions implemented using MCM can be validly applied under several conditions: (i) the function f is continuous with respect to X_i in the neighbourhood of the best estimates x_i of the X_i; (ii) the distribution function for Y is continuous and strictly increasing; (iii) the PDF for Y is continuous over the interval for which this PDF is strictly positive, unimodal and strictly increasing (or zero) to the left of the mode and strictly decreasing (or zero) to the right of the mode; (iv) $E(Y)$ and $V(Y)$ exist; (v) a sufficiently large value of M is used.

Compared to the GUM approach, the MCM departs from it for some main features:

- PDFs are explicitly assigned to all input quantities X_i, (rather than associating standard uncertainties with estimates x_i of X_i) based on information concerning these quantities. The classification into Type A and Type B evaluations of uncertainty is not needed;
- the sensitivity coefficients are not an inherent part of the approach and the calculation or numerical approximation of the partial derivatives of the model with respect to the X_i is not required;
- a numerical representation of the distribution function for Y is obtained that is defined completely by the model and the PDFs of the X_i, and is not restricted to a Gaussian distribution or scaled and shifted t-distribution;

- since the PDF for Y is not general symmetric, a coverage interval for Y is not necessarily centred on the estimate of Y.

In general, MCM can improve the estimate of Y and the standard uncertainty associated with the estimate of Y for non-linear models, especially when the X_i are assigned with non-Gaussian PDFs. MCM can also provide a coverage interval corresponding to a stipulated coverage probability when the PDF for Y cannot adequately be approximated by a Gaussian distribution or a scaled and shifted t-distribution. Such an inadequate approximation can arise when the PDF assigned to a dominant X_i is not a Gaussian distribution or a t-distribution, the model in non-linear or the approximation error incurred in using the Welch-Satterthwaite formula for effective degrees of freedom is not negligible. Finally, with MCM a coverage factor is not required for determining a coverage interval.

Supplement 1 gives guidance on the assignment, in some common circumstances, of the PDFs to the input quantities X_i, in the formulation stage of uncertainty evaluation.

The value of M, i.e. the number of model evaluations to be made, needs to be selected. It can be chosen *a priori*, with no direct control over the quality or the numerical results provided by MCM. Otherwise, a procedure that selects M adaptively, i.e. as the trials progress, can be used. The number of trials needed to provide these results to a prescribed numerical tolerance will depend on the "shape" of the PDF for the output quantity and on the coverage probability required.

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CHAPTER 4

ANALYSIS OF ORGANIC MICROPOLLUTANTS IN FOOD

4.1 The concept of food safety

Food safety is, nowadays, a matter of fundamental relevance, for the consumer health, the food industry and the entire economic field.

The contamination of food products can have considerable fallouts both at the social and the economic level, for communities and their national health services. Indeed, foodborne diseases are a problem for public health at the international level, and regard also the most developed countries, where the problem has reached particular relevance (e.g. the dioxins contamination of Bufala mozzarella in Italy in 2008 and the *E. coli O104* contamination of food in Germany and France in 2011). In this framework, the need of having powerful and reliable instruments to protect the consumers from adverse health effects appears clear. One of the challenges which are of greatest importance in these days for the assessment and management of food safety risks is to carry out accurate and efficient controls. The methods used must avoid an excessive increase of the costs for the industries and, as a consequence, for the consumers, while the quality of food products should not be lowered. There are many micro-organisms and chemical substances which must be monitored and detected in all the production steps of food, in order to guarantee the safety and quality of these products.

A definition for "safe food" is necessary to clarify the food safety goals of governments and industries and to measure progress toward achieving the goals. A safe food is intended as one that does not cause harm to the consumer when it is prepared and/or eaten according to its intended use [1].

The concept of food safety has been proposed to provide a target for operational food safety management. The concept helps to better relate operational food safety management to public health goals, i.e. to an appropriate level of protection. Today, with important changes in lifestyles and demographic compositions and with food markets becoming increasingly more "global", the food supply is growing rapidly in size and diversity. In this framework, it has been necessary to adapt and improve the food safety management

systems on a continuous basis. In recent years the control over the safety and quality of food has become tighter and tighter. Food safety management systems such as Hazard Analysis and Critical Control Points (HACCP) and the pre-requisite systems Good Manufacturing Practice (GMP) and Good Hygiene Practice (GHP) have provided the professional players in the food supply chain with excellent tools.

Risk assessment methods to derive human safe of exposure have been developed by scientists and public-health agencies; risk assessment has been divided into four sequential steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation. In practice, once a chemical has been identified, its content in food measured through validated analytical techniques, its biological (toxicological) effects characterised and a safe level derived, one can relate exposure to biological effects for human risk assessment.

The concerns in the food safety field can regard both microbiological and chemical aspects; indeed human beings are exposed to a wide range of micro-organisms and chemicals, the uptake of which by the human body is mainly through food, water, air and dermal contact [2]. Despite the significant efforts by all parties involved, there is still a considerable burden of foodborne illness, in which micro-organisms play a prominent role. Microbes can enter the food chain at different steps, are highly versatile and can adapt to the environment allowing survival, growth and production of toxic compounds.

Man-made contaminants of importance include persistent organic pollutants (POPs), [i.e. dioxins, polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs)], melamine, phtalates, perfluoroalkyl acids, a large number of pharmaceuticals and natural toxins (i.e. mycotoxins, marine biotoxins and plant toxins). Other contaminants in food are produced from the Maillard reaction during frying and cooking at high temperature (i.e. acrylamide) or as a reaction product between ethanol and precursors (cyanide). A large class of chemical also added intentionally to food are food additives and food-contact materials (e.g. from packagings), whereas chemicals resulting from intentional treatment of raw commodities include pesticides, biocides and veterinary residues [2].

Monitoring of contamination in the food chain, combined with surveillance of human illness and epidemiological investigations of outbreaks and sporadic cases continue to be important sources of information. At present HACCP programs and GMP are mainly used to manage microbial hazards in food [3]. Specific concepts have been developed in the food safety management, i.e. microbiological criteria, control measures and process criteria. In addition, stakeholders in food safety management such as governments, trade or

sector organisations, have developed guidelines, best practice advice, regulations and food safety standards [4].

Governments meet their food safety goals by being the overseers of the total food supply from production or harvesting to consumption. This involves a variety of activities, such as inspections, documenting the burden of foodborne disease, identifying food safety problems through epidemiology, conducting research to understand the problems, and educating food handlers at all levels in proper procedures. Industry meets its safety goals by establishing policies and procedures that can ensure the safety of its products. This can be accomplished only through knowledge of the processing conditions on the safety of the food. It also depends on a thorough understanding of the product and its intended use. After companies have confidence that their established processes and management systems result in safe products, then they will have to make adjustments to ensure compliance with regulatory requirements [1].

The EU has issued different directives and regulations concerning the analysis of chemical residues in food and feed, among which:

- Council Directive 96/23/EC of 29 April 1996, on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC, corresponding to the Italian D. Lgs. n. 336 of the 4th August 1999;
- Regulation (EC) n. 882/2004 of the European Parliament and of the Council of 29 April 2004, on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules;
- Directive 2004/41/EC of the European Parliament and of the Council of 21 April 2004 repealing certain directives concerning food hygiene and health conditions for the production and placing on the market of certain products of animal origin intended for human consumption and amending Council Directives 89/662/EEC and 92/118/EEC and Council Decision 95/408/EC, corresponding to the Italian D. Lgs. n. 193 of the 6th November 2007;
- Commission Directive 2000/42/EC of 22 June 2000 amending the Annexes to Council Directives 86/362/EEC, 86/363/EEC and 90/642/EEC on the fixing of maximum levels for pesticide residues in and on cereals, foodstuffs of animal origin and certain products of plant origin, including fruit and vegetables respectively;

- Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market, implemented by the Plant Protection Products Regulations in 2003, limited more types of pesticides to detectable level residues in certain food samples tested.

The European Food Safety Authority (EFSA) was created in 2002 to assess the risk of the hazards (both microbial and chemical) to human health, when they are ingested via food.
Concerning chemical occurring in food, it is necessary to define xenobiotics substances, which are contaminants that have been intentionally added to food or raw commodities, and can be classified into broad categories, according to their relevance in terms of food safety.
On a worldwide perspective, some years ago the World Health Organisation (WHO) started the "Initiative to Estimate the Global Burden of Foodborne Diseases – a Growing Risk" (2008) in order to provide reliable, accurate estimates of the global burden relate to foodborne diseases, caused by chemicals, parasites and enteric infections. This program is expected to estimate and compare on a common scale the respective burden for human health of various hazards from different origins [5].
Once contaminants have been identified and quantified in food, and human exposure is known, the biological activity or toxicity of chemical arises from two basic processes:

- what the body does to the chemical – toxicokinetics (TK);
- what the chemical does to the body – toxicodynamics (TD).

Evaluation of TK and TD are usually performed within the context of risk assessment or pure safety assessment, using data from epidemiological or toxicological studies.
In addition, when considering toxicological effects from a regulatory perspective, two basic mechanisms have been retained for chemical risk assessment, namely:
- whether or not the chemicals are genotoxic carcinogens;
- derivation of health-based guidance values for humans, that differ according to the difference in mode of action (MOA).

The term "genotoxic" refers to a substance (or its active metabolite) which affects cellular DNA through a direct DNA-reactive MOA, involving covalent binding in target cells to cause pre-carcinogenic mutations.
For such genotoxic carcinogens, the Margin of Exposure (MOE) approach has recently been applied to a number of contaminants (i.e. aflatoxins, ethylcarbamate, polyaromatic hydrocarbons and acrylamide) by the Joint Food and Agricultural Organisation of the

UN/WHO (FAO/WHO) Expert Committee on Food Additives (JECFA) and EFSA. Such MOEs are derived using dose-response or dose-effect data using the model-based evaluation of a benchmark dose and its lower confidence limit [2].

4.2 Analysis of pesticide residues in food products

4.2.1 Introduction

Pesticides are a numerous and diverse group of chemical compounds, which are used to eliminate pests in agriculture and households. They help to limit the many human diseases transmitted by insects or rodent vectors. However, pesticides are some of the most toxic, environmentally stable and mobile substances in the environment. Due to their environmental stability, ability to bioaccumulate and toxicity, pesticides may place the human body at a greater risk of disease and poisoning. Pesticides enter the environment in various forms and are of enormous importance in increasing the yields and quality of agricultural products. They are used to:

- control the numbers of pests destroying whole plants or their parts;
- increase the production of animal and plant biomass;
- combat microorganisms causing farm produce to rot and to decay;
- combat animal pests damaging crops;
- stimulate or inhibit plant-growth processes;
- kill harmful organisms in farm buildings, houses, hospitals, stores, vehicles.

The widespread use of pesticides not only contaminates water, soil, and air, but also causes them to accumulate in crops. Pesticides are transported mainly by rain and wind from their points of application to neighbouring crops and lands. The amounts of pesticides in any particular region depend to a large extent on the intensity of pesticide application and the types of crops grown there. The diversity of their chemical structures, actions and applications makes any classification of pesticides difficult. There are a number of criteria according to which they can be categorised:

1) toxicity;
2) purpose of application;
3) chemical structure;
4) environmental stability;
5) the pathways by which they penetrate target organisms.

Structurally, they can be divided into inorganic and organic compounds; the inorganic include arsenic insecticides, fluoride insecticides, inorganic herbicides and inorganic fungicides, while the organic comprise organochlorine, organophosphorus and organonitrogen pesticides.

Organophosphorus pesticides (OPPs) are the principal group of compounds used to protect plants. They include all organic compounds containing phosphorus and usually have an ester structure, decomposing fairly easily on the surface and interiors of plants, and in the soil. Their toxicity depends on inhibiting the activity of enzymes controlling the functions of the nervous system, mainly acetilcholinesterase. Organonitrogen pesticides (ONPs) also play a major part in combating pests. Even though they are less stable in the environment than organochlorine pesticides (OCPs), they can get into the human digestive system, posing a health hazard. Some carbamate insecticides can be teratogenic in large doses and nitrosated to form strongly carcinogenic nitroso-compounds.

OCPs, including aldrin, chlordane, lindane and DDT, have been withdrawn from use in many countries, because they are very toxic, they have a considerable stability in the environment (as long as 30 years) and may be transported by air or water over long distances.

Plant foods can be contaminated by pesticides under a great variety of circumstances and at different times preceding their consumption. Many factors can reduce such contamination (e.g. rainfall, wind, chemical reactions induced by oxygen, moisture, light or plant enzymes). The structure of the plant in question is also important because, for example, OCPs accumulate in the waxy layer of the rind of many fruits, especially citrus fruits. It is a matter of urgency that pesticide residues in fruit and vegetables are monitored, because they can put human health at greater risk of various diseases [6].

OCPs were intensively used in agriculture to protect cultivated plants in mid-twenty century and the use of pesticides in the USA doubled from 1960 to 1980. DDT (1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane) is one of the common OCPs and was used to prevent the spreading of malaria and other vector-borne diseases. Due to their relative stability and bioaccumulation properties, these persistent chemical can be transferred and magnified to higher trophic levels through the food chain. Consequently, OCP residues are present in fatty foods, both foods of animal origin and of plant origin. Human exposure occurs still primarily via low level food contamination, even if these chemicals are widely distributed in the environment, which provides another route of unwanted intake in

humans. Since their mode of action is by targeting systems or enzymes in the pests which may be identical or very similar to systems or enzymes in human beings, the OCPs pose risks to human health and the environment.

The most persistent organic pollutants (POPs) are OCPs, namely aldrin, endrin, chlordane, DDT, heptachlor, mirex, toxaphene and hexachlorobenzene (HCB). They have been banned for agricultural or domestic uses in Europe, North America and many countries of South America in accordance with the Stockholm Convention. However, some OCPs are still used (e.g. DDT is used to control the growth of mosquito that spread malaria. Besides, the most commonly used acaricide, dicofol, is made of DDT) and residues of OCPs have been detected in breast milk in contaminated areas.

Recently, the scope of POPs was extended to include nine plus one chemicals. Among these new POPs, there are several OCPs: chlordecone, lindane, α-HCH, β-HCH, pentachlorobenzene (PeCB) and endosulfan. In order to fulfil the requirements of the Stockholm Convention, the participating countries have to develop their own implementation plan to monitor the background level and collate exposure data [7].

The intensive development of agriculture means that more and more toxic organic and inorganic compounds are entering the environment. Because of their widespread use, stability, selective toxicity and bioaccumulation, pesticides are among the most toxic substances contaminating the environment. They are particularly dangerous for fruit and vegetables, by which people are exposed to them, and it is therefore crucial to monitor pesticide residues in fruit and vegetables, using suitable analytical techniques. Pesticides have many advantages, but they also do much harm to the environment. Each year, 140000 t of pesticides are sprayed onto crops in European Union (EU) alone. Fruit and vegetables are the crops most likely to be contaminated by pesticides, particularly grapes, citrus fruits and potatoes. According to data from the EU's Pesticide Action Network (as of 2008) some 350 different pesticides were detected in food produced in the EU. More than 5% of products contained pesticides at levels exceeding the EU's maximum permitted levels (MPL).

Even though pesticides facilitate improvement in crop yields and quality, they do pose a risk to consumers and this is why international organisations have established maximum residue levels (MRLs) of pesticides in food. Any assessment of the state of contamination of fruit and vegetables by pesticides requires the knowledge of MRLs laid down by the EU. EU member states are obliged to organise effective monitoring of food with the aim of assessing its safety. In the case of pesticides residues, this task is carried out in the form of

monitoring programs and official inspections of food to ensure compliance with MRLs. The aim of controlling pesticide residues is to protect consumer health from their possible side effects. Safe food should have above all an appropriate nutritious value and contain the least possible amounts of substances that could be hazardous to health [7].

For monitoring purpose, MRLs would be set for particular pesticides in particular food matrices. Moreover, the Codex Alimentarius Commission has established extraneous maximum residue limits (EMRLs) for some of the OCPs residues in foods. The EMRL refers to the maximum pesticide residue level arising from environmental sources other than the use of the pesticide directly or indirectly on the commodity itself. It is the maximum concentration of a pesticide residue that is recommended by the Codex Alimentarius Commission to be legally permitted or recognised as acceptable in or on a food, agricultural commodity or animal feed and is temporary, regardless of the status of the Acceptable Daily Intake (ADI), until required information has been provided and evaluated. MRL/EMRL has to be considered before developing an appropriate method for routine monitoring. Table 4.1 summarises the definition of OCPs that have Codex's MRL/EMRL [7]. However, some of the residues have not been included in the list of monitoring chemicals of the Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) of World Health Organisation (WHO).

OCP	Codex residue definition	GEMS/Food monitoring targets	Codex MRL/EMRL (mg/kg on fat basis)		
			Milk	Meat	Poultry meat
Aldrin and Dieldrin	Sum of HHDN (aldrin) and HEOD (dieldrin)	Aldrin and dieldrin	0.006	0.2	0.2
Chlordane	Plant commodities: sum of cis- and trans-chlordane (fat-soluble) Animal commodities: sum of cis- and trans-chlordane and "oxychlordane"	Chlordane (α- and γ-, trans-nonachlor + oxychlordane)	0.002	0.05	0.5
DDT	Sum of p,p′-DDT, o,p′-DDT, p,p′-DDE and p,p′-TDE (DDD)	Sum of p,p′-DDT, o,p′-DDT, p,p′-DDE and p,p′-TDE (DDD)	0.02	5	0.3
Dicofol	Plant commodities: Dicofol (sum of o,p′ & p,p′ isomers) (fat-soluble) Animal commodities: Sum of dicofol and 2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol (p,p′-FW152) calculated as dicofol	Dicofol (sum of o,p′ and p,p′ isomers)	0.1	3[a]	0.1
Endosulfan	For compliance with the MRL and for estimation of the dietary intake: sum of alpha-endosulfan, beta-endosulfan and endosulfan sulphate. This definition applies to plant and animal commodities	Endosulfan (sum of α- and β-isomers)	0.01[b]	0.2	0.03
Endrin	Sum of endrin and delta-keto-endrin	Endrin (sum of α- and β-isomers)	–	–	0.1
Heptachlor	Sum of heptachlor and heptachlor epoxide	Sum of heptachlor and heptachlor epoxide	0.006	0.2	0.2

[a] Cattle meat.
[b] On whole weight basis.

Table 4.1: Summary of Codex Alimentarius definitions of OCPs listed in GEMS/Food chemical list [7].

4.2.2 Determining pesticides in real samples

The analysis of pesticides in biological samples continues to present challenges to analysts. The main problems in the analysis of pesticide residues are:

1) complexity and the diversity of matrices in biological materials;
2) low concentrations of pesticides in food samples.

Target analytes must be isolated from the matrices and enriched before the final determination.

It is extremely important that the several stages of the analytical procedure and the procedure as a whole are validated in order to ensure compliance with the requirements defining the procedure and to assess its usefulness [6-7]:

- Preparation of samples for analysis: it is extremely important that the sample is homogeneous and representative. A representative sample has a chemical composition which resembles as closely as possible the average composition of the whole analysed material. Any operation during the sample preparation (washing, desiccation, grinding, and homogenization) should be controlled and carried out in order to avoid losses of the analytes or contamination of the samples.
- Extraction of pesticides from the samples: isolation and preconcentration mean the transfer of analytes from the primary matrix to a secondary one with the simultaneous removal of interferents and increase in target-analyte concentrations to levels above the Limit of Detection (LOD) of the analytical technique applied. Usually, the solid matrix has to be replaced by a liquid one, by using a suitable extraction method. Common extraction methods are, among others: liquid-liquid extraction (LLE), accelerated solvent extraction (ASE), microwave-assisted solvent extraction (MAE); Soxhlet and Soxtec extraction; ultrasound-assisted extraction (UAE); supercritical fluid extraction (SFE); matrix solid-phase dispersion (MSPD).
  LLE does not need expensive and complicated apparatus, but it requires large amounts of toxic solvents and is poorly selective. MAE and ASE (also known as pressurised liquid extraction, PLE) allow speeding up sample preparation, by using high temperature and pressure, to heat the sample-solvent mixture. MAE is a process of heating with microwave energy the solvent in contact with a sample to partition compounds of analytical interest from the sample matrix into the solvent. The relatively new technique ASE extracts samples under elevated temperature,

while elevated pressure ensure that volatile extractants remain liquid. This technique can be completely automated and it employs very small extractant volumes, with extraction times of less than 1 h. Soxhlet extraction is a classical technique that ensures intimate contact of the sample matrix with the extraction solvent. The extraction procedure in the Soxhlet apparatus is simple to carry out and allows isolation and concentration of water-insoluble or slightly water soluble organic compounds, but the main drawbacks are the long extraction times, the need of large volumes of extraction solvent and the possibility to obtain a sample extract at a time. UAE is carried out using several devices such as waterbaths, probes and sonoreactors, but, in general, UAE of pesticides in food has been mainly performed in an ultrasonic bath at room temperature without temperature control. UAE is primarily used in the extraction of pesticides from solid samples using appropriate solvents. It has been also applied in the LLE of fungicides and OPP insecticides from beverages (e.g. wine and must). A complete review of the applications of UAE to the determination of contaminants in food and soil samples is presented in [8].

SFE has the advantages of efficiency, selectivity, short extraction time and low solvent volume but is difficult to optimise for different matrices and large amount of unwanted matrix substances are also co-extracted. MSPD is based on the solid phase dispersion of the sample matrix for the subsequent isolation of various analytes. By blending a liquid or solid food sample with irregular shaped particles (silica or polymer-based solid support) with lipid solubilising capacity of a support-bound polymer (octadecylsilyl (C_{18})), a semi-dry material is obtained. In this way, the analytes can be isolated by elution with organic solvents of different elution powers and polarities. This technique is simple, rapid and allows several steps to be performed in the sample preparation simultaneously.

- Clean up of the extract: extraction yields not only the target analytes but also interferents (e.g. sugars, fats and chlorophyll) which may distort the results of the analysis. Extract clean up is essential and should always precede the analysis of the extract. The usual techniques for cleaning up food extracts are: solid-phase extraction (SPE), solid-phase microextraction (SPME), gel-permeation chromatography (GPC), adsorption chromatography, stir-bar sorption extraction (SBSE), matrix solid-phase dispersion extraction (MSPDE).

SPE is the most popular clean up technique. As the sample passes through a column of sorbent, the target analytes are adsorbed on the sorbent particles. The compounds retained are than liberated with a solvent and analysed. Sorbents used for SPE include C18, polymers, graphitized non-porous carbon and ion-exchangers.

In SPME, analytes are adsorbed on a fiber coated with a suitable solid phase that is pushed out from a micro-syringe. The analyte is then thermally desorbed and transferred to the injector of a gas-chromatograph. Depending on where the fibre is placed in relation to the sample, SPME can divided into "direct immersion" (DI-SPME) and "headspace" (HS-SPME). Many attempts had been conducted to combine extraction, enrichment and sample introduction into one single step by using SPME and moderate success was achieved in the application to the analysis of pesticides on certain food matrices. Examples of applications are reviewed in [9-11]. Extraction efficiencies using SPME for pesticides determination were found to drop drastically when the lipids content of the samples was increased and this demonstrated that the matrix and, in particular, the lipids content of the sample extracts would have significant effect on the adsorption dynamics of the OCPs to the SPME fiber.

New techniques for the analysis of pesticide residues are defined with the acronym Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) which is a combination of liquid-liquid extraction (LLE) with SPE. The consumption of sample and toxic solvents with the QuEChERS method is minimal. By applying QuEChERS to the determination of pesticides in fruit and vegetables, matrix effects are eliminated and high recoveries of target analytes are possible. The method can be modified depending on the type of sample and the target analytes.

A review of different clean-up techniques for the purification of samples containing OCPs is given in [7]. In particular, for complex food matrices, the first clean up that is needed is the removal of lipids. Indeed, the fatty substances are highly soluble in organic solvents and tend to absorb in the GC system, resulting in poor chromatographic performance.

- Identification and determination of the analytes: the last stage is the identification of compounds and their quantitative determination, using appropriate instrumentation. The choice of the final technique depends, above all, on the properties of the analytes. Pesticides cannot be treated as a homogeneous group of specific environmental contaminants, because they differ in physicochemical

properties. The main detection techniques include: capillary gas-chromatography (GC) and high-performance liquid chromatography (HPLC), for pesticides that are unsuitable for determination by GC. Pesticides to be determines by GC should be volatile and thermally stable. The choice of the chromatographic column is extremely important for separating analytes and for their qualitative and quantitative determination. The column should be highly efficient and resistant to changes in the parameters of the separation process. The stationary phase should be thermally stable and selective to the constituents of the mixture being analysed.

The multi-residue determination of pesticides in food matrices (particularly vegetables and fruit) is generally carried out by gas chromatography coupled with mass spectrometry (GC-MS), as it assures efficient chromatographic separation, sensitivity and confirmation power based on electron-impact ionization (EI) mass spectra. Liquid chromatography coupled with mass spectrometry (LC-MS) also allows rapid determination of many compounds determined with difficulty by GC or conventional LC procedures, such as polar, non-volatile and/or thermally labile pesticides (e.g. chlordecone). Generally, OCPs are non-polar compounds, and are not ionised efficiently with atmospheric chemical (APCI), or electrospray ionisation (ESI) mode of LC-MS. The development of atmospheric pressure photoionisation (APPI) technology has expanded the range of compounds detected by LC-MS, including non-polar compounds.

Improved peak resolution and smaller influence of the matrix on the final result can be achieved with two-dimensional GC (GCxGC). This system uses two orthogonal capillary columns with different retention mechanisms and the advantage is that the separation mechanisms are independent from each other, so that constituents that were co-eluted from the first column can be separated by means of the second one. GCxGC is widely used because of its high resolving power, greater sensitivity and chromatograms showing much sharper peaks, with smaller width and higher peak intensity. The enhancement in sensitivity is of around one order in terms of peak height. GCxGC coupled to time-of-flight mass spectrometry (TOF-MS) or other detectors has been applied to pesticide residue analysis in food.

Fast GC is another technique frequently used to shorten the time of analysis and to obtain better peak resolution. It requires short capillary columns, compared to classical GC, with smaller diameters and thinner solid phase films, as well as, faster flow rate and high pressure of the carrier gas.

Finally, typical detection techniques for pesticides determination are: mass spectrometry (MS), suitable for the determination of pesticides of various classes; electron-capture detector (ECD), highly sensitive for compounds containing electronegative atoms and generally used for the quantification of OCPs; flame-photometric detector (FPD), applied in the determination of OPPs; nitrogen-phosphorus detector (NPD), for the simultaneous determination of ONPs and OPPs; thermionic specific detector (TSD), for the determination of compounds containing nitrogen or phosphorus.

Tandem mass spectrometry (MS^2) is a detection technique which combines two analysers and improves sensitivity and selectivity of analytical methods. Ions are separated in the first analyser and then are fragmented and analysed in the second one. With this kind of technique, the chromatogram background is reduced, the signal value enhanced with respect to noise and the LOD of the target analytes is lowered.

4.2.3 Endosulfan: properties, behaviour and fate in the environment

Endosulfan is an OCP that has wide spread use in many parts of the world, including the European Union, India, Indonesia, United States, Mexico and Central America, Brazil and China. It has been in use for almost 5 decades and is effective against a broad number of insect pests and mites. This pesticide is applied to a wide number of crop types, including cotton, cereals, fruit trees and plantation crops (such as tea and coffee). Due to its semi-volatility and relative persistence, endosulfan is a ubiquitous environmental contaminant occurring in many environmental compartments. Concentrations of endosulfan in air, soil, water and vegetation have been reported in a wide number of different environments. For example, endosulfan is one of the most commonly detected pesticides in surface waters of the United States and is one of the most abundant OCPs in air. Concerns arises due to the ubiquitous occurrence of endosulfan, and the physical-chemical properties, which are analogous to those of the "legacy" OCPs now included in the Stockholm Convention on Persistent Organic Pollutants (http://chm.pops.int/). Endosulfan is present in remote locations and has a propensity to undergo long range transport; indeed is routinely detected in arctic air, being one of the most abundant pesticides in this environment. The chemical

and its major degradate, endosulfan sulphate, undergo uptake in the biota and there is evidence for bioconcentration/bioaccumulation in Arctic marine foodwebs.

Technical grade endosulfan is commercially available as a mixture typically containing >95% of the two diasteroisomers, known as α-endosulfan (or I) and β-endosulfan (or II) in ratios from 2:1 to 7:3, depending on the technical mixture. Since its introduction as a broad spectrum insecticide in 1954 by Fabwerke Hoechst, Germany, endosulfan has become an important agrochemical and pest control agent resulting in its global use to control a range of insect pests for a number of diverse applications. Common examples of endosulfan use include the control of tsetse fly in tropical countries and the use as agent for wood treatment.

The annual global production volume of endosulfan has been calculated by Li and Macdonald (2005) to be 12800 t, with India estimated to be the largest producer with six plants producing about 5400 t/y, with a total use of 113000 t from 1958 to 2000, followed by United States with 26000 t from 1954 to 2000. In China, annual use is estimated to average 2800 t/y during the period 1998 to 2004. The cumulative global use of endosulfan in agriculture was estimated to be 308000 t (1950 to 2000). While endosulfan use appears to have declined in the northern hemisphere over this period, use in the southern hemisphere has increased (e.g. South America, Australia) maintaining an annual average global use of 12450 t over the period 2000 to 2004 [12].

The isomers of endosulfan, shown in figure 4.1, are semi-volatile, with similar vapour pressures to other chlorinated pesticides, making them susceptible to volatilisation to the atmosphere with subsequent atmospheric transport and deposition.

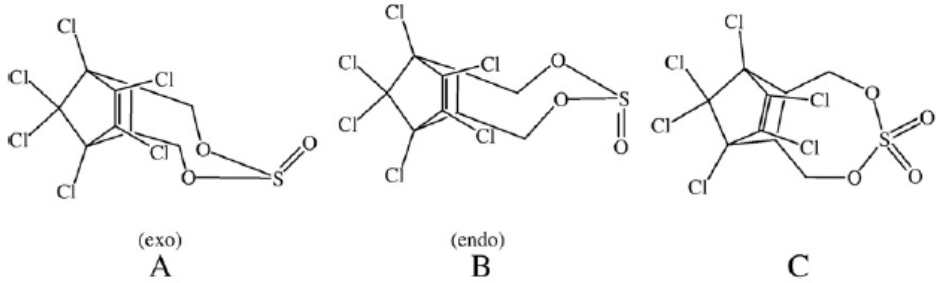

Figure 4.1: chemical structures of (A): α-endosulfan (or I), (B): β-endosulfan (or II), and (C): endosulfan sulphate [12].

The vapour pressures of the α- and β-isomers are similar, while endosulfan sulphate is almost 4-fold lower. The aqueous solubility of the β-isomer is markedly higher than the α-isomer (~10-fold) and as a result the β-isomer has a lower Henry's law constant (*H*) and

will therefore partition to aqueous phases more readily. Both β-isomer and the sulphate show relatively higher vapour scavenging from the atmosphere by precipitation than the α-isomer and are more susceptible to vapour dissolution to surface waters (e.g. marine surface waters) during long range transport. In general, physical-chemical property data for endosulfan sulphate are lacking or have a high degree of uncertainty.

One of the criteria for designation of a chemical as a POP is that it has a logK_{ow}[1]>5 [12]. Both endosulfan isomers do not exceed this value, but are close to it, suggesting a potential for bioaccumulation. In addition, the relatively high K_{ow} values indicate a propensity for partitioning to the organic carbon fraction in soils and sediments for both endosulfan isomers and the sulphate. In the atmosphere, endosulfan is found predominantly (>95%) in the gas phase; once in the atmosphere, endosulfan is subject to atmospheric transport and deposition. In addition, it is relatively stable in the atmosphere, with wet and dry deposition playing an important role in its removal. Vapour dissolution and wet deposition are likely to be significant sources of endosulfan to large, fresh water lakes and ocean surfaces.

Significant conversion of the β-isomer to the α-isomer has been reported from a number of studies and the physical basis for irreversible conversion of the β- to the α-isomer has been established, whereby physical-state transitions, such as volatilisation which causes asymmetry in the β-isomer, increasing the potential for transformation to the α-isomer. Isomeric conversion (β- to α-) has been demonstrated to occur at the solid-water interface as well as at the air-water interface. The average ration of environmental (air, water, soil, sediment, and vegetation) α- and β-endosulfan is 2.4.

Endosulfan is subjected to both biotic and abiotic degradation in the environment that may result in oxidation to the corresponding sulphate or hydrolysis in acquatic systems to endosulfan diol. The diol may in turn degradate further to endosulfan ether, endosulfan α-hydroxyether or endosulfan lactone. The degradation for the isomers is shown schematically in figure 4.2.

[1] K_{ow} is the octanol-water partition coefficient.

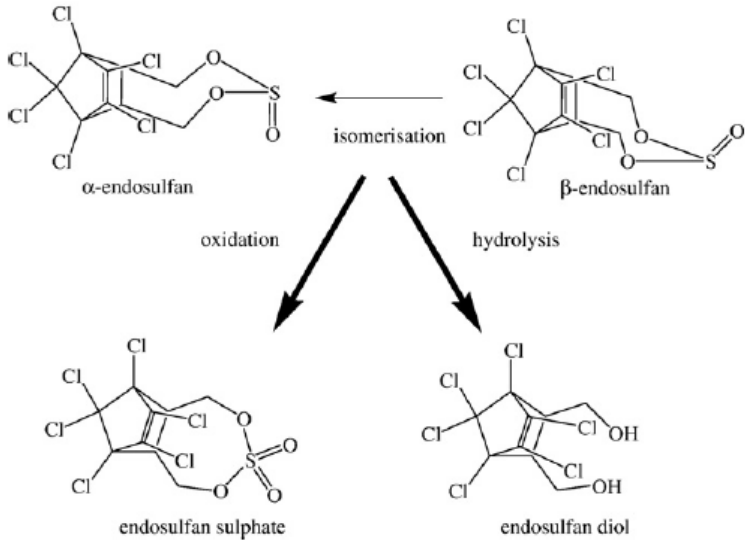

Figure 4.2: Transformation pathways of α- and β-endosulfan in the environment [12].

Both endosulfan isomers were found to degradate twice as quickly in non-sterile sediments compared to sterile conditions indicating the importance of biotic degradation with endosulfan sulphate being the only detectable metabolite. Degradation rates strongly depend on the soil conditions (particularly soil water content and ambient temperature). Endosulfan sulphate has been identified as the main metabolite of endosulfan degradation in soil and sediments and has been observed on plant surfaces. Endosulfan sulphate degradates at a slower rate and hence is more persistent than the parent isomers, although it has been observed to have lower aquatic toxicity on select aquatic biota. However, a combination of the α- and β-isomers and endosulfan sulphate appeared to be more potent than any single endosulfan isomer.

Hydrolysis is the dominating abiotic degradation process, resulting in the formation of endosulfan diol; a positive correlation between hydrolysis rate and pH has been found, being base-driven hydrolysis a predominant degradation process in slightly alkaline waters. Aqueous endosulfan is stable compared to α- and β-isomers. It was also reported that direct photolysis is of little importance on the environmental fate of aqueous endosulfan as photolysis with environmentally relevant UV-A light had no statistically significant effect on endosulfan degradation compared to experiments performed in the dark. However these studies, conducted under controlled conditions, do not necessarily mirror environmental conditions as the persistence of both isomers increases in the presence of humic acids and other dissolved constituents.

For biotic (microbial) degradation, it was found that extensive degradation of endosulfan (>85%) by microbial populations under anaerobic conditions; α-endosulfan has a high

potential of biodegradation in low-oxygen containing environments, while in a natural aerobic aquatic environment, endosulfan sulphate is likely to represent the predominant residue of technical grade endosulfan over time. Aquatic half-lives ($t_{1/2}$) have been reported to be 23-27 h and 22-27 h for α- and β-isomer respectively, depending on the initial nominal concentration. These values are much lower than the persistence criteria designated for a POP [13] (i.e. aqueous $t_{1/2}$>2 months), although in the colder marine waters at high latitudes (i.e. Arctic Ocean) base-driven hydrolysis half-lives for endosulfan are likely to be greatly extended (e.g. several months), compared to warmer waters in temperate or tropical regions (e.g. hours to days).

Endosulfan is ubiquitous and has been detected in a variety of environmental media across the globe, with the abundance of reported data on the order of α- >β- >sulphate. Reviewing the global occurrence of endosulfan, data are categorised according to the following: source, where either production or direct application occurs; regional, with short to medium range transport as a transfer pathway; and remote, areas such as the Polar Regions requiring long-range transport processes.

Endosulfan has been shown to exhibit widespread distribution in vegetation, displaying relatively high concentrations in tree bark samples collected from a large number of countries, with concentrations akin to hexachlorocyclohexanes (HCHs) and *p,p'*-DDE.

Endosulfan is susceptible to long-range atmospheric transport with detectable quantities (similar to long-range atmospheric transport with detectable quantities (similar to other OCPs) in air and water in remote regions. For example, endosulfan has been reported in remote mountainous regions and in the Arctic. The Arctic has been subjected to numerous studies on persistent organic pollutants and can be regarded as a "sentinel" region with which to assess the persistence of chemical contaminants and their ability to undergo long-range transport. Furthermore, where systematic measurements have been conducted, datasets can be used to assess baseline trends of key contaminants and examine bioaccumulation in remote foodwebs.

Finally, endosulfan concentrations in biota and freshwater sediments were reported to be lower than those of the DDTs and PCBs, with concentrations in biota found in the range of 10-1000 ng/g_{lipid} for "background" locations, with higher concentrations in selected biota close to urbanised areas of Australia, South Africa and South America [12].

4.3 Melamine adulteration of food

4.3.1 Introduction

Food adulteration is recognised as a worldwide phenomenon with high chance of adverse biological consequences. Contaminated foodstuff is recognised as a cause of serious health hazard, even in recent times. Food adulteration is not unique to food for human consumption; animal (pet) foods have been contaminated with unwanted substances. With the wide spread growth of food adulteration, food safety has emerged as an important concept.

In 2007 and 2008, illegal adulteration of pet food, livestock food, fish feeds, and raw milk used for infant formula to falsely boost the apparent protein levels with either melamine alone or "scrap" melamine containing cyanuric acid resulted in illness and death in infants and companion animals due to the nephrotoxicity associated with the accumulation of melamine-uric acid or melamine-cyanuric acid crystals in the kidneys.

Melamine induced nephrotoxicity is emerging as a global epidemic and the unique reason for this can be linked to melamine adulteration of foodstuffs for profit-earning reasons. Melamine is added to foodstuff, including milk, due to its high nitrogen content, and because it produces false-positive results for proteins when estimated by the Kjeldhal or Dumas methods. Both methods are widely used to examine the protein content of milk, and rely on the liberation of ammonia from the proteins by concentrated sulphuric acid or heat treatment, respectively. Because melamine contains a high percentage of non-protein nitrogen, it causes a false-positive result, particularly when proteins are measured by ammonia-liberating assay, making melamine an attractive milk adulterant.

Melamine adulteration of food has been a well-known phenomenon worldwide over the past thirty years, especially after the recent melamine scandal reported in China in 2008. However, melamine adulteration of milk is still reported from other parts of the world [14].

Melamine (2,4,6-triamino-1,3,5-triazine) is an organic compound that is commercially synthesized from urea and is produced in large amounts mainly for the use in the synthesis of melamine-formaldehyde resins for the manufacture of laminates, plastics, coatings, commercial filters, glues and adhesives, dishware and kitchenware. The analogues of melamine (cyanuric acid, ammeline and ammelide) can also be produced as impurities during the manufacturing process of melamine (Fig. 4.3).

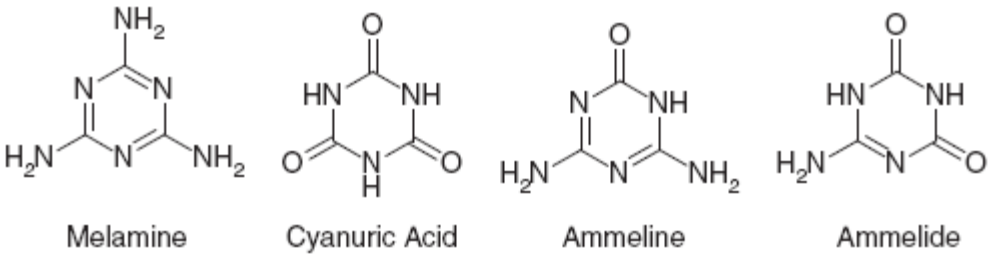

Figure 4.3: structures of melamine and its analogues [17].

In addition, the bacterial metabolism of melamine may contribute to the production of these analogues, if the melamine is not completely metabolised to ammonia and carbon dioxide.

In 2008, the World Health Organization (WHO) reported on the toxicity, preliminary risk assessment and guidance on levels of melamine and its analogue cyanuric acid in food. The Codex Alimentarius Commission (CAC) has promulgated the maximum limits of 1 mg/kg in powdered infant formula and 2.5 mg/kg in other food and feed in 2010 and 0.15 mg/kg for melamine in liquid infant formula finally adopted in 2012.

In general, the limits of 1 mg/kg and 2.5 mg/kg of melamine in food have been considered suitable by many countries for judging unacceptable adulteration. The WHO Expert Meeting concluded that these limits suggest a sufficient margin of safety from any dietary exposure to melamine which could produce a health risk.

Intensified food safety concern over melamine in infant formula in 2008 has prompted national authorities and countries to assess its Tolerable Daily Intake (TDI) for protection of general population including young children. The dietary exposure to melamine and the risk from melamine-tainted infant formula have also been assessed.

The TDI is defined as "the estimated maximum amount of an agent to which individuals in the population may be exposed daily over their lifetimes without appreciable health risk" (WHO, 2004). The estimation of a TDI for melamine was initially conducted by the United States Food and Drug Administration (US FDA), to respond the critical situation of melamine crisis in contaminated pet food. The FDA rapidly estimated a TDI value of 0.63 mg/kg bw[2]/day for melamine using the data from a selected animal toxicity assay performed by the National Toxicology Program (NTP) of the US Department of Health and Human Services (NTP, 1983). The TDI value originally set by the FDA was based on the results of a 13-week rat study, and includes a 100-fold safety factor (SF). Other national food safety authorities have acknowledged the TDI value originally set by the

[2] body weight

FDA, for examples WHO set a TDI at 0.5 mg/kg bw/day. Considering the increased toxicity that results from combined exposure to melamine and cyanuric acid, the FDA subsequently applied an additional 10-fold SF to give a TDI/10 value of 0.063 mg/kg bw/day, to compensate for these uncertainties.

4.3.2 Metabolism and toxicology of melamine

Melamine and cyanuric acid are quickly adsorbed and excreted in an unmetabolised form in the urine of mono-gastric animals. Some toxicokinetic studies demonstrated melamine was predominantly restricted to blood or extracellular fluid and is not extensively distributed to most organs or tissues. Melamine is primarily eliminated by renal filtration in rats and does not undergo substantial metabolism. Considering the toxicity of cyanuric acid and other melamine analogues, it is important to evaluate the toxicity potential of cyanuric acid and a combination of melamine and cyanuric acid.

Toxicological studies showed that the dietary addition of cyanuric acid and melamine could induce kidney damage, and the effects were harmful when the ratio of cyanuric acid/melamine was 1:3 [15]. The qualitative and quantitative methods for the determination of melamine in kidney stones have been established and optimised after the incident in China in 2008. Using Fourier transform infrared spectroscopy (FTIR), the composition of kidney stones caused by melamine-contaminated formula could be characterised as a mixture of uric acid dehydrate and ammonium acid urate. A further study used HPLC to determine the contents of melamine in urinary stones, scanning electron microscope (SEM) to observe the configuration of stones, and FTIR spectrum and X-ray powder diffraction (XRD) analysis to reveal the chemical components of the obtained stone samples

Also a good understanding of the pharmacokinetic profiles of melamine and cyanuric acid and their combinations is essential to define properly the risk associated with different exposure scenarios. In addition, complex like the melamine-cyanurate complex can significantly alter the toxicokinetics of individual melamine or cyanuric acid, with reduced bioavailability of compounds, delayed peak concentrations, and prolonged elimination half-lives [16].

Melamine is known to stimulate inflammatory response and reactive oxygen species (ROS) production in human embryonic kidney cell line and macrophage like cell line. and this oxidative stress can account for melamine-induced toxicity. Such melamine-induced

toxicity in kidney cells, observed in gut microflora of rats, may work in human or other subhuman species, but concrete evidence of this issue is presently lacking.

Melamine combines with cyanuric acid and uric acid to form crystals that are known to be nephrotoxic. By forming such crystals, melamine ca contribute to the formation of renal stones. Melamine also contributes to chronic kidney inflammation and bladder cancer, causes sperm cell abnormality without any observed evidence of genotoxicity in prokaryotic or eukaryotic cells. In vitro studies have suggested the binding of melamine with DNA by electrostatic interactions and by hydrogen bond formation. Many laboratory animals also exhibit reproductive toxicity due to melamine in a dose-dependent manner that is enhanced in the presence of cyanuric acid. In vitro studies have also shown the potential for transplacental transfer of melamine, and such conclusions are also derived from in vivo studies from in vivo studies in animal model systems. Although melamine toxicity has been reported in humans, its specific dose-dependent reproductive toxicity has not yet been documented in humans. Research is urgently needed in this area because there is a high potential for reproductive toxicity in humans exposed to melamine [14].

Carcinogenic effects observed with melamine are considered to be secondary to irritation caused by stones. However, there are few data on melamine analogues other than cyanuric acid. The WHO International Agency for Research on Cancer (IARC) has concluded that there is sufficient evidence in experimental animals for the carcinogenity of melamine under conditions in which it produces bladder calculi (IARC, 2008). At the moment, melamine is classified as a category III carcinogen by the IARC, due to insufficient animal data and lack of work on the carcinogenesis of melamine in humans. Results of genotoxicity studies showed that melamine has no mutagenic effect on prokaryotes or eukaryotes and does not induce malignant cell transformation after long-term exposure. In conclusion, melamine is not considered to be genotoxic, teratogenic (i.e. it causes developmental damages) or a reproductive toxicant (WHO, 2008) [16].

A detailed review of toxicology for melamine and its structural analogues in laboratory animals, in companion and farm animals and in humans is given in [17], together with an overview of the adulteration incidents and the risk assessment. In these review, a particular focus is given to the recent EFSA risk assessment addressing impacts on animal and human health of background levels of melamine and structural analogues in animal feed. A possible mechanism of the toxicity of melamine is described in figure 4.4 and figure 4.5 [17]:

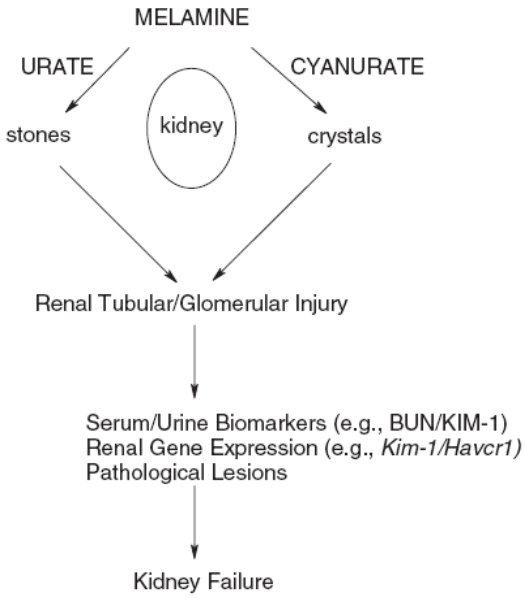

Figure 4.4: Induction of kidney toxicity by melamine through either stone formation with urate or crystal formation with cyanurate and the experimental methodology for its detection [17].

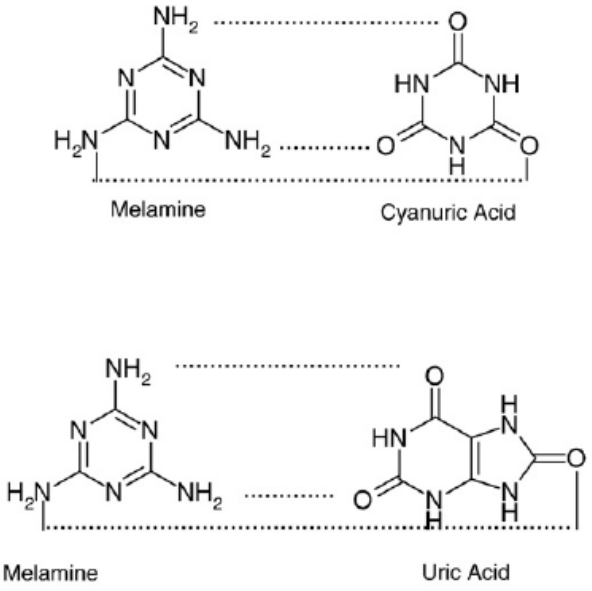

Figure 4.5: hydrogen-bonded complex formation between melamine and either cyanuric acid (MCA) or uric acid [17].

4.3.3 Analytical chemistry of melamine and its analogues

The adulteration of milk and milk products in China has promoted analytical methods validation and sample investigation worldwide. A wide range of fit-for-purpose analytical methods for the quantification of melamine was reported, which mainly included enzyme-

linked immunosorbent assay (ELISA), capillary electrophoresis (CE), high-performance liquid chromatograpy (HPLC) and various mass spectrometry techniques (mainly gas chromatography coupled with mass spectrometry, GC-MS).

Commercial ELISA kits were developed by various manufacturers for the semi-quantitative determination and this technique offers a solution for high throughput screening of samples. ELISA was sensitive for melamine and ammeline, while ammelide and cyanuric acid showed cross-reactivity. Although the sensitivity of ELISA was adequate for high throughput analysis, the main weakness of this assay was the significant cross-reaction demonstrated when interference compounds structurally close to melamine were present in milk samples. Alternatively, some HPLC coupled with ultra violet detection (UV) or HPLC coupled with diode-array detection (DAD) methods have bee validated for the quantification of melamine in infant formula or milk products. Both ELISA and HPLC were evaluated as reliable methods for semi-quantitative determination of melamine in milk products, but both of these techniques are limited in terms of specificity; also, UV and DAD for HPLC separated samples have poor selectivity because many organic compounds absorb in the wavelength range between 200 and 270 nm.

Capillary electrophoresis (CE) has some advantages compared to other chromatographic methods, such as high separation efficiency, high speed, low consumption of solvent and sample. CE has been used as an effective method of analysis of melamine and related compounds, although many disadvantages, i.e. the lack of sensitivity and the low reproducibility, but many options can be found in literature to avoid these problems.

Concerning GC-MS, the US FDA initially developed a screening method in which melamine in sample extracts was derivatized with a chemical agent (i.e. trimethylsilyl). This method (FDA, 2008) has been improved by the application of gas chromathography coupled with tandem mass spectrometry (GC-MS/MS), the limit of detection (LOD) of which could reach 2 μg/kg.

In addition, several LC-MS/MS techniques that employed labelled melamine as the internal standard to improve the precision of the method were developed. Melamine is analysed in positive electrospray ionisation mode, while analogues are analysed in the negative ionisation mode.

Responding to melamine incident of infant formula in China in 2008, some institutions reported the organisation of proficiency test (PT) programs to the testing communities. These PTs shared the common objective of evaluating the capabilities of laboratories and their degree of equivalence in melamine testing.

The Government Laboratory of Hong Kong (GLHK) organised an inter-laboratory comparison program in late September 2008 in which participants were requested to determine three samples with different melamine levels (0.05-4.5 mg/kg) and a blank sample. Afterwards, such a PT program was run at an international level. The European Commission Institute of Reference Material and Measurements (IRMM) in Belgium organised an international scheme on melamine testing in powdered milk in January 2009. The participants comprised 114 laboratories from 31 countries all over the world and 21 Member States of the European Union. Organisation of PT programs provided good evidence to facilitate laboratories accreditation in accordance with ISO/IEC 17025 requirements [16].

Performance and validation studies of different methods were carried in recent years, following the adulteration incidents occurred in China. Different screening and confirmatory methods for the determination of melamine in cow milk and milk-based powder infant formula are described in [18], based on ELISA, HPLC-UV, GC-MS and LC-MS. These methods were tested in the framework of internal and European PTs, coordinated by JRC/IRMM. All these techniques have been predominantly used in PT programs by laboratories worldwide. In [19] the general performance of four techniques (ELISA, GC-MS, LC-MS/MS and HPLC-UV) for melamine analysis in food and feed samples on the basis of the results from two international PT programs organised in 2009 is discussed.

From the melamine crisis of infant formula in 2008, countries all over the world, especially China, learnt a lot about food safety. In the chain of food safety, the role of national authorities is critical to protect the consumer against unsafe food.

The construction of a food safety system not only depends on the responsibility from the national authorities, but also relies on the support from the food manufacturers and consumers [16].

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EXPERIMENTAL SECTION

CHAPTER 5

ORGANOCHLORINE PESTICIDES DETERMINATION IN FOOD MATRICES

5.1 Introduction

INRiM activity concerning the determination of organic micro-pollutants at trace concentrations in various matrices (food, environmental samples), aims at establishing metrological traceability to the International System of Units (SI) and correctly evaluating the measurement uncertainty for the results of these measurements. The possibility of analysing these substances with accurate and metrologically traceable methods is a goal of fundamental importance, both in the food safety and the environmental fields, due to the potential toxic effects that these substances can act on human health and on natural ecosystems.

The activity consists in the development and validation of suitable analytical methods in order to establish correct metrological traceability chains. The development of such traceability chains might be not easy, due to several problems: presence of a great amount of analytes in the samples at various concentrations (even at trace levels), complexity of the environmental samples and matrix effects, lack of primary methods, applicable to routine measurements, which allow the direct reference to a measurement standard. A fundamental aspect regards the evaluation of measurement uncertainty considering all the significant contributions.

The analytes of interest in the PhD research are classified as Persistent Organic Pollutants (POPs) by the United Nation Environment Programme [1], in the framework of the Stockholm Convention.

The classes of compounds considered until now at INRiM are Polychlorobyphenils (PCBs), Polycyclic Aromatic Hydrocarbons (PAHs), some organochlorine pesticides (OCPs) and melamine.

This chapter focuses on the activity which I carried out at INRiM during the PhD on two organochlorine pesticides (namely endosulfan and its metabolite endosulfan sulphate, (see

Chapter 4 for details). This activity consisted in the set up of a metrological procedure for the determination of the pesticides in a matrix of green tea, in order to establish metrological traceability of the measurement results and correctly evaluating the associated measurement uncertainty.

5.2 Analysis of organochlorine pesticides in tea

We started an activity regarding the analysis of some organochlorine pesticides (OCPs) in food matrices, for the participation in an international comparison of measurement in the framework of the *Comité Consultatif pour la quantité de matière* (CCQM), namely the "Pilot Study CCQM-P136 Mid-Polarity Analytes in Food Matrix: Mid-Polarity Pesticides in Tea", which concerned the determination of the mass fractions of two pesticides, β-endosulfan and its metabolite endosulfan sulphate in a food matrix, i.e. green tea powder, between 100 μg/kg to 1000 μg/kg.

Endosulfan is a broad-spectrum insecticide, widely used in agricultural practices, which was banned in 2011 as it is a strong neurotoxic agent, both on insects and on mammals, including humans. In addition endosulfan is an endocrine disruptor (agent that can "mime" the activity of some hormones) and many studies have documented its reproductive and developmental toxicity. Endosulfan can also bioaccumulate in the food chain, displaying high toxicity [2]. All these features supported its inclusion in the POPs classification in 2011.

The CCQM-P136 pilot study (and the parallel Key Comparison K95), co-organised by Government Laboratory of Hong Kong (GLHK - Hong Kong) and National Institute of Metrology (NIM - China), required the development of a procedure which involved extraction, clean-up, analytical separation and selective detection of the analytes in the food matrix.

The CCQM-K95 study provides the means for assessing measurement capabilities of participating NMIs and Designated Institutes (DIs) for measuring analytes in the mass fraction range from 100 to 1000 μg/kg of analytes with molecular mass range 100-600 and intermediate polarity (-log K_{ow} in range -5 to -1) in plant matrices. The comparison was carried out within the scope of the Organic Analysis Working Group (OAWG) of the CCQM. The CCQM Key Comparison-K95 and Pilot Study-P136 were carried out in

parallel, i.e. the same study materials was used in both studies. We decided to take part in the Pilot Study CCQM P-136 as we had never dealt with this kind of analytes before and we had to set up a completely new *ad hoc* analytical procedure.

GLHK and NIM took responsibility for the development and operation of the comparison, including preparation and distribution of samples, initial data analysis and evaluation of results to facilitate OAWG discussions, draft reports, and communications with participants.

The study material was prepared by the coordinating laboratories as follows: about 10 kg of dried green tea leaves was purchased from the local market for the study. The material was confirmed to contain trace levels of β-endosulfan and endosulfan sulphate. The material was powdered, sieved, homogenized and disinfected by γ-irradiation at a dose of about 1 kGy. The homogenized powder was independently dispensed into clean amber glass bottles with screw caps, about 20 g each, which were then sealed in polypropylene bags under vacuum.

About 400 bottles of test samples were prepared and homogeneity study (with a sample size of about 1.0 g) of the testing material was performed at GLHK by Isotope Dilution MS using gas-chromatography coupled with mass spectrometry (GC-MS) in negative chemical ionisation mode (GC-NCI-MS). Besides, stability study of the testing material was conducted using the same method and continued to cover the period of the entire exercise. Random samples were analysed at least in duplicate after sample storage at room temperature (about 20°C) and at elevated temperatures (about 30°C or above) for monitoring the stability of analytes before distribution of samples and after submission of results. Two bottles of sample were sent to the participants, one for the method development and another for the analysis with the preferred method. To avoid variations in results due to varying quantities of moisture in samples, mass fractions were to be reported on a dry mass basis.

5.3 Method development

For the method development, the following steps had to be carried out:

- set up of the analytical procedure for the extraction of the pesticides from the matrix and preparation of the samples for the quantification step (clean-up, concentration); (par. 5.3.1)
- set up of the best analytical conditions for the quantification by means of GS-MS; (par. 5.3.3)
- evaluation of measurement uncertainty.

The last step, concerning measurement uncertainty, requires additional explanation and is discussed in details in par. 5.4.

5.3.1 Set up of the analytical procedure

The extraction of the analytes from the green tea matrix was carried out by means of Soxhlet extraction, using two devices in parallel in order two prepare to samples at the same time. The extractors used at INRiM are made of pyrex glass, have an internal diameter of 30 ml, are equipped with 100 ml glass round bottomed flasks, in which the extracted samples are collected. Extractors with small dimensions like the ones used at INRiM allow reducing the extraction time and the volume of solvent required. For the set up of the extraction procedure the Environmental Protection Agency (EPA) method 3540c guidelines [3] were followed. This method concerns the extraction of non volatile or semi-volatile organic compounds from solid matrices, e.g. soils, sludge and wastes. Method [3] reports as extraction solvents the following reagents:

1. acetone/n-hexane 1:1 v/v, CH_3COCH_3/C_6H_{14}
2. dichlorometane/acetone 1:1 v/v, CH_2Cl_2/CH_3COCH_3
3. dichlorometane, CH_2Cl_2
4. toluene/methanol (10:1 v/v), $C_6H_5CH_3/CH_3OH$

The first two mixtures are recommended for soil or sediment samples and for aqueous wastes. For other types of samples the use of the two latter options is suggested.
For the extraction of the pesticides from a vegetal matrix the choice was the mixture acetone/n-hexane 1:1 v/v, as this is the less toxic choice and the less problematic from the environmental point of view. Although during the past years many new solventless extraction techniques have emerged such as supercritical fluid extraction (SFE), solid-phase micro-extraction (SPME), solid-phase extraction (SPE), and matrix solid-phase dispersion (MSPD), liquid-liquid extraction and solid-liquid extraction using organic

solvents such as ethyl acetate, acetone, dichloromethane, n-hexane, acetonitrile or 2-propanol-petroleum ether are still used [4]. For a detailed description of different extraction techniques see chapter 4.

The solvent used to prepare the extraction mixture are the following:

- n-hexane (Fluka Analytical), for pesticide residue analysis;
- Acetone Chromasolv Plus for HPLC, for pesticide residue analysis (Sigma – Aldrich), purity grade ≥ 99,9%.

The volume of solvent was chosen taking into account the guidelines in [3]; in this method, a volume of about 300 ml is recommended for a round-bottomed flask having a volume of 500 ml and an extractor of internal diameter (ID) 40 mm. A volume of 60 ml was used for the extraction, to keep the ratio given in the EPA method.

Before starting the extractions the duration of the extraction was determined by counting the number of cycles per hour performed by the extractors. A complete cycle of the Soxhlet extractor is completed in 4-5 minutes and the number of cycles per hour is approximately 12-15, as the speed of the cycles increases with the increasing temperature of the solvent. In [3] an extraction time of 16-24 hours is prescribed but it is referred to bigger Soxhlet extractors which use major amounts of solvents and carry out longer cycles. The total number of cycles should be around 96 in order to obtain a quantitative extraction of the analytes from the samples [3]. Thus 8 hours of extraction are needed to guarantee the number of cycles prescribed from EPA, as extractors used at INRiM carry out about 12 cycles per hour. In order to reduce the risks of contamination of the samples and possible "memory effects", the Soxhlet extractors are conditioned by refluxing an aliquot of the solvent mixture for several hours the day before the extraction. In addition, at the end of each extraction, Soxhlets are accurately washed with a detergent and then rinsed with acetone to remove any residues of surfactants. A the end of the extraction, the sample is let to cool to room temperature, and stored at 4 °C overnight. Afterwards, the samples are filtered onto cellulose filters and concentrated by means of a rotary evaporator IKA RV 05 basic equipped with a thermostatic bath HB4 basic IKA-WERKE, until a final volume of about 1 ml. The concentrated extract is subsequently purified by using Florisil SPE cartridges (Supelclean ENVI™ Florisil SPE tubes, 6-ml (1 g), Supelco) to remove any possible interfering species using a mixture of hexane:ethyl acetate (80:20 v/v) for the elution of the purified fraction. The recovered fraction is then concentrated to dryness under a pure nitrogen stream and finally re-dissolved in hexane to a final volume of about

0.4-0.5 ml. The extracts were analysed the day after the preparation procedure, in order to minimise possible degradation phenomena and were stored in the dark at 4 °C. The exact volume of each extract was determined at the end of the concentration step and was done gravimetrically by weighing the mass of the vial before and after the addition of the extract. The mass of each extract, determined by difference, was then converted into a volume by multiplying this value for the density of the extract (which has been proved to be equal to the density of hexane). The developed method is summarised in fig. 5.1:

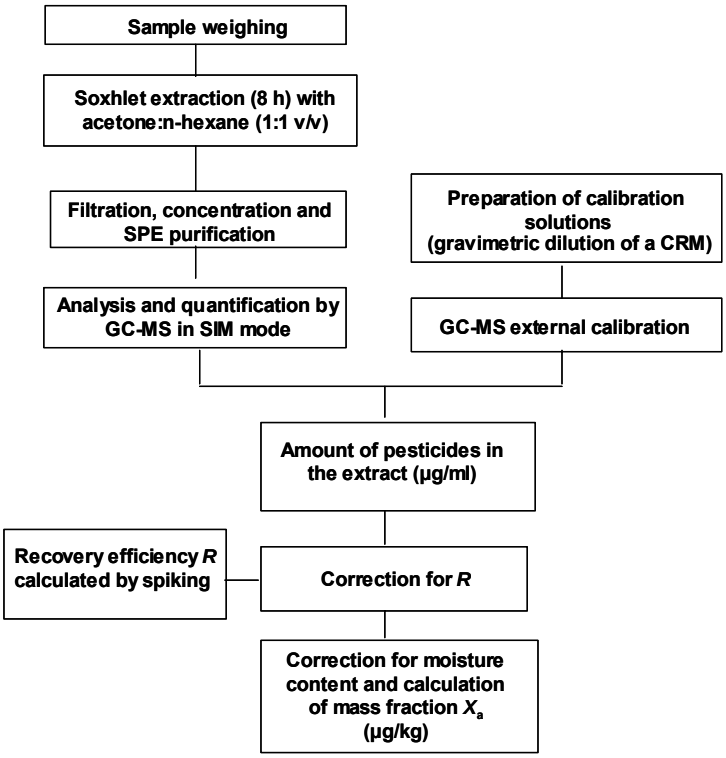

Figure 5.1: Method for the quantification of β-endosulfan and endosulfan sulphate in tea.

5.3.2 Recovery efficiency evaluation

The recovery efficiency *R* of the method was determined preparing some extracts by spiking commercial green tea with known amounts of pesticides in order to obtain mass fractions values of 500 µg/kg and 1000 µg/kg for *β*-endosulfan and endosulfan sulphate. For the evaluation of *R* the NIST SRM 2275 "Chlorinated pesticides solution-II in iso-octane" was used: aliquots of 250 µl and 500 µl of the SRM were added to 1 g of commercial green tea to obtain the two theoretical mass fractions chosen. The samples were extracted by Soxhlet for 8 hours and processed as the unknown samples. Finally the extracts were analysed by using the same calibrated GC-MS used for the samples of the comparison and the concentrations in the final extracts were determined.

The mean values obtained for R were 47% for β-endosulfan and 59% for endosulfan sulphate. In general, the values of R should be comprised between 80-120% and values lower than 70% indicate problems in the sample preparation of the extraction procedure. We checked the different steps of the procedure to exclude losses of the analytes but, as the recoveries were not satisfactory, we investigated the possible reason of these values. The problem could be related to the necessity of soaking the samples before the extraction from the matrix. This particular was given by the organisers of the key comparison after the submission of the results. The recovery R has to be checked by using a CRM (a solution or a matrix CRM) and is expressed by the eq. (5.1):

$$R = \frac{C_{\text{calc}}}{C_{\text{theor}}} \cdot 100 \tag{5.1}$$

where:

R = recovery efficiency expressed in %

C_{calc} = analyte concentration measured in the spiked extract

C_{theor} = theoretical concentration in the spiked sample

5.3.3 Set up of the analytical conditions for GC-MS quantification of the extracts

For the quantification of the pesticides a Thermofisher Scientific single quadrupole GC-MS Focus GC DSQ II was used. Before starting the quantification step, several quantitative analyses were carried out in order to set up the best chromatographic conditions for the identification of the isomers α-endosulfan, β-endosulfan and the endosulfan sulphate in the extracts. Preliminary tests were done using commercial solutions purchased from Dr. Ehrenstorfer GmbH, containing the single pesticides with a nominal concentration of 10 µg/ml in cyclohexane. In figure 5.2 an example of gas-chromatogram of α-endosulfan is shown together with the corresponding mass spectrum.

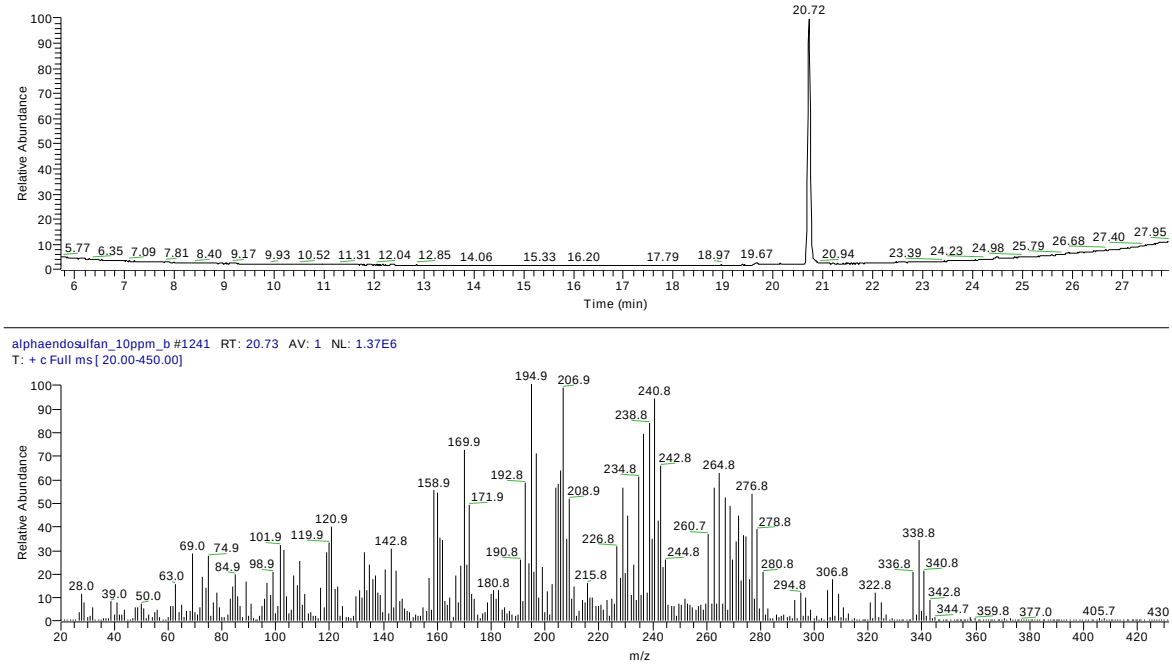

Figure 5.2: example of gas-chromatogram of α-endosulfan with the corresponding mass spectrum.

The gas-chromatographic column Thermo Scientific TR-5ms was used, with the following technical specifications:

- internal film thickness: 0.25 µm
- internal diameter: 0.25 mm
- lenght: 30 m
- stationary phase: (5 % phenyl) - polymethyl – siloxane

Since the calibration solutions were prepared by gravimetric dilution of NIST SRM 2275, which contains several pesticides, preliminary tests were conducted to optimise the analytical separation of the analytes. The analytical method is the following:

- column temperature:	isotherm 160 °C (for 1 min.)
	ramp of 6 °C/min to 320 °C (for 0 min)
- injector temperature:	275 °C
- injection mode:	splitless 1 min
- carrier gas:	helium
- carrier gas rate:	1.2 ml/min
- transfer line temperature:	270 °C
- scan range:	30-430 m/z

- source temperature: 250 °C
- injected volume : 1 µl

The qualitative analyses were carried out in Full Scan mode, while the quantification was done by using the Single Ion Monitoring (SIM) mode, and the selected ions were: 241 m/z and 277 m/z (as confirmation ion) for β-endosulfan, 387 m/z and 422 m/z (as confirmation ion) for endosulfan sulphate. For the quantification of the two pesticides an internal standard (IS) was used to normalize the areas of the chromatographic peaks and to minimise, in this way, the variability of the different chromatographic analyses. Pyrene-d_{10} was used as IS because the perdeuterated compound chosen as IS for the quantification, i.e. β-endosulfan-d_4, showed some instability problems with the native β-endosulfan and could not be used. Pyrene-d_{10} is a Polycyclic Aromatic Hydrocarbon and was contained in the NIST SRM 2270, a certified solution of 5 perdeuterated PAHs in hexane/toluene (96:4 v/v). The mass chosen for pyrene-d_{10} was 212 m/z.

5.3.4 Preparation of the calibration solutions

The quantification of endosulfan and endosulfan sulphate was carried out calibrating the GC-MS with standard solutions with known concentrations prepared by gravimetric dilution of the NIST SRM 2275, in order to have different concentrations of the analytes in the range of interest.

The SRM is a certified solution containing some organochlorine pesticides and it allowed guaranteeing the metrological traceability both for the calibration step and the extraction step (as it was used to determine the recovery efficiency *R*). The solutions were prepared in three subsequent steps, weighing the empty volumetric flasks and after the addition of the aliquots of solution and solvent. The solutions were prepared in n-hexane, as this is the solvent of the final extracts. For the weighing, the balance Mettler H51AR was used with calibrated mass standards from the set of weights Häfner class E2. The weighing of the volumetric flasks war carried out following the scheme:

$$C - I - C{+}m - I{+}m$$

where:

C = calibrated mass standards

I = unknown sample (volumetric flask)

$C + m$ = calibrated mass standards + sensitivity masses

$I + m$ = unknown sample + sensitivity masses

This cycle is repeated four times, for each preparation step of the solutions. The first cycles is generally carried out to warm up the balance and is rejected. The interval between two subsequent weighings is 30 seconds. In addition, it was observed that injecting in the flasks a volume of solvent bigger than the volume of the solution, it was recommended to wait some minutes before weighing the flasks, for the solution to reach the thermal equilibrium. The solution n. 1 was obtained by 1:2 dilution of the SRM and the solution n. 2 by 1:4 dilution of the SRM. In table 5.1 the mass fractions and concentrations of β-endosulfan and endosulfan sulphate for the SRM and for each solutions are summarised.

	Mass fraction (μg/g)	**Expanded uncertainty *U*** (μg/g)	**Amount of substance concentration** (μg/ml)	**Expanded uncertainty *U*** (μg/ml)
NIST SRM 2275 (parent solution)				
β-endosulfan	2,943	0,069	2,031	0,048
Endosulfan sulphate	2,926	0,087	2,019	0,060
SOLUTION N. 1				
β-endosulfan	1,519	0,018	1,025	0,012
Endosulfan sulphate	1,510	0,022	1,019	0,015
SOLUTION N. 2				
β-endosulfan	0,762	0,009	0,509	0,006
Endosulfan sulphate	0,758	0,011	0,506	0,008

Table 5.1: mass fractions and concentrations of endosulfan and endosulphan sulphate in the NIST SRM 2275 and in each calibration solution.

The calibration solutions were analysed in increasing order of concentration, injecting each solution for three times, in order to take into account the instrumental repeatability, according to the following scheme:

- solution n. 2 x 3 times
- solution n. 1 x 3 times
- unknown extract x 3 times
- SRM 2275 x 3 times

A known volume of the IS was added both to the calibration solutions and to the extracts to be quantified, in order to obtain a constant concentration of IS in each solution to be injected in the GC-MS. Aliquots of 1 µl of the SRM solution 2270 were added to 100 µl of each standard solutions and of the samples quantified, at the beginning of each measurement series.

In figure 5.3 the gas-chromatogram of the NIST SRM 2275 is shown, while in figure 5.4 the gas-chromatogram of an extract (extract n. 2) is reported, with the mass chromatograms for β-endosulfan (retention time t_r = 18.87 min - brown line) and for endosulfan sulphate (retention time t_r = 20.11 min - green line).

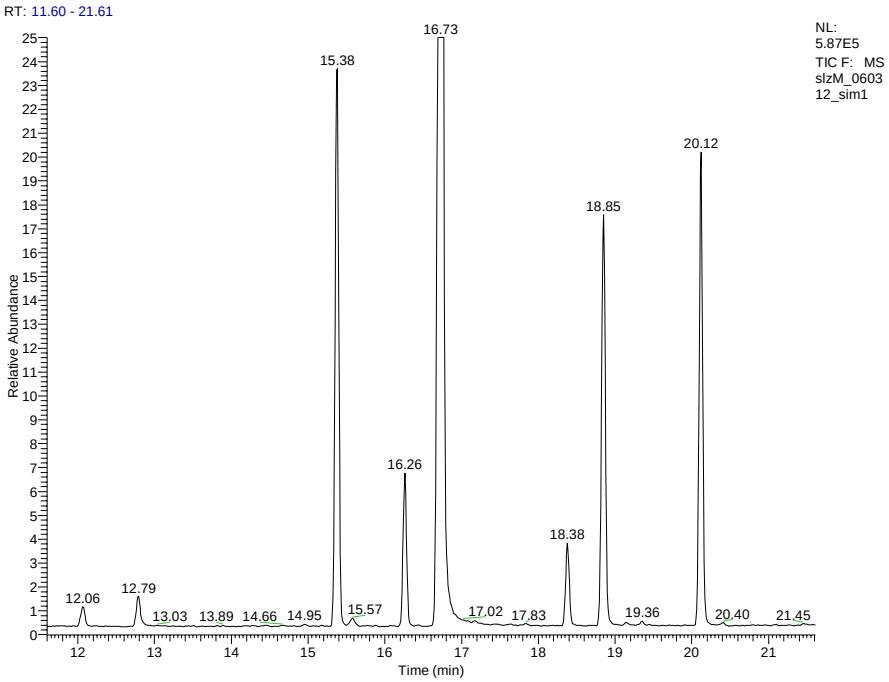

Figure 5.3: gas-chromatogram of NIST SRM 2275 (sim masses 212-241-277-387-422 m/z). β-endosulfan is visible at 18.85 min, endosulfan sulphate at 20.12 min, while pyrene-d_{10} at 16.73 min)

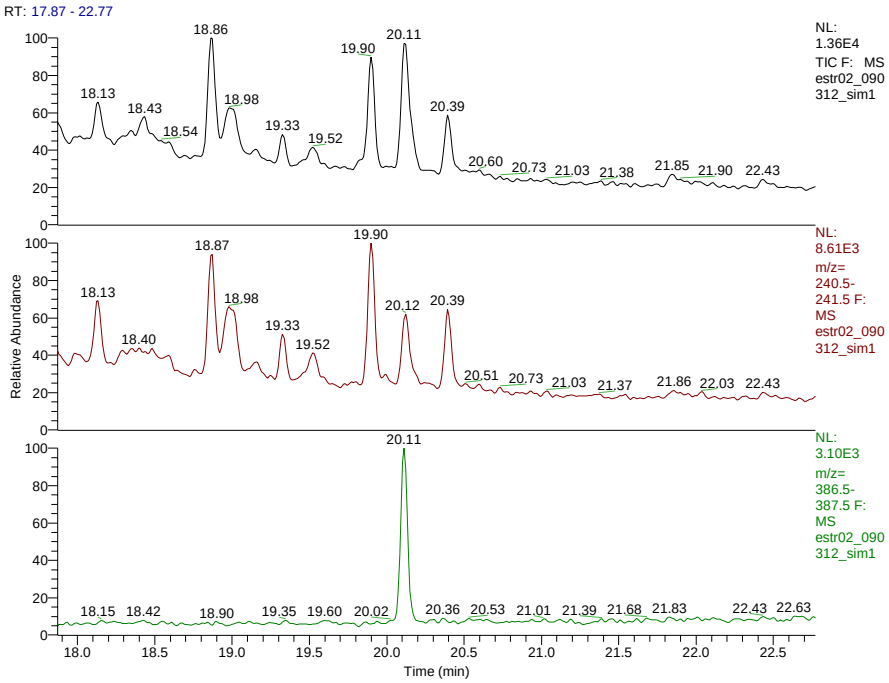

Figure 5.4: gas-chromatogram of extract n. 2, with the mass chromatogram of β-endosulfan (t_r: 18.87 min - brown line) and endosulfan sulphate (tr: 20.11 min - green line)

5.4 Uncertainty evaluation

The evaluation of measurement uncertainty was carried out starting from the model equation (eq. 5.2), taking into account all the significant sources for each input quantity. The major contributions to the combined standard uncertainty $u_c(\chi_a)$ are related to the analyte concentration determined by GC-MS $u(C_a)$ and the recovery efficiency $u(R)$.

5.4.1 Model equation

The model equation defined to determine the mass fraction of β-endosulfan and endosulfan sulphate is the following one:

$$\chi_a = \frac{C_a \cdot V_E}{R \cdot m_h \cdot f_h} \tag{5.2}$$

where:

χ_a: mass fraction of each analyte in µg/kg

C_a: is the analyte concentration in µg/ml determined in the sample extract by GC-MS quantification

V_e: is the final volume of the extract in ml, determined by weighing

R: is the recovery factor

m_h: is the weighed mass of the sample in kg

f_h: is the correction factor for moisture content

For the uncertainty evaluation, two approaches were followed and the results obtained were compared. The first was the classical GUM approach in which the law of propagation of uncertainty was applied [7]. In the second approach, the Monte Carlo method was used to evaluate the measurement uncertainty, as described in Supplement 1 to the GUM [8].

For the evaluation of measurement uncertainty, as the model equation comprises only quotients or products of quantities, e.g. $y = (p \times q \times r \times ...)$ or $y = p/(q \times r \times ...)$, the combined standard uncertainty $u_c(y)$ can be simplified to the following equation [7,9]:

$$u_c(y) = y\sqrt{\left(\frac{u(p)}{p}\right)^2 + \left(\frac{u(q)}{q}\right)^2 + ...} \tag{5.3}$$

where $u(p)/p$ e $u(q)/q$ are the relative standard uncertainties of the parameters of the model equation, expressed as relative standard deviations. The final expression of $u_c(\chi_a)$ is the following:

$$u_c(\chi_a) = \chi_a \cdot \sqrt{\frac{u^2(C_a)}{C_a^{\,2}} + \frac{u^2(V_E)}{V_E^{\,2}} + \frac{u^2(R)}{R^2} + \frac{u^2(m_h)}{m_h^{\,2}} + \frac{u^2(f_h)}{f_h^{\,2}} + 2 \cdot \frac{u(C_a, R)}{C_a \cdot R}} \tag{5.4}$$

The last term under the square root represents the covariance between quantities C_a and R, as the term C_{calc} (which derives from the definition of R in eq. 5.1) is determined by using the same procedure as C_a.

In fig. 5.5, a cause-effect diagram (fishbone diagram) summarises the uncertainty contributions of the input quantities to the combined standard uncertainty of χ_a.

The evaluation of the uncertainty of each input quantity is described in details in the following paragraphs.

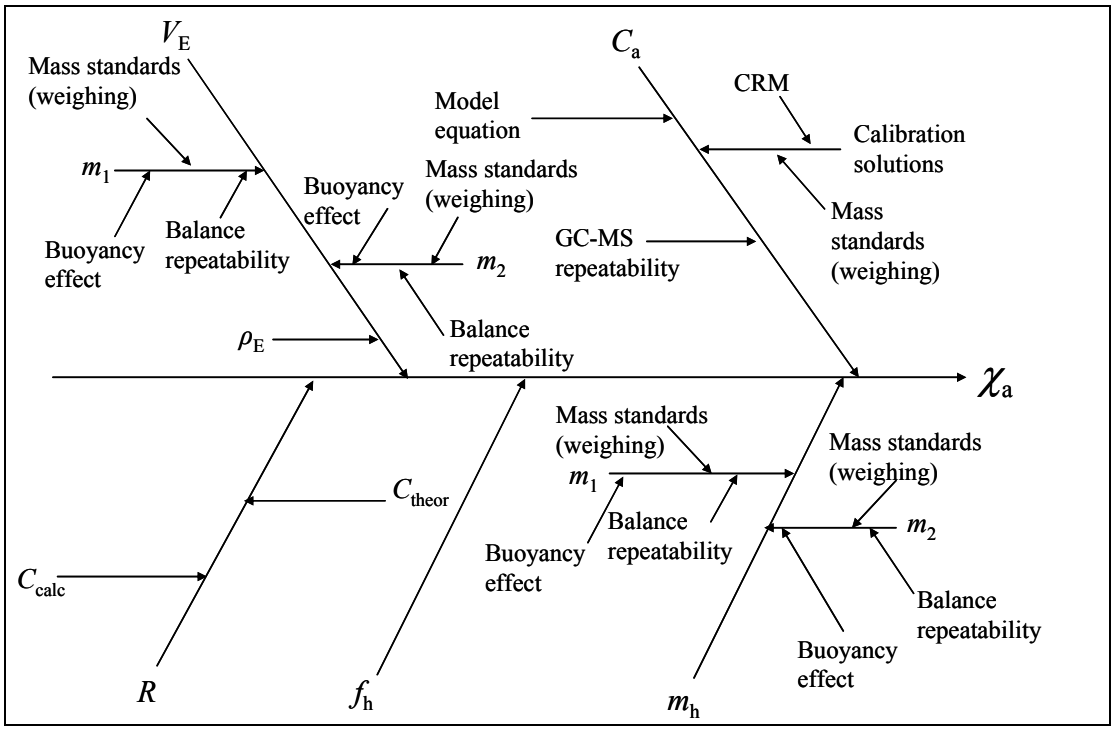

Figure 5.5: cause-effect diagram of the uncertainty contributions of χ_a.

5.4.2 Evaluation of $u(C_a)$

The calibration curves for the determination of c_a were obtained by means of an algorithm developed at INRiM, based on the Weighted Least Squares method [5,6], which calculates a linear correction to be applied to the instrument readings according to the following equation:

$$x = y + d(y) = y + \alpha_0 + \alpha_1 \cdot y \tag{5.5}$$

where:

x = concentration of the analyte in the standard solutions

y = instrument output (normalised areas of the chromatographic peaks)

$d(y)$ = correction to be applied

This calibration procedure allows building a linear model which takes into account the adherence of the mathematical model to the experimental data. This model [5,6] can introduce a contribute to the uncertainty of the final concentration.

The measurands are the polynomial coefficients α_0 and α_1 which are ordered in vector $\boldsymbol{\alpha}$:

$$\boldsymbol{\alpha} = \begin{pmatrix} \alpha_0 \\ \alpha_1 \end{pmatrix} \tag{5.6}$$

The estimation algorithm takes care of different sources of uncertainty: the standard solutions uncertainty, the repeatability of the instrument, the lack of fit, the instrument resolution. Being the standard solutions prepared from the same SRM, a correlation coefficient of 0.9 was adopted in the calculation. The calibration solutions and the sample extracts were analysed in triplicate by GC-MS to take into account instrument repeatability.

After the calibration parameters α_0 and α_1 being known, if a set of n_r instrument readings, arranged in a vector $\boldsymbol{r}$, are to be corrected by the calibration algorithm, the matrix $\boldsymbol{R}$ can be defined, whose columns are the first two powers of $\boldsymbol{r}$:

$$\boldsymbol{R} = (\boldsymbol{r}^0\ \boldsymbol{r}) \tag{5.7}$$

which can be also written as:

$$\boldsymbol{R} = \begin{pmatrix} 1 & r_1 \\ 1 & \vdots \\ 1 & r_n \end{pmatrix} \tag{5.8}$$

The correction vector $\boldsymbol{d}(\boldsymbol{r})$ can be computed from eq. 5.9, where $\boldsymbol{\alpha}$ is the vector of the coefficients in 5.6:

$$\boldsymbol{d}(\boldsymbol{r}) = \boldsymbol{R}\,\boldsymbol{\alpha} \tag{5.9}$$

with:

$$\boldsymbol{d}(\boldsymbol{r}) = \begin{pmatrix} d_1 \\ \vdots \\ d_n \end{pmatrix} \tag{5.10}$$

The algorithm gives the combined standard uncertainty for the coefficients α_i, $u_c(\alpha_i)$, from which the value of the expanded uncertainty can be calculated. The confidence level chosen is about 95%, for a coverage factor $k = 2$.

The vector $\boldsymbol{d}(\boldsymbol{r})$ in eq. (5.9) gives the corrections for each reading r_i. The corrected readings indicated with $\boldsymbol{q}$, are:

$$\boldsymbol{q} = \boldsymbol{d}(\boldsymbol{r}) + \boldsymbol{r} \tag{5.11}$$

The covariance matrix of the readings is expressed as:

$$\boldsymbol{\Psi}_r = s^2 \boldsymbol{I} \tag{5.12}$$

where s is the repeatability standard uncertainty of the instrument and $\boldsymbol{I}$ an identity matrix of dimension (n_r x n_r).

The covariance matrix of the corrections d, $\boldsymbol{\psi}_d$, can be estimated starting from the law of propagation of uncertainty:

$$\boldsymbol{\psi}_d = \nabla_\alpha(\boldsymbol{d})\boldsymbol{\psi}_\alpha\nabla_\alpha(\boldsymbol{d})^{\mathrm{T}} + \nabla_r(\boldsymbol{d})\boldsymbol{\psi}_r\nabla_r(\boldsymbol{d})^{\mathrm{T}} \tag{5.13}$$

where the symbol $\nabla_z(\boldsymbol{w})$ means the Jacobian matrix, i.e. the matrix derivative, of the vector $\boldsymbol{w}$ with respect to the vector $\boldsymbol{z}$ and $\boldsymbol{\psi}_\alpha$ is the variance-covariance matrix of the coefficients α_0 and α_1.

The algorithm uses an iterative process for the calculation of the variance-covariance matrix of the calibration data, $\boldsymbol{\Psi}_d$, starting from a covariance matrix in which the contribution of the calibration model is not considered, with respect to the contributions given by the calibration solutions and the instrumental repeatability. Indeed, from

$$u^2{}_c(q) = u^2(d(r)) + u^2(r) \tag{5.14}$$

it follows that the combined standard uncertainty of a result derives from a term due to the correction obtained by the calibration curve and from a term due to instrument repeatability:

For the definition of $u(C_a)$, a fundamental contribution derives from the uncertainty of the concentrations of the calibration $u(C_{\mathrm{fin}})$, gravimetrically prepared and used for the quantification of the pesticides in the extracts. The concentrations of each pesticide in the calibration solutions were calculated from the equation:

$$C_{\mathrm{fin}} = C_{\mathrm{in}} \cdot \frac{m_2 - m_1}{m_3 - m_1} \tag{5.15}$$

where:

C_{fin} = pesticide concentration in the final solution (μg/g)

C_{in} = pesticide concentration in the solution to be diluted (μg/g)

m_i = mass (g) of the pesticide determined in each of the three weighing steps (1: tare, 2: tare+ solution to be diluted; 3: tare + solution + solvent).

The uncertainty budget for the pesticides in the calibration solutions was developed taking into account all the possible uncertainty sources, coming from the CRM concentration used

for the preparation of the solutions and from the weighing process (calibrated mass standards, balance repeatability, buoyancy effect).

In addition the possible covariances between the input quantities were taken into account, i.e. the covariances between the mass values m_1, m_2 and m_3. The various contributions were combined by applying the law of propagation of uncertainty [7]:

$$u^2(C_{\text{fin}}) = \left(\frac{\partial C_{\text{fin}}}{\partial C_{\text{in}}}\right)^2 u^2(C_{\text{in}}) + \left(\frac{\partial C_{\text{fin}}}{\partial m_1}\right)^2 u^2(m_1) + \left(\frac{\partial C_{\text{fin}}}{\partial m_2}\right)^2 u^2(m_2) + \\ + \left(\frac{\partial C_{\text{fin}}}{\partial m_3}\right)^2 u^2(m_3) + 2\sum_{i=1}^{2}\sum_{j=2}^{3}\frac{\partial C_{\text{fin}}}{\partial m_i}\frac{\partial C_{\text{fin}}}{\partial m_j}\text{cov}(m_i, m_j) \tag{5.16}$$

As an example, the uncertainty budget of β-endosulfan in the calibration solution n. 1 is reported in table 5.2. The main contribution to the uncertainty of C_{fin} derives from the CRM, while the contributions of the weighings have minor relevance.

Uncertainty component $u(x_i)$	**Uncertainty source**	**Standard uncertainty, $u(x_i)$**	**$\delta C_{\text{fin}}/\delta x_i$**	**Contribution to $u(C_{\text{fin}})$ $\lvert\delta C_{\text{fin}}/\delta x_i\rvert \cdot u(x_i)$**
$u(C_{\text{in}})$	Concentration of β-endosulfan in the solution to be diluted	0.035 μg/g	0,52	0.018 μg/g
$u(m_1)$	Tare	$5.3\cdot10^{-04}$ g	-1,1 μg/g^2	$5.6\cdot10^{-04}$ μg/g
$u(m_2)$	Mass tare + solution to be diluted	$7.4\cdot10^{-04}$ g	2,2 μg/g^2	$1.6\cdot10^{-03}$ μg/g
$u(m_3)$	Massa tare + solution to be diluted + solvent	$7.4\cdot10^{-04}$ g	-1,1 μg/g^2	$8.4\cdot10^{-04}$ μg/g
		$cov(x_i,x_j)$	**$\delta C_{\text{fin}}/\delta x_i \cdot \delta C_{\text{fin}}/\delta x_j$**	**Contribution to $u^2(C_{\text{fin}})$ $\lvert\delta C_{\text{fin}}/\delta x_i\rvert \lvert\delta C_{\text{fin}}/\delta x_j\rvert \cdot cov(x_i,x_j)$**
$cov(m_1,m_2)$	Covariance between m_1 e m_2	$2.8\cdot10^{-07}$ g^2	-2.3 μg^2/g^4	$6.6\cdot10^{-07}$ μg^2/g^2
$cov(m_1,m_3)$	Covariance between m_1 e m_3	$2.8\cdot10^{-07}$ g^2	1.2 μg^2/g^4	$3.4\cdot10^{-07}$ μg^2/g^2
$cov(m_2,m_3)$	Covariance between m_2 e m_3	$5.5\cdot10^{-07}$ g^2	-2.5 μg^2/g^4	$1.4\cdot10^{-07}$ μg^2/g^2
C_{fin} = 1,519 μg/g				
$u_c(C_{\text{fin}})$ = 0,018 μg/g				

Tab. 5.2: Uncertainty budget of β-endosulfan in the calibration solution n. 1.

5.4.3 Evaluation of $u(V_e)$

The final volume of the extract used for the quantification of the pesticides was determined by weighing according to the double substitution scheme (A-B-B-A) by comparison with calibrated mass standards. The weighted mass of the extract was converted into volume using the density of n-hexane. The value of $u(V_e)$ was determined by combining the uncertainty contributions deriving from the mass standards, the weighing process, the buoyancy effect and the density of n-hexane.

The same weighing procedure was adopted for the determination of the masses of the samples to be extracted (see paragraph 5.4.4) and for the evaluation of the correction factor for moisture content f_h (par. 5.4.5).

The model equation used for the evaluation of the uncertainties on the volumes of the extracts is simpler than the model used for the calibration solutions (eq. 5.15), as the final mass of the extract is calculated by difference between the mass of the filled (m_2) and empty vial (m_1):

$$m_{fin} = m_2 - m_1 \tag{5.17}$$

The uncertainty of the masses of the extracts $u(m_{fin})$ was calculated by applying the law of propagation of the uncertainty considering all the significant sources coming from the weighing process (mass standards, balance repeatability, buoyancy effect). The resulting equation is:

$$u^2(m_{fin}) = \left(\frac{\partial m_{fin}}{\partial m_1}\right)^2 u^2(m_1) + \left(\frac{\partial m_{fin}}{\partial m_2}\right)^2 u^2(m_2) + 2\frac{m_{fin}}{m_1}\cdot\frac{m_{fin}}{m_2}\mathrm{cov}(m_1, m_2) \tag{5.18}$$

The standard uncertainty $u(m_{fin})$ is converted into a relative standard uncertainty, $u(m_{fin})/m_{fin}$, and in volume unit (ml) by multiplying the relative uncertainty for the calculated volumes of the extracts, thus obtaining $u(V_E)$.

5.4.4 Evaluation of $u(m_h)$

The value of the mass of the samples to be processed m_h was determined by weighing as described in 5.4.3. The uncertainty takes into account the contributions deriving from the mass standards, the weighing process and the buoyancy effect.

5.4.5 Evaluation of $u(f_h)$

The correction factor for moisture content f_h was determined on 3 aliquots of the sample, of approximately 1 g each, which were heated at 105 °C to constant weight. The value of f_h is the mean of these 3 values and its uncertainty $u(f_h)$ is their standard deviation.
The evaluation of the moisture content of the sample was performed in order to determine the mass fractions of the pesticides on a dry mass basis, as requested by the protocol of the comparison. The moisture content of green tea samples was determined by weighing some aliquots of tea before and after heating them to constant weight, thus obtaining by difference the content of humidity in the samples.

5.4.6 Evaluation of $u(R)$

The recovery R was determined by spiking samples of green tea of approximately 1 g each with known amounts of the two pesticides in order to obtain theoretical mass fractions of 500 and 1000 μg/kg of each analyte. These samples were processed in the same way of the samples of the comparison in order to take into account all the possible sources of loss during the whole sample preparation process. The spiked samples were also analysed by GC-MS in the same conditions of the comparison samples.
The recovery was evaluated starting from eq. 5.1 where C_{calc} was determined as described in 5.4.2 and C_{theor} is the theoretical concentration spiked in the sample.
The uncertainty $u(R)$ was determined combining the uncertainty contributions of C_{calc} and C_{theor} following the uncertainty propagation law.

5.4.7 Uncertainty budget of χ_a

The following tables report some examples of uncertainty budget for the quantification of β-endosulfan and endosulfan sulphate in different samples analysed for the comparison. In tables 5.3, 5.4, 5.5, 5.6, the values of the input quantities, their standard uncertainties and the contributions to the final combined standard uncertainty are reported. The contribution of the covariance between the quantities C_a and R is also reported.

Input quantity x_i	**Uncertainty component $u(x_i)$**	**Uncertainty source**	**Input quantity value x_i**	**Standard uncertainty value $u(x_i)$**	**Contribution to $u_c(\chi_a)/\chi_a$ $u(x_i)/x_i$**
C_a	$u(C_a)$	Analyte concentration determined by GC-MS	0,418 µg/mL	0,020 µg/mL	0,048
V_E	$u(V_E)$	Final volume of the sample extract	0,442mL	0,001 mL	0,0030
R	$u(R)$	Recovery	0,47	0,02	0,05
m_h	$u(m_h)$	Weighted mass of the tea sample	$9{,}856 \cdot 10^{-4}$ kg	$1{,}3 \cdot 10^{-6}$ kg	0,0014
f_h	$u(f_h)$	Moisture content	0,916	0,003	0,004
			$cov(x_i, x_j)$		$cov(x_i, x_j)/ x_i \cdot x_j$
	$cov(C_a,R)$	Covariance between the analyte concentration C_a determined by GC-MS and recovery R	$6{,}3 \cdot 10^{-5}$ µg/mL		-0,00032
χ_a = 440 µg/kg					
$u_c(\chi_a)$ without $cov(C_a,R)$ = 30 µg/kg					
U without $cov(C_a,R)$ = 60 µg/kg (k = 2)					
$u_c(\chi_a)$ with $cov(C_a,R)$ = 28 µg/kg					
U with $cov(C_a,R)$ = 56 µg/kg (k = 2)					

Table 5.3: uncertainty budget for β-endosulfan in extract n.2

Input quantity x_i	**Uncertainty component $u(x_i)$**	**Uncertainty source**	**Input quantity value x_i**	**Standard uncertainty value $u(x_i)$**	**Contribution to $u_c(\chi_a)/\chi_a$ $u(x_i)/x_i$**
C_a	$u(c_a)$	Analyte concentration determined by GC-MS	0,414 µg/mL	0,037 µg/mL	0,089
V_E	$u(V_E)$	Final volume of the sample extract	0,442mL	0,001 mL	0,003
R	$u(R)$	Recovery	0,59	0,06	0,09
m_h	$u(m_h)$	Weighted mass of the tea sample	$9{,}856 \cdot 10^{-4}$ kg	$1{,}3 \cdot 10^{-6}$ kg	0,0014
f_h	$u(f_h)$	Moisture content	0,916	0,003	0,004
			$cov(x_i, x_j)$		$cov(x_i, x_j)/ x_i \cdot x_j$
	$cov(C_a,R)$	Covariance between the analyte concentration C_a determined by GC-MS and the recovery R	$1{,}0 \cdot 10^{-4}$ µg/mL		-0,00041
χ_a = 345 µg/kg					
$u_c(\chi_a)$ without $cov(C_a,R)$ = 45 µg/kg					
U without $cov(C_a,R)$ = 90 µg/kg ($k = 2$)					
$u_c(\chi_a)$ with $cov(C_a,R)$ = 44 µg/kg					
U with $cov(C_a,R)$ = 88 µg/kg ($k = 2$)					

Table 5.4: uncertainty budget for endosulfan sulphate in extract n.2

Input quantity x_i	**Uncertainty component $u(x_i)$**	**Uncertainty source**	**Input quantity value x_i**	**Standard uncertainty value $u(x_i)$**	**Contribution to $u_c(\chi_a)/\chi_a$ $u(x_i)/x_i$**
C_a	$u(C_a)$	Analyte concentration determined by GC-MS	0,497 µg/mL	0,024 µg/mL	0,049
V_E	$u(V_E)$	Final volume of the sample extract	0,392 mL	0,001 mL	0,0030
R	$u(R)$	Recovery	0,47	0,02	0,05
m_h	$u(m_h)$	Weighted mass of the tea sample	$1{,}0223 \cdot 10^{-3}$ kg	$1{,}3 \cdot 10^{-6}$ kg	0,0013
f_h	$u(f_h)$	Moisture content	0,916	0,003	0,004
			$cov(x_i, x_j)$		**$cov(x_i, x_j)/ x_i \cdot x_j$**
	$cov(C_a,R)$	Covariance between the analyte concentration $C_a$determined by GC-MS and the recovery R	$6{,}3 \cdot 10^{-5}$ µg/mL		-0,00027
χ_a = 448 µg/kg					
$u_c(\chi_a)$ without $cov(C_a,R)$ = 31 µg/kg					
***U* without $cov(C_a,R)$ = 62 µg/kg (*k* = 2)**					
$u_c(\chi_a)$ with $cov(C_a,R)$ = 29 µg/kg					
***U* with $cov(C_a,R)$ = 58 µg/kg (*k* = 2)**					

Table 5.5: uncertainty budget for β-endosulfan in extract n.4

Input quantity x_i	**Uncertainty component $u(x_i)$**	**Uncertainty source**	**Input quantity value x_i**	**Standard uncertainty value $u(x_i)$**	**Contribution to $u_c(\chi_a)/\chi_a$ $u(x_i)/x_i$**
C_a	$u(C_a)$	Analyte concentration determined by GC-MS	0,578 µg/mL	0,038 µg/mL	0,066
V_E	$u(V_E)$	Final volume of the sample extract	0,393 mL	0,001 mL	0,0030
R	$u(R)$	Recovery	0,59	0,06	0,09
m_h	$u(m_h)$	Weighted mass of the tea sample	$1{,}0223 \cdot 10^{-3}$ kg	$1{,}3 \cdot 10^{-6}$ kg	0,0013
f_h	$u(f_h)$	Moisture content	0,916	0,003	0,004
			$cov(x_i, x_j)$		**$cov(x_i, x_j)/ x_i \cdot x_j$**
	$cov(C_a,R)$	Covariance between the analyte concentration C_a determined by GC-MS and the recovery R	$1{,}0 \cdot 10^{-4}$ µg/mL		-0,00029
χ_a = 412 µg/kg					
$u_c(\chi_a)$ without $cov(C_a,R)$ = 47 µg/kg					
U without $cov(C_a,R)$ = 94 µg/kg ($k = 2$)					
$u_c(\chi_a)$ with $cov(C_a,R)$ = 46 µg/kg					
U with $cov(C_a,R)$ = 92 µg/kg ($k = 2$)					

Table 5.6: uncertainty budget for endosulfan sulphate in extract n. 4.

5.5 Monte Carlo simulation for measurement uncertainty evaluation

For the uncertainty evaluation, we decided to apply also another approach, described in Supplement 1 to the GUM [8], i.e. the Monte Carlo method (MCM) for probability density function propagation. This numerical method, described in detail in chapter 3, can be very useful when the conditions of applicability of the GUM uncertainty framework are not satisfied. The MCM is a tool which allows combining and propagating probability density functions (PDFs) and not only statistical uncertainties. It consists in a random numerical generation to simulate the values of random variables.

The GUM uncertainty framework can be expected to work well in many circumstances, but it is not always straightforward to determine whether all the conditions for its application hold. Since these circumstances cannot readily be tested, Supplement 1 suggests that any case of doubt should be validated. Since the domain of validity of MCM is broader than that of the GUM uncertainty framework, both the GUM uncertainty framework and MCM could be applied and the results compared [8].

For this reason, we decided to implement the MCM to evaluate the uncertainty of β-endosulfan and endosulfan sulphate. Suitable probability density functions (PDFs) were assigned to each input quantity, starting from the equation model (eq. 5.2). According to prescriptions of Supplement 1, Gaussian PDFs were assigned to all input quantities, considering that the available information on the quantities were their best estimate x_i and the associated standard uncertainty $u(x_i)$. For the simulations, the software R was used [10]. The chosen number of MCM trials was 10^6.

The expanded uncertainties and coverage intervals obtained for β-endosulfan and endosulfan sulphate with the MC simulations are lower than those obtained within the GUM uncertainty framework, as it can be seen in table 5.7. In figures 5.6 and 5.7 the simulated PDFs of the mass fractions of β-endosulfan and endosulfan sulphate in sample extract n. 2 are reported as an example. Similar results were obtained for other two extracts (n. 4 and n. 6) chosen for the simulations, and for this reason the graphical PDFs representations are not reported.

Sample n.	Mass fraction χ_a	$u_c(\chi_a)$ GUM	$u_c(\chi_a)$ MCM	GUM Coverage interval (k = 2)	MCM Coverage interval ($I_{95\%}$)
	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
β-endosulfan					
2	440	30	21	[380, 500]	[398, 481]
4	448	31	22	[386, 510]	[404, 491]
6	559	47	39	[465, 653]	[481, 634]
Endosulfan sulphate					
2	345	45	31	[255, 435]	[284, 405]
4	412	47	27	[318, 506]	[358, 465]
6	523	67	45	[389, 657]	[434, 610]

Table 5.7: combined standard uncertainties for β-endosulfan and endosulfan sulphate obtained with the classical GUM approach and MCM, with the associated coverage intervals at a confidence level of 95%.

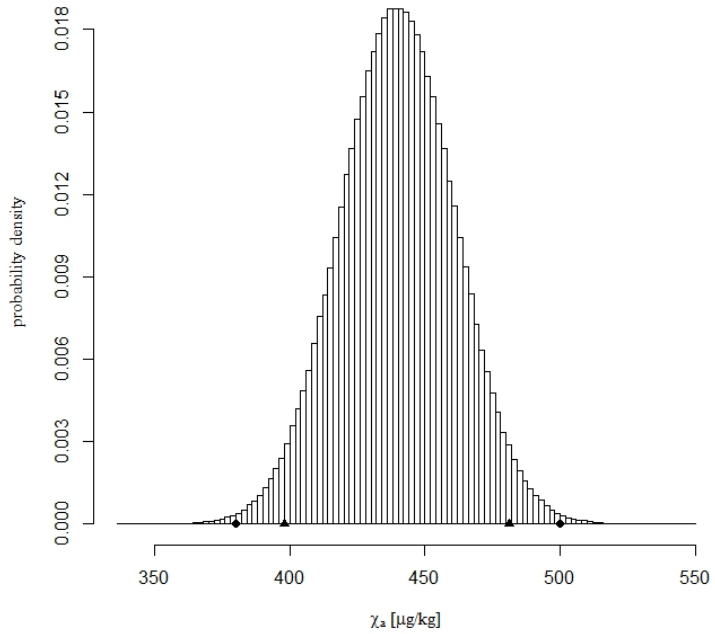

Figure 5.6: probability density function for β-endosulfan in extract n. 2, obtained with a MCM simulation. The black triangles (▲) represent the extremes of the MCM coverage interval *I* corresponding to a 95% coverage probability while the black dots (◆) are the extremes obtained with the classical GUM approach (expanded uncertainty *U* for $k = 2$ and a confidence level of about 95%).

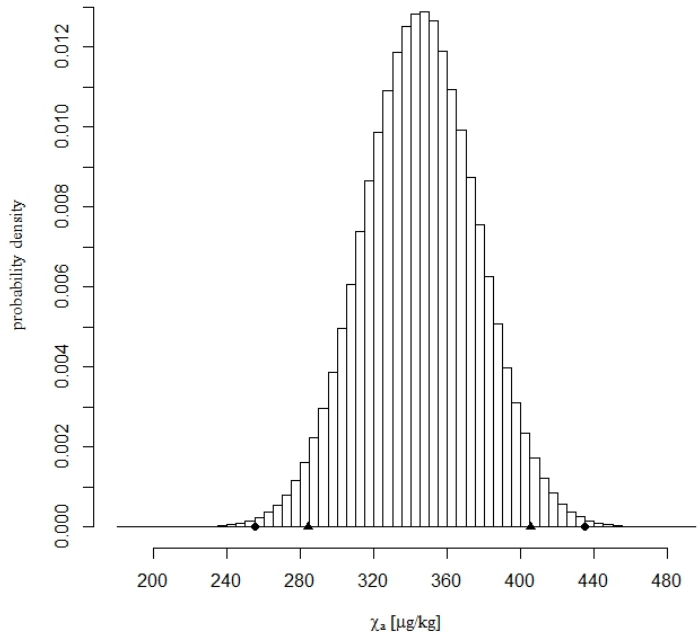

Figure 5.7: probability density function for endosulfan sulphate in extract n. 2, obtained with a MCM simulation. The black triangles (▲) represent the extremes of the MCM coverage interval *I* corresponding to a 95% coverage probability while the black dots (◆) are the extremes obtained with the classical GUM approach (expanded uncertainty *U* for $k = 2$ and a confidence level of about 95%).

From table 5.7, it can be seen that results obtained with the MC approach are comparable with the uncertainties obtained by applying the classical GUM approach.

However, as can be clearly seen in figures 5.6 and 5.7, the extremes of the coverage intervals obtained with the classical GUM approach are wider with respect to those obtained with the MC approach.

An explanation for the obtained results can be related to the limitations of the GUM uncertainty framework. As already explained, this approach is based on the law of propagation of uncertainties, after the identification and the quantification of the uncertainties of the input quantities (bottom-up approach). This approach implies some key features:

- Linearization of the model: the law of propagation of uncertainties derives from the use of the first-order expansion of the Taylor series and this represents a linear approximation to the model, while in some cases higher order terms could be necessary;

- Assumption of the normality of the measurand y: in general, in routine analysis, the distributions of the results are assumed normal and, as a consequence, the expanded uncertainty $U(y)$ is calculated as the product of the combined standard uncertainty for the coverage factor k of a normal distribution. Usually, a coverage factor $k = 2$ is assumed, which corresponds to a confidence level of about 95% (the exact value is 95,45%).
- Evaluation of the effective degrees of freedom ν_{eff}: the calculation of ν_{eff} might represent a problem, for examples for the Type B uncertainties, which contributes with an infinite number of ν_{eff}.

The MC simulation can give more reliable results with respect to the classical GUM approach, in particular when dealing with non-linear models. This is the case of the quantification of the mass fractions χ_a of β-endosulfan and endosulfan sulphate in tea, as expressed in the model equation 5.2. The discrepancies between the uncertainties determined with the classical GUM approach and the MCM, can be related to the approximations at the basis of the application of the law of propagation of uncertainties which correspond to wider coverage intervals, while the MCM automatically generates these intervals starting from the PDFs assigned to the input quantities x_i, without making assumptions on the output PDF and without introducing degrees of freedom or coverage factors. The only limitation of the MC simulation can be the choice of the PDFs assigned to each input quantity, which depends on the degree of knowledge of the physical phenomena underlying each input quantity.

The MC approach could also be very useful when dealing with concentrations very close to the limit of detection of the analytical techniques, as it allows only the extractions of positive values of concentration obtaining coverage intervals that are always positive and, as a consequence, avoiding senseless results from a physical point-of-view, such as negative concentrations of the analytes. The MCM could represent, in this sense, a valid alternative to the classical GUM approach, based on the assumption of symmetric PDFs for the measurand, in the field of organic micropollutants analysis at trace levels.

5.6 Final results and conclusions

The final concentrations of β- endosulfan and endosulfan sulphate are reported in table 5.8. The final results were determined by calculating the weighted mean [11] of the concentrations obtained from 6 different samples.

Sample	β-ENDOSULFAN			ENDOSULFAN SULPHATE	
	Mass fraction µg/kg	***u*** µg/kg		**Mass fraction** µg/kg	***u*** µg/kg
1	619	41		508	60
2	440	30		345	45
3	483	35		478	49
4	448	31		412	47
5	351	27		329	39
6	559	47		523	67

Table 5.8: mass fractions (µg/kg) of β-endosulfan and endosulfan sulphate and associated combined standard uncetainties.

As the results were largely scattered, the combined standard uncertainty of the final value χ_a was evaluated by enlarging the standard uncertainties of each input value by a factor (2,7 for β-endosulfan and 1.7 for endosulfan sulphate) determined using the chi-squared test. The obtained values are in agreement with the standard deviation of the mean of the results (i.e. 39 µg/kg for β-endosulfan and 34 µg/kg for endosulfan sulphate) hence confirming the followed calculation approach.

The described method represents an example of metrological traceability establishment for the analysis of organic micro-pollutants in complex matrices. This method allowed obtaining good results for endosulfan sulfate quantification, while β-endosulfan showed some stability problems during the quantification process, which are under investigation.

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CHAPTER 6

DETECTION OF MELAMINE IN MILK BY SURFACE ENHANCED RAMAN SCATTERING (SERS)

6.1 Introduction

The scope of the work presented in this chapter was the development of a rapid and sensitive method to detect melamine in cow milk based on Surface Enhanced Raman Scattering (SERS) spectroscopy, exploiting the selective binding of gold nanoparticles (AuNPs) with this analyte. This interaction promotes the aggregation of the AuNPs inducing a huge enhancement of the melamine signals in the Raman spectrum due to the formation of SERS "hot spots". An external standard calibration method was employed for quantitative analysis and the method was validated for linearity, repeatability, limit of detection, limit of quantitation and recovery, obtaining a good linearity (R^2=0.99) in the concentration range of 0.31– 5.0 mg/l in milk with a limit of detection (LOD) of 0.17 mg/l. This method was developed with the aim of detecting melamine in milk matrix in accordance with the European law limits. Considering the potential toxicity of melamine, the Codex Alimentarius Commission has set a limit of 1 mg/l for powder infant formula and 2.5 mg/l for other foods and animal feed [1].

The method developed is based on the use of of spherical AuNPs, which guarantees high sensitivity and gives a linear response in a range of concentrations useful for practical applications. We used selectively tested spherical AuNPs with dimensions chosen to obtain the highest SERS effect. Moreover, the AuNPs concentration was previously tuned to reach the linearity in the selected melamine range. For the calibration of the Raman spectrophotometer the acetonitrile (ACN) Raman band was used to normalize the Raman intensity of melamine, minimizing possible variations due to laser power, focal distance and environmental parameters (temperature, humidity). The method developed proved to be simple and not requiring a long extraction procedure, with a total analysis time of about 30 minutes.

As previously described in chapter 4, melamine is an important industrial material that is mainly used for resin production, for thermosetting plastic and for polymer manufacturing in general [2]. Its fame, unfortunately, came out recently because it was used as a food adulterant in milk, pet and animal feed [3-4]. As a high rich-nitrogen molecule, melamine was intentionally added into food ingredients to produce an incorrectly high reading in the measurement of the protein content based on total nitrogen. The main concern on melamine, as a food additive, is the ability of combining with its analogues, such as cyanuric acid, leading to the formation of insoluble crystals which were responsible for kidney failures and even death in infants in China [5-7].

6.2 State of the art

Currently, gas chromatography (GC) or liquid chromatography (HPLC) coupled with mass spectrometry (MS) [8-9], matrix-assisted laser desorption/ionization MS [10], ELISA [11] and IR spectroscopy [12], represent the major categories of techniques for melamine detection. However, these methodologies usually require expensive instrumentations and long sample preparation procedures are needed mainly due to analyte extraction steps. Recently, several methods to detect melamine based on gold nanoparticles (AuNPs) have been developed [13-15]. Some of these methods were based on a colorimetric visual inspection of the nanoparticle solution colour change upon melamine interaction. Melamine interaction with modified or unmodified gold nanoparticles decreases the stability of the AuNPs provoking the formation of aggregates and inducing a shift of the surface plasmon resonance with a consequent variation of the color solution from red to blue, that can be easily monitored by UV/Vis absorption measurements. However, in the presence of interferent substances in milk, such as other organic molecules or even positively charged ions competing with melamine for AuNPs binding, a change in the AuNPs aggregation state can be seen, even in absence of the analyte and thus leading to a false positive response. In order to avoid these problems, Raman spectroscopy was used since it can provide a fingerprint of the melamine molecule in the Raman spectrum. Raman spectroscopy together with the help of gold or silver nanoparticles offers a very high sensitivity due to the Surface Enhanced Raman Scattering (SERS) effect that occurs when a molecule is adsorbed or grafted on a rough metallic surface. The Raman signal of the molecule can be enhanced theoretically up to a 10^{13} factor for potential single molecule detection. Different methodologies for the detection of melamine based on the SERS effect were developed. Most of them were based on the fabrication of SERS substrates [16-18],

usually prepared by metallic nanoparticles deposition on silicon or glass or by photolithography techniques. SERS substrates demonstrated to achieve a very high sensitivity (detection limit in the μg/l range) but they usually suffered of lack of reproducibility and homogeneity of the molecule distribution on the SERS substrate, leading to problems in the quantification. Other SERS analysis were developed in liquid, mainly based on silver nanoparticles, achieving very good results for melamine detection in milk [19]. As for gold nanoparticles, instead, only few works have been published. Lou, Wang, Peng, Xiong and Chen (2011) [20] developed a very sensitive indirect method (LOD 0.1 μg/l) to detect melamine in milk by SERS using 4-mercaptopyridine-modified AuNPs. However, the linearity response of this method is between 0.5-100 μg/l which might affect the practical application of this assay in routine analysis. Moreover, the melamine quantification is done by using a Raman reporter and not by the melamine itself. Another interesting work was proposed by Yazgan, Boyac, Topcu and Tamer (2012) [21] who developed a rapid and sensitive method to detect melamine in milk by using spherical magnetic-core gold-shell nanoparticles and rod-shaped gold nanoparticles labeled with a Raman-active compound. They reached the limits of detection (LOD) and quantification (LOQ) of 0.38 mg/l and 1.27 mg/l, respectively.

6.3 Raman SERS spectroscopy analysis

Raman spectroscopy is a technique used to observe vibrational, rotational, and other low-frequency modes in a system. It is based on the measurement of inelastic scattering (or Raman scattering), of monochromatic light produced by a laser radiation and the atoms (or molecules) of a substance. The laser light (usually in the visible, near infrared or near ultraviolet range) interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the vibrational modes in the system. In figure 6.1, the energy level diagram showing the states involved in Raman signal is presented. The line thickness is roughly proportional to the signal strength from the different transitions.

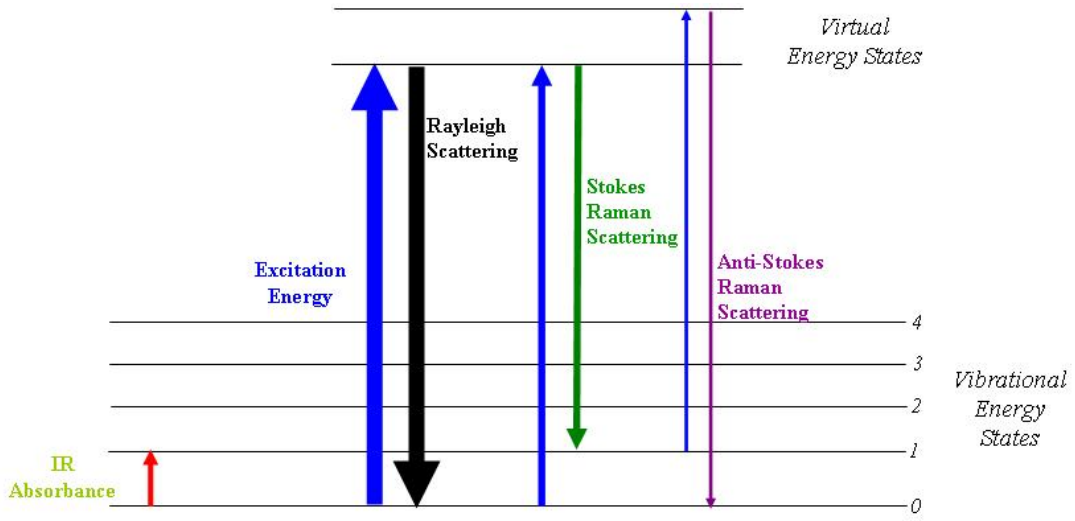

Figure 6.1: energy level diagram showing the states involved in Raman signal [22].

Typically, a sample is illuminated with a laser beam. Light from the illuminated spot is collected with a lens and sent through a monochromator. Wavelengths close to the laser line due to elastic Rayleigh scattering are filtered out while the rest of the collected light is dispersed onto a detector.

For the spontaneous Raman effect, a photon excites the molecule from the ground state to a virtual energy state. When the molecule relaxes it emits a photon and it returns to a different rotational or vibrational state. The difference in energy between the original state and this new state leads to a shift in the emitted photon frequency away from the excitation wavelength. If the final vibrational state of the molecule is more energetic than the initial state, then the emitted photon will be shifted to a lower frequency in order to balance the total energy of the system. This shift in frequency is called Stokes shift. If the final vibrational state is less energetic than the initial state, then the emitted photon will be shifted to a higher frequency, and this is called anti-Stokes shift.

However, spontaneous Raman scattering is typically weak and, as a result, the main difficulty of Raman spectroscopy is separating the weak inelastically scattered light from the intense Rayleigh scattered laser light.

Raman spectroscopy is very useful for the analysis of gaseous, liquid and solid (crystalline or amorphous) samples, providing information on molecular composition, chemical bonds, crystalline phases and structures.

A particular feature of this technique is the so-called SERS effect which increases the analytical performances of Raman spectroscopy. Indeed, when the sample is put in contact with a metallic irregular surface or constituted of metallic nanoparticles (gold or silver), a

considerable intensity enhancement (by a factor of 10^6-10^{10}) of the Raman signal can be observed.

The use of this technique is becoming more and more popular in the scientific community, as it allows getting detailed information from complex matrices like biological samples (cells, tissues) or inorganic materials (plastic matrices, polymers), assuring high accuracy and sensitivity. It can be used in various analytical fields: electrochemistry, bio-sensing, environmental analysis and, in general, all the analytical chemistry fields.

The application presented in this chapter concerns melamine analysis in milk. As introduced in chapter 4, melamine can be added into food as an adulterant to increase its apparent protein content. Several papers proposed gold nanoparticles (AuNPs) as useful substrates for melamine detection since the colour of the particles changes after the interaction with the melamine in solution. AuNPs are usually fabricated by using the sodium citrate method which allows an easy tunability of particles dimensions just by changing the gold precursor salt and the sodium citrate molar ratio. The sodium citrate, indeed, works both as a reducing agent for gold nucleation and as stabilizing agent by coating the surface of the nanoparticles preventing their aggregation in solution. When the melamine is injected in the system, hydrogen bonds between the melamine amino groups and citrate ions occur, decreasing the electrostatic repulsion between individual AuNPs and finally resulting in the aggregation of AuNPs and a change in the colour of the solution. This process is schematically shown in figure 6.2:

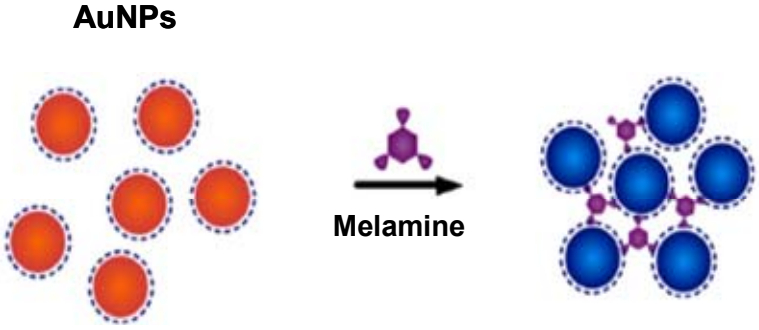

Figure 6.2: AuNPs aggregation mechanism in the presence of melamine [23].

As a result of the aggregation, the solution colour changes from wine-red to blue-gray and the absorbance peak of AuNPs (530 nm) decreases and a new absorption band around 700 nm shows up.

The SERS effect takes place in presence of “hot spot” due to the formation of gold aggregates and its enhancement efficiency can be maximised when the plasmon resonance

peak of the aggregates is in resonance with the laser source (780 nm). It is reasonable to infer that a strong polarization occurs at the surface of these AuNPs and where the electric field increases strongly, and this can result in both an increased enhancement factor, and in a change of the vibrational Raman selection rules, which allow the appearance of forbidden Raman bands.

6.4 Method development

6.4.1 Gold nanoparticles preparation

All glassware used in the experiment was soaked in aqua regia (HCl:HNO_3 3:1 v/v), rinsed thoroughly in water and dried with nitrogen prior to use. AuNPs were synthesized according to Frens method (1973). For the preparation of 40 nm AuNPs, 5 ml of a 1% aqueous solution of trisodium citrate was rapidly injected into 500 ml boiling solution of $HAuCl_4$ (0.01% v/v). The mixture was further refluxed for 10 min and then cooled to room temperature under continuous stirring and a wine-red color solution of AuNPs was obtained. AuNPs solution was stored at 4 °C before use.
The AuNPs had been previously characterised by UV-Vis absorption measurements and by Scanning Electron Microscopy (SEM) imaging. UV-Vis absorption spectra were collected with an Evolution 60s spectrophotometer (Thermo Scientific) and the surface plasma resonance peaks of AuNPs solutions were measured for AuNPs dimensions of 10, 40 and 80 nm, respectively. SEM characterization was carried out at the Nanofacility Piemonte (Torino, Italy). The characterisation of AuNPs was not part of the PhD work.
Hydrogen tetrachloroaurate trihydrate (HAuCl4 $3H_2O$, ≥99%), trisodium citrate dihydrate (≥99%), melamine (99 %) were purchased from Sigma-Aldrich. Sodium hydroxyde (NaOH, 97%), hydrochloric acid (HCl, 37%), nitric acid (HNO_3, 68%), absolute ethanol (99.99%) and acetonitrile (ACN, >99.5%) were obtained by Carlo Erba Reagents. All solutions were prepared with Milli-Q quality water (18 MΩcm). Semi-skimmed milk used for the assays was purchased in a local supermarket in Torino, Italy.

6.4.2 Melamine standard solutions

Melamine stock standard solution was prepared by accurately dissolving 50 mg of standard in 50 ml of ethanol/H_2O (50:50 v/v), to reach a concentration of 1000 mg/l. Melamine

standard solutions were prepared by subsequent dilutions from the stock solution in water to reach the following concentrations: 100, 20, 10, 5, 1, 0.5, 0.2, 0.1 mg/l. These pure melamine standards were used to set up the analytical procedure. Aliquots of the melamine standards were mixed in a 1:1 ratio with AuNPs stock solutions, mixed with vortex for 3 s and subsequently analyzed by UV-Vis and the Raman spectrophotometer.

Melamine standard solutions in non-spiked milk extract were also prepared for the external calibration of the Raman spectrophotometer, as explained in the par. 6.6. Consecutive dilutions were made starting from 10 mg/l to reach the following concentrations in matrix: 1, 0.50, 0.25, 0.125, 0.063 mg/l. These solutions were mixed with AuNPs (1:1) and analyzed by Raman spectroscopy to build the calibration curve.

6.4.3 Detection of melamine in liquid milk by SERS

Aliquots of the 100 mg/l melamine stock solution were added to milk to obtain concentrations of 0.5, 1, 3, 5 and 10 mg/l. Melamine-free milk was processed as the spiked milk and used to prepare blank samples. The extraction procedure was carried out by first adding 200 µl of 1 M HCl to 4 ml of spiked milk and vigorously mixing by vortex for 10 s. The samples were then transferred into 1.5 ml centrifuge tubes and centrifugated for 30 min at 14000 rpm. Supernatants from the same sample were collected and filtered with a 0.22 µm PTFE filter. The pH of the filtered solution was adjusted at 4.7 by adding 60 µl of 1 M NaOH. 10 ml of pure ACN were then added inducing the precipitation of most of the proteins in solution. A final centrifugation step was carried out at 14000 rpm for 30 min in order to remove any aggregates. 250 µl of the resulting supernatant was mixed in a 1:1 ratio with a 10-fold concentrated 40 nm AuNPs solution and immediately analyzed by Raman spectroscopy. The 10-fold concentrated 40 nm AuNPs solution was obtained by centrifugating the AuNPs stock solution at 4000 rpm for 30 min and subsequently resuspending in a proper amount of water solution.

SERS spectra were recorded using a Thermo Scientific DXR Raman equipped with a microscope, excitation laser source at 780 nm, a motorized microscope stage sample holder, and a charge-coupled device (CCD) detector. Spectra of samples were collected using a 20x long working distance microscope objective with a 24 mW laser power and a spectral range from 200 to 1800 cm^{-1}. The acquisition time was of 20 s with 1s exposure time.

In figure 6.3, the analytical method for the analysis of melamine in milk samples is shown.

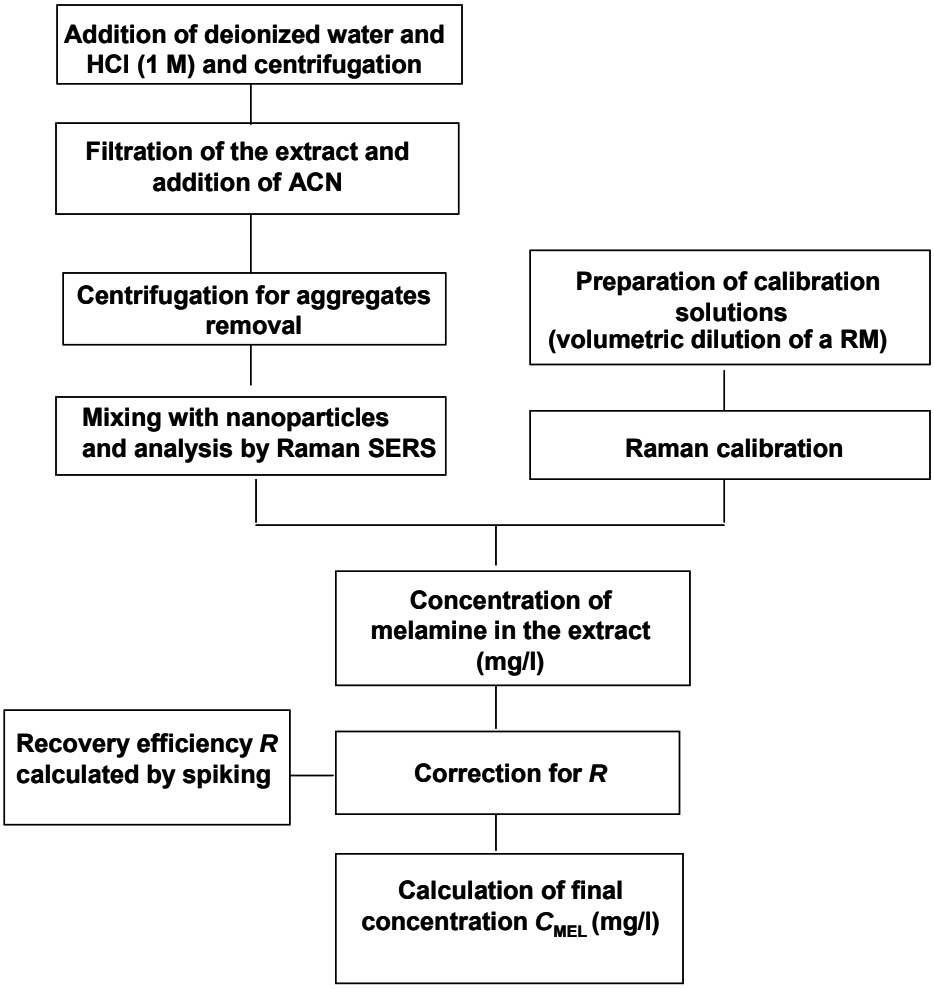

Figure 6.3: Scheme of the analytical method developed for melamine in milk samples.

6.5 Validation of the method

The validation of the method was performed by calculating linearity, repeatability, mean recovery, limit of detection (LOD) and limit of quantitation (LOQ).

The instrumental linearity was evaluated from four calibration curves with 5 levels of melamine concentrations in non-spiked milk extract, representative of the analysed matrix: 0.031, 0.063, 0.125, 0.25, 0.50 mg/l corresponding in the matrix to values from 0.31 – 5.0 mg/l. The linearity was estimated by the determination coefficient R^2 and the acceptability criteria to assume the linearity of response was R^2>0.99. A linear regression was found between the normalized Raman signal at 715 cm^{-1} and melamine concentration with a good determination coefficient (R^2) of 0.99.

The recovery (%) was calculated by the average concentration values (n=6) obtained for melamine spiked samples (at 1 mg/l and 3 mg/l concentration levels corresponding to 0.1

mg/l and 0.3 mg/l in the analyzed milk extracts) and it was found to be 96.7 % and 96.8% respectively.

The intra-day repeatability and bias of the method were determined for milk spiked samples with 1 and 3 mg/l melamine. Repeatability is expressed as relative standard deviation (RSD%) and the RSD values were 10.1 % and 3.8 % for 1 and 3 mg/l spiked samples, respectively. The bias, determined as the difference between the mean concentration of melamine found in the extracts and the "true" concentration spiked in the samples, was calculated and reported in relative terms. Each spiked sample was analyzed six times in the same day, in order to test the repeatability of the method.

The LOD was experimentally detected on blank samples (n=10) and calculated by the equation [24]:

$$LOD = \frac{3s_b}{b} \tag{6.1}$$

where s_b is the standard deviation of the areas of the blank samples in the Raman spectrum at 715 cm^{-1}, and b is the slope of the calibration curve. Indeed, the LOD of melamine concentration in the matrix (mg/l) was evaluated by using the standard calibration curve previously built (715 cm^{-1} band area versus concentration in matrix). The LOQ, was estimated with the following equation:

$$LOQ = \frac{10s_b}{b} \tag{6.2}$$

The LOQ is not univocally defined in the IUPAC publications. However, it is generally accepted that the LOQ refers to the smallest concentration or mass, which can be quantitatively analyzed with reasonable reliability by a given procedure. In addition, it is accepted that the LOQ is calculated as 10 times the standard deviation of the repeated analyses of the blanks, performed for the calculation of a reliable LOD [25].

According to equations (6.1) and (6.2) the LOD and LOQ were 0.017 mg/l and 0.057 mg/l respectively in the milk extracts which correspond to values of 0.17 mg/l (LOD) and 0.57 mg/l (LOQ) in the milk matrix. The method is then suitable for melamine quantification in the concentration range of 0.57 – 5.0 mg/l in milk matrix in accordance with the European law limits of 1 mg/l and 2.5 mg/l in dairy products for infants and other food and animal feed, respectively [1]. The validation parameters are summarized in Table 6.1.

Linearity range (melamine in milk matrix) (r^2=0.99)	0.31 – 5.0 mg/l	
Quantification range	0.57 – 5.0 mg/l	
LOD (melamine in milk matrix)	0.17 mg/l	
LOQ (melamine in milk matrix)	0.57 mg/l	
	1 mg/l	**3 mg/l**
Mean recovery (%)	96.7	96.8
Repeatability (RSD %)	10.1	3.8
Bias (%)	-3.3	-3.2

Table 6.1: quantification range and validation parameters calculated for melamine determination in milk.

6.6 Calibration of the Raman spectrophotomer and analysis of the samples

In order to demonstrate a practical application in the food analysis field, we decided to detect melamine in liquid raw milk and we started to develop a measurement procedure based on 40 nm AuNPs building the SERS substrates. Food samples are complex matrices that are difficult to analyze because of their protein and carbohydrate content. Detecting low levels of melamine in food is not easy because melamine can be bound with the milk constituents due to its strong tendency to form hydrogen bonds. Thus, prior to the analysis, extraction of melamine from milk is a fundamental step. An acidic extraction was first carried out with hydrochloric acid (1 M) in order to precipitate caseins from milk. Further purifications steps, such as filtrations and solvent extraction using ACN, resulted to be necessary to induce proteins precipitations and to extract melamine simultaneously. Since this method of analysis is based on the covalent bonding of free amino groups ($-NH_2$) in melamine with the AuNPs, the removal of any source of free amino groups is important to increase the efficiency of the detection method, reducing the interfering molecules. Moreover, the pH of the solution was adjusted to maximize the melamine-AuNPs interaction. The solution pH influences both the surface charge on AuNPs and the protonation state of melamine amino groups. Considering the pK_a values of the three carboxylic moieties of the citrate ions (pK_a = 3.13, 4.76 and 6.34) and the melamine pK_a value around 5, setting a solution pH of 4.7 results in a reduction of repulsive forces between AuNPs and it induces the protonation of the melamine molecule. Thus, a pH value

around 5 was found to be the best compromise to foster AuNPs aggregation together with their interaction with melamine and it resulted in a chemical enhancement by charge transfer complexes and/or localized surface plasmons, that yield an enhancement of the melamine Raman signal.

The initial set up of the analytical procedure was performed by spiking semi-skimmed milk with melamine to obtain concentrations in the range of interest for practical applications. As we already mentioned, limit value of melamine has been set in Europe at 1 mg/l in infant formula and 2.5 mg/l in liquid milk and food in general. Various concentrations of melamine were spiked in milk and subsequently extracted and analyzed by SERS. As shown in Fig.6.4, five levels (0.5, 1, 3, 5 and 10 mg/l) of melamine in liquid milk were analyzed.

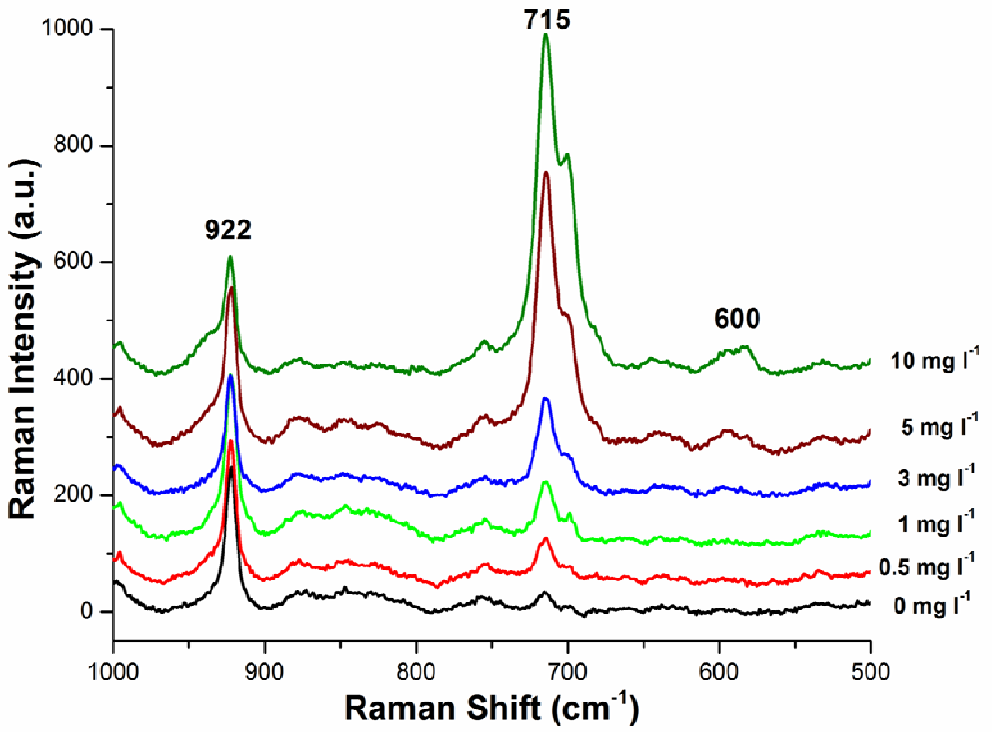

Figure 6.4: Raman spectra of five concentration levels (0.5, 1, 3, 5 and 10 mg/l) of melamine in milk and of a blank milk sample.

By monitoring the highest intense melamine Raman peak at 715 cm^{-1}, it was found that the area of this peak was enhanced with increasing concentration of melamine.

To optimize the method several concentrations of AuNPs were tested (data not shown) and the best results were obtained with a 10 folds concentrated AuNPs solution which guarantees a linear detection response of the melamine Raman signal in the selected

concentrations range. As shown in Fig. 6.4, the melamine peaks corresponding to each concentration of melamine in the spiked samples are well separated and the method gives good responses in the concentration range studied.

Moreover, since the final extraction of melamine from the matrix is done in ACN and the solvent concentration is the same for every analyzed sample, the area of the ACN peak at 922 cm^{-1} was established as a internal reference to normalize the area of the peak at 715 cm^{-1} and to correct the Raman signal in order to eliminate the effects of the matrix and other factors, such as environmental parameters (temperature, humidity) or instrumental settings like the focal distance.

An external calibration procedure was chosen for the melamine quantification in milk. In a first step the calibration was carried out by using a method based on Partial Least Square (PLS) provided by the software of the Raman spectrophotomer (OMNIC). In a second step another method was used, based on weighted total least-squares (WTLS) [26] and this algorithm was also used for the evaluation of the measurement uncertainty of the final results (the method is described in details in par. 6.7.2.). The WTLS algorithm is able to deal with any desired fitting model for regression problems with uncertain and correlated variables. A typical application concerns the determination of calibration curves especially when the uncertainties on the independent variables x_i (i.e. the concentrations of the calibration solutions) cannot be considered negligible with respect to those associated with the dependent variables y_i (i.e. the analytical response) and when correlation exists among x_i and y_i. Considering uncorrelated values x_i and uncorrelated data y_i may be a strong assumption, for example when the standards used for the calibration are traceable to a common reference standard or when the instrument responses need to be corrected for the estimate of a common quantity. It is important to take into account such correlations both for estimating the fitting parameters and evaluating the associated uncertainty.

Taking into account the dilution factor for the melamine concentration from the starting matrix through the extraction procedure, five levels of melamine concentrations (0.031, 0.063, 0.125, 0.25 and 0.50 mg/l) were chosen for the calibration curve, which correspond to melamine values in the range of 0.31 – 5.0 mg/l in the milk samples. Melamine-free milk was used to prepare the blank samples, processing it in the same way as the spiked milk. SERS spectra of melamine standard solutions in milk extracts are shown in figure 6.5.

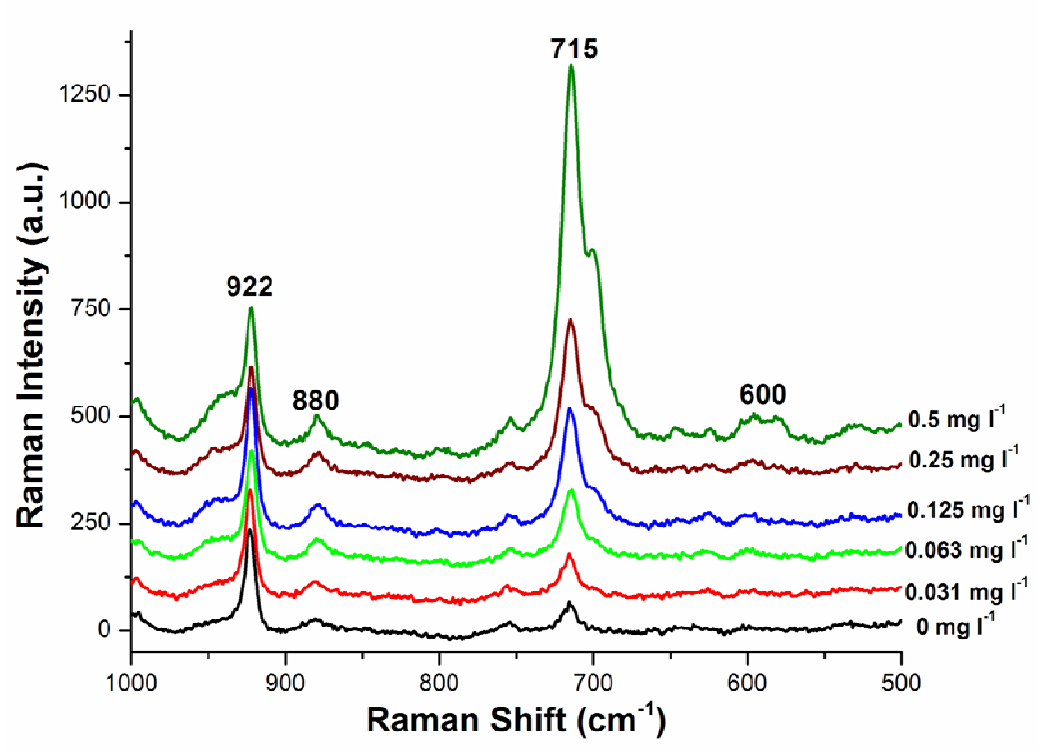

Figure 6.5: SERS spectra of melamine standard solutions in milk extracts having concentrations 0.031, 0.063, 0.125, 0.25 and 0.50 mg/l.

An example of calibration curve obtained for the Raman spectrophotometer and used for the quantification of melamine in the spiked samples of milk is shown in figure 6.6, in which the vertical bars are the expanded uncertainty bars for the fitted values on the calibration curves.

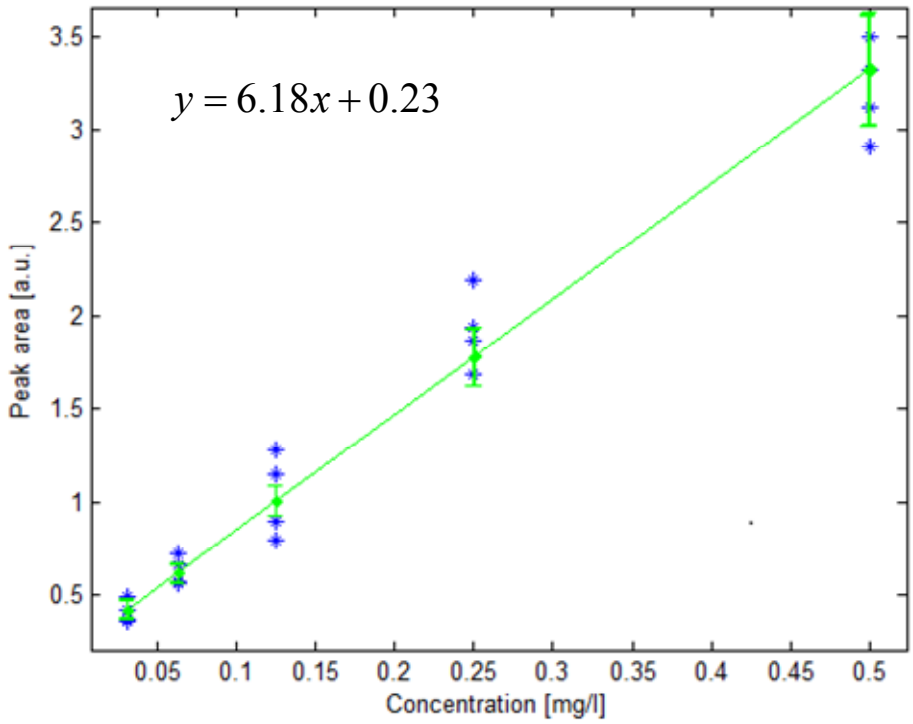

Figure 6.6: calibration curve obtained for the Raman spectrophotometer, used for the quantification of melamine in the spiked samples of milk.

6.7 Uncertainty evaluation

6.7.1 Model equation

The complete model equation for the calculation of melamine concentration (in mg/l) C_{mel} is the following:

$$C_{mel} = \frac{C_a \cdot f_d}{R} \tag{6.3}$$

where:

C_a = concentration of melamine determined by Raman SERS (mg/l)

f_d = dilution factor (extraction procedure)

R = recovery efficiency

The recovery efficiency can be defined as the ratio:

$$R = \frac{C_{\text{calc}}}{C_{\text{theor}}} \tag{6.4}$$

The evaluation of the uncertainty of the quantity R was carried out taking into account the contributions of the quantities C_{theor} and C_{calc}, respectively.

By combining eq. (6.3) and eq. (6.4) we obtain the explicit model equation, which can be used for the calculation of the concentration of melamine C_{mel} in real contaminated samples:

$$C_{\text{mel}} = \frac{C_{\text{a}} \cdot f_{\text{d}} \cdot C_{\text{theor}}}{C_{\text{calc}}} \tag{6.5}$$

The activity carried out on melamine and described in the previous paragraphs concerned the quantification of this analyte in spiked milk samples, and not on real contaminated samples. The validated procedure will be used for the detection and quantification of melamine in real contaminated milk samples.

In figure 6.7, the cause-effect diagram showing the contributions of the input quantities to the final uncertainty of C_{mel}, $u_c(C_{\text{mel}})$ is presented.

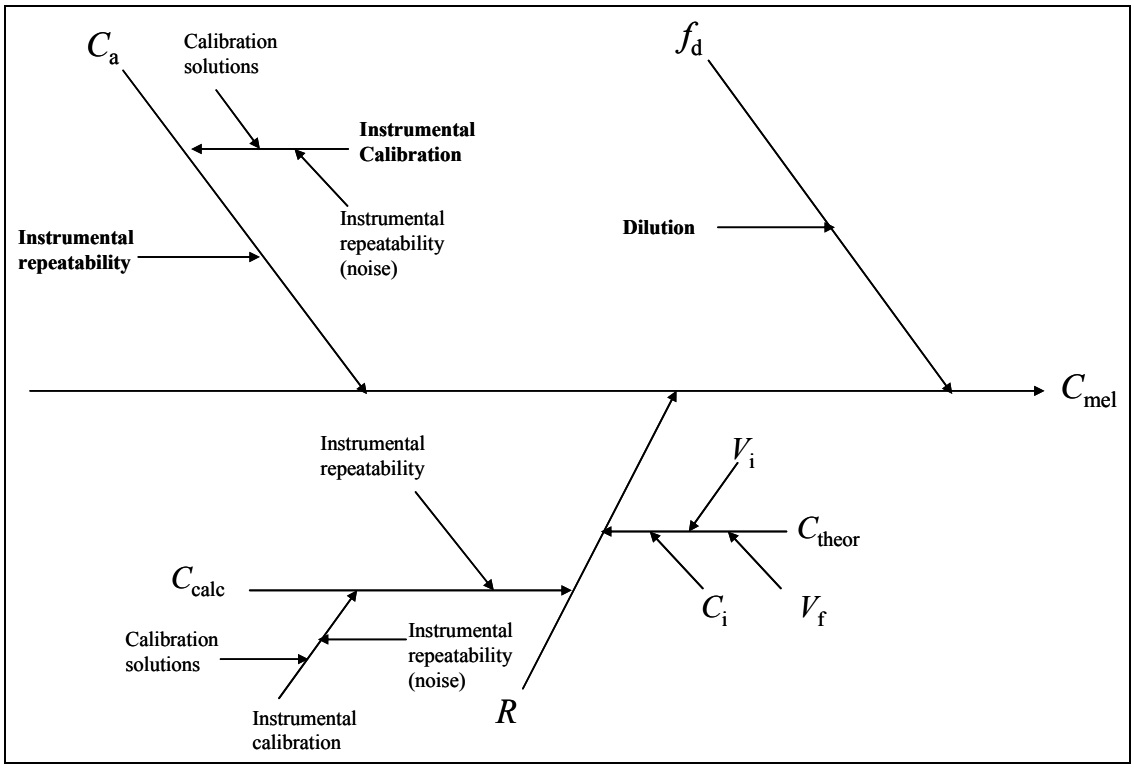

Figure. 6.7: cause-effect diagram for the combined standard uncertainty of C_{mel}, which shows all the contributions to the combined standard uncertainty $u_c(C_{mel})$.

6.7.2 Evaluation of $u(C_{calc})$

For the evaluation of $u(C_{calc})$ the WTLS method [26] was used. In order to fit n experimental data pair (x_i, y_i) for $i = 1, \ldots, n$, whose components have uncertainties $u(x_i)$ and $u(y_i)$, respectively, a general fitting model can be considered:

$$y_i = f\left(x_i, \boldsymbol{p}\right) \tag{6.6}$$

where $\boldsymbol{p} = [p_1,\ldots, p_k]$ is the vector of the parameters to be estimated. The function to be minimised is:

$$\chi^2 = \sum_{i=1}^{n}\left[\frac{(x_i - X_i)^2}{u_{x_i}^2} + \frac{(y_i - f(X_i, p))^2}{u_{y_i}^2}\right] \tag{6.7}$$

where X_i are further n parameters to be adjusted jointly with $\boldsymbol{p}$.

A more general approach, involving also any possible covariances $cov(x_i, x_j)$ and $cov(y_i, y_j)$, is expressed in the matrix form:

$$\chi^2 = \boldsymbol{dx}\boldsymbol{U}_x^{-1}\boldsymbol{dx}^{\mathrm{T}} + \boldsymbol{dy}\boldsymbol{U}_y^{-1}\boldsymbol{dy}^{\mathrm{T}} \tag{6.8}$$

where ***dx*** and ***dy*** are the residual vectors, and $\boldsymbol{U}_x$ and $\boldsymbol{U}_y$ are the covariance matrices of the experimental data. $\boldsymbol{U}_x$ and $\boldsymbol{U}_y$ can be covariance matrices of any form, not necessarily diagonal. When $\boldsymbol{U}_x$ and $\boldsymbol{U}_y$ are diagonal, eq. 6.8 reduces to eq. 6.7.

In general, the function 6.8 is non-linear in its parameters $\boldsymbol{X} = [X_1,...,X_n]^{\mathrm{T}}$ and ***p***, and a numerical solution is necessary for its minimisation. The implemented algorithm provides minimisation for multidimensional and non-linear functions, using a function (fminunc.m) in MATLAB ambient.

Function 6.8 is written in a m-file, which is passed to fminunc.m as an input, together with the starting (vector) point [$\boldsymbol{X}_0$, $\boldsymbol{p}_0$] for the parameter estimates. Among the outputs provided by fminunc.m, the most relevant are the estimates vector [$\hat{\boldsymbol{X}}$, $\hat{\boldsymbol{p}}$], which is the optimal solution, and the value of the objective function χ^2 at the solution, that is, $\chi^2_{\min}$.

This algorithm was used for the calibration of the Raman spectrophotometer and for the determination of the concentrations of melamine in the spiked milk extracts. The analysis curve gives the values of the generic unknown concentration x in each spiked extract analysed by Raman SERS:

$$x = \frac{(y-a)}{b} \tag{6.9}$$

In figure 6.8, the same calibration curve shown in figure 6.6 is presented, together with the estimates of two spiked extracts at 1 mg/l and 3 mg/l of melamine in milk matrix obtained by eq. 6.9. The vertical error bars represent the repeatability uncertainty of the areas of the melamine peaks of the samples analysed. The horizontal bars are the expanded uncertainty bars of the estimates, obtained by applying the law of propagation of uncertainty to eq. 6.9.

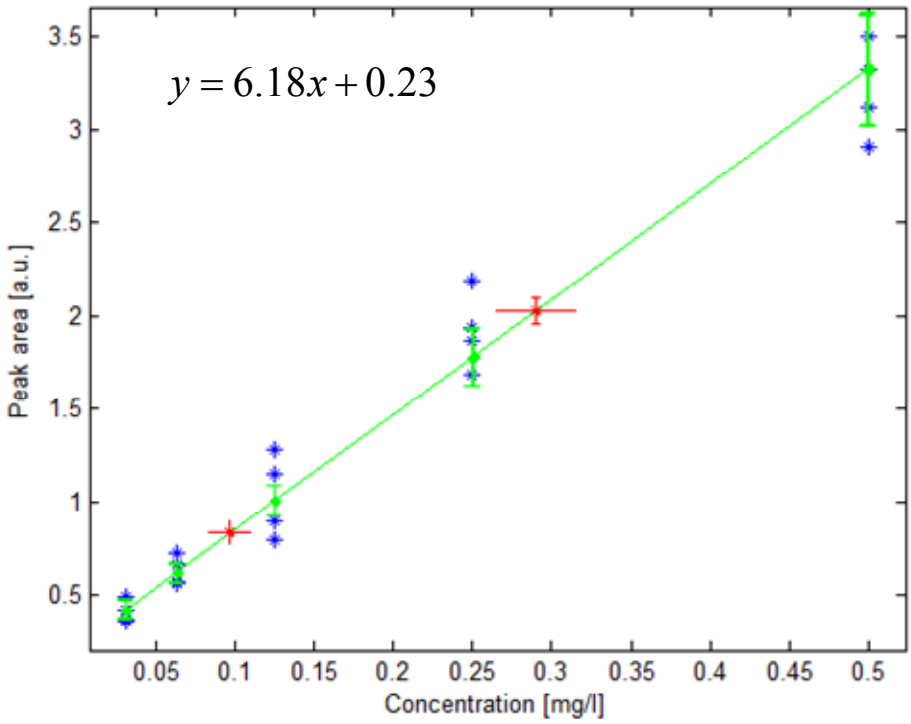

Figure 6.8: calibration curve of the Raman spectrophotomer and calculated concentrations for the spiked milk samples at 1 mg/l and 3 mg/l, with associated expanded uncertainties.

An important contribution to the uncertainty $u(C_{calc})$ is related to the uncertainty of the calibration solutions of melamine. As the standard solutions were prepared by dilution of a stock solution prepared from pure melamine, the following model equation for the dilution process was considered, where C_k is the concentration of a generic calibration solution (in mg/l):

$$C_k = C_0 \cdot \left(\frac{V_0}{V_0 + V_s} \right)^k \tag{6.10}$$

where:

C_0 = concentration of the solution to be diluted expressed in mg/l

V_0 = volume of the solution to be diluted expressed in l

Vs = volume of the solvent added for the dilution expressed in l

The uncertainty $u(C_k)$ is obtained by applying the law of propagation of uncertainty:

$$u^2(C_k) = \left(\frac{V_0}{V_0 + V_s}\right)^{2k} u(C_0)^2 + \left[kC_0\left(\frac{V_0}{V_0 + V_s}\right)^{k-1} \frac{V_s}{(V_0 + V_s)^2}\right]^2 u(V_0)^2 + \\ + \left[-kC_0\left(\frac{V_0}{V_0 + V_s}\right)^{k-1} \frac{V_0}{(V_o + V_s)^2}\right]^2 u(V_s)^2 \quad (6.11)$$

No correlations were considered between V_0 and V_s, as the pipettes used for taking the aliquots of melamine solution and solvent in each dilution step were different.

The same approach described for $u(C_{calc})$ applies to the evaluation of $u(C_a)$, i.e. the uncertainty of the concentration of melamine in real contaminated extracts, not analyzed in this work.

6.7.3 Evaluation of $u(C_{theor})$

The theoretical concentration of melamine in each spiked extract was obtained by a dilution process, analogue to that used to prepare the calibration solutions. The model equation describing the process is the following:

$$C_{theor} = \frac{C_i \cdot V_i}{V_f} \quad (6.12)$$

where:
C_i = initial concentration of the melamine solution to be added to the milk sample expressed in mg/l
V_i = initial volume of the melamine solution expressed in l
V_f = final volume of the spiked milk sample expressed in l

As the final volume V_f can be expressed as the sum of the initial volume V_i of the melamine solution and the volume of the milk in which V_i is diluted (i.e. to the volume of the dilution "solvent" V_s), eq. 6.12 can be written as:

$$C_{theor} = \frac{C_i \cdot V_i}{(V_i + V_s)} \quad (6.13)$$

Analogue considerations may be done for the evaluation of the uncertainty of the dilution factor, f_d. This factor comes from the extraction procedure and appears in the model equation 6.3. The analytical concentration of melamine in real contaminated extracts C_a has to be multiplied for f_d, in order to obtain the final concentration C_{mel} in real contaminated samples. The evaluation of $u(f_d)$ was not carried out in this work as we did not analyze real contaminated samples.

6.7.4 Uncertainty budget of *R*

The uncertainty of R, $u(R)$, was evaluated by applying the law of propagation of uncertainty. A covariance term between quantities C_{calc} and C_{theor} was taken into account as the value of C_{calc} is the analytical response of the Raman spectrophotomer obtained by analysing the spiked samples having a theoretical concentration C_{theor}.
In tables 6.2 and 6.3, the uncertainty budgets for the spiked milk samples at 1 mg/l and 3 mg/l are reported.

Input quantity	**Uncertainty component**	**Uncertainty source**	**Input quantity value**	**Standard uncertainty value**	**Relative standard uncertainty value**
x_i	$u(x_i)$		x_i	$u(x_i)$	$u(x_i)/x_i$
C_{calc}	$u(C_{calc})$	Calculated concentration for spiked extracts	0,0967 mg/l	0,0070 mg/l	0,073
C_{theor}	$u(C_{theor})$	Theoretical concentration for spiked extracts	0,1	0,0022 mg/l	0,022
	$cov(C_{calc},C_{theor})$	Covariance C_{calc} -C_{theor}		0,0025 mg^2/l^2	0,026
R_1 (at 1 mg/l) = 96,7 %					
$u_c(R_1)$ = 6,4 %					
$U(R_1)$ = 12,8 % (k=2)					

Table 6.2: uncertainty budget for melamine concentration in the spiked milk extract at 1 mg/l.

Input quantity	Uncertainty component	Uncertainty source	Input quantity value	Standard uncertainty value	Relative standard uncertainty value
x_i	$u(x_i)$		x_i	$u(x_i)$	$u(x_i)/x_i$
C_{calc}	$u(C_{calc})$	Calculated concentration for spiked extracts	0,290 mg/l	0,013mg/l	0,043
C_{theor}	$u(C_{theor})$	Theoretical concentration for spiked extracts	0,3 mg/l	0,0036 mg/l	0,012
	$cov(C_{calc},C_{theor})$	Covariance $C_{calc} - C_{theor}$		0,0025 mg^2/l^2	0,029
R_3 (at 3 mg/l) = 96,8 %					
$u_c(R_3)$ = 4,2 %					
$U(R_3)$ = 8,4 % (k=2)					

Table 6.3: uncertainty budget for melamine concentration in the spiked milk extract at 3 mg/l.

6.8 Conclusions

A sensitive and rapid method to detect melamine in milk was developed by using AuNPs and Raman spectroscopy. Melamine in milk is able to promote the formation of AuNPs aggregates which behave as Raman "hot spots" and enhance the melamine Raman signal allowing melamine detection in the mg/l range. SERS is emerging as a new technique for analytical methods that can be suitable for high throughput screening analysis and could become a valid alternative to the classical analytical methodologies based on GC or HPLC coupled with mass spectrometry. The method here developed is sensitive, fast and it can be applied in routine analysis for melamine detection. Adequate purification steps are required to obtain a good selectivity, as previously described. SERS analysis can be also performed with a motorized sample holder stage together with a 96 wells plate in order to make the method automatic and to reduce the total volume of the analysis. The whole extraction procedure can be carried out in less than 30 minutes without substantially affecting the sensitivity of the methodology and the reliability of the quantification step.

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CHAPTER 7

CONCLUSIONS

In the presented work, an overview of the PhD activities carried out in the framework of the food analysis field is presented. Food safety is, nowadays, a matter of fundamental importance, for the consumer health, the food industry and the entire economic field.

The contamination of food products may have considerable fallouts both at the social and the economic level, for communities and their national health services. Indeed, foodborne diseases are a problem for public health at the international level, and regard also the most developed countries, where the problem has reached particular relevance (e.g. the dioxins contamination of Bufala mozzarella in Italy in 2008 and the *E. coli O104* contamination of food in Germany and France in 2011). In this framework, the need of having powerful and reliable instruments to protect the consumers from adverse health effects appears clear. One of the challenges which are of greatest importance in these days for the assessment and management of food safety risks is to carry out accurate and efficient controls. The methods used must avoid an excessive increase of the costs for the industries and, as a consequence, for the consumers, while the quality of food products should not be lowered. There are many micro-organisms and chemical substances which must be monitored and detected in all the production steps of food, in order to guarantee the safety and quality of these products.

In the described work two food matrices are considered, i.e. green tea and milk, which are common beverages used all over the world. The PhD activity concerned the set up of metrological traceable analytical methods for the identification and the quantification of two micropollutants that could be present in those matrices.

The analytes determined in green tea powder were two organochlorine pesticides (OCPs), namely β-endosulfan and endosulfan sulphate, its metabolite. OCPs have great toxicological relevance as they may cause harmful effects to human beings health. For this reason, they have been banned for agricultural or domestic uses in Europe, North America and many countries of South America in accordance with the Stockholm Convention. However, some OCPs are still used, e.g. DDT is used to control the growth of mosquito that spread malaria.

Endosulfan was included in the Stockholm Convention on Persistent Organic Pollutants (POPs) (http://chm.pops.int/) in 2011 and in this framework it appears clear the need of having available and reliable procedure for the detection of this micropollutant in food matrices.
The participation in the International Comparison of measurement "Pilot Study CCQM-P136 Mid-Polarity Analytes in Food Matrix: Mid-Polarity Pesticides in Tea" allowed the set up of a suitable procedure for the extraction, clean-up, and quantification of endosulfan (and of other pesticides having similar polarity) in vegetable matrices, establishing metrological traceability for the mass fractions of the pesticides in real matrices, and correctly evaluating the measurement uncertainty of the final results. This approach may be applied in the future also for the evaluation of other micropollutants in similar matrices, assuring traceability to this kind of measurements.

The other analyte considered was melamine, an organic molecule widely used in the industrial field but used in recent years for the fraudulent adulteration of milk and pet feed, and known in particular for the Chinese scandal of infant formula adulteration occurred in 2008. Melamine can have adverse effects on kidneys functionality especially in children and many cases of poisoning of Chinese babies were reported.
Melamine determination was carried out by applying a non conventional analytical technique for this kind of measurements, i.e. Raman SERS spectroscopy, which exploits the selective binding between melamine molecules and gold nanoparticles, for the direct determination of this micropollutant in real food matrices.
The developed method was validated and tested using in-house contaminated milk samples, i.e. commercial uncontaminated milk spiked with known amounts of melamine. Good results were obtained by applying the developed method to the analysis of spiked samples, and thus it may be used to analyse and evaluate the possible contamination of real milk samples and to support the activity of the laboratories which carry out measurements of this micropollutant both at the regional and national levels.

In conclusion, two applications of metrological traceability are presented in this work and the importance of carrying out reliable and comparable measurements in food analysis was highlighted. Further and constant efforts are needed in this field with the aim of guaranteeing food safety and, as a consequence, the consumer health. A fundamental role is committed to the scientific community, which has to improve the analytical procedures

from which reliable analytical data are obtained, in order to support epidemiological studies and to monitor the trends of micropollutants in the food webs and in the environment.

APPENDIX A

EXTRACTION OF PAHs FROM PARTICULATE IN WATER

1. Introduction

From 13th May to 26th July 2013 I carried out a training period at the *Laboratoire National de Métrologie et d'Essais* (LNE) in Paris under the supervision of Dr. Béatrice Lalere and Dr. Julie Cabillic. The main theme of the research activity was the development of an analytical procedure for the extraction of Polycyclic Aromatic Hydrocarbons (PAHs) from particulate suspended matter in water.

PAHs are persistent ubiquitous environmental contaminants, which have high carcinogenic and mutagenic effects. Therefore, they are part of the 33 priority water pollutants which are of major concern for European Waters and have been established by the European Water Framework Directive (WFD) [1].

According to the WFD, a "good chemical status" for a water body is obtained when the concentration of the priority substances in water, sediments or biota are below the Environmental Quality Standards (EQSs). WFD implementation strongly depends on the availability of accurate and reliable analytical methods to ensure confidence in the results given by the testing laboratories.

For reliable measurements of contaminants at the EQS level, the Directive 2009/90/EC (QA/QC Directive) [2] requires analytical methods for laboratory tests which can reach a limit of quantitation (LOQ) equal to or lower than 1/3 of EQS, with measurement uncertainty less than 50 % at EQS.

International standardised methods for analysing PAHs in surface waters are available but they are not compatible with the WFD in terms of LOQ and uncertainties. Therefore there is a need of methods to achieve the WFD environmental objectives.

In this framework, one of the main goals is to develop a method for PAHs in whole water (dissolved and particulate phases). A possible approach is the analysis of the two phases separately. The development of the extraction of the dissolved phase is currently underway at LNE and it consists of solid phase extraction followed by gas-chromatography coupled

with mass spectrometry (GC-MS). The development of a procedure for PAHs extraction from the particulate phase with Accelerated Solvent Extraction (ASE) [3] followed by gas chromatography coupled with isotopic dilution-mass spectrometry (IDMS) was carried out during my training period at LNE. ASE technique, also known as Pressurised Fluid Extraction (PFE) allows the extraction of solid and semisolid matrices using pressurised solvents or mixtures of solvents at high temperatures to increase the efficiency of the extraction process. IDMS allows the measurement of a wide range of organic and elemental analytes in various matrices. It is based on isotope ratio mass spectrometry measurements of a sample with unknown amount content before and after the addition of a known amount of an enriched (isotopically-labelled) "spike" material. The amount of substance in the original sample is calculated from the measured isotope ratios and the amount of spike added. IDMS fulfils the definition of primary method for the measurement of amount of substance developed by the *Comité Consultatif pour la quantité de matière* (CCQM) and play an important role in providing traceability to the SI for a wide range of chemical analyses [4].
The activity was articulated in different steps, in order to set up the best conditions for the extraction, purification and analysis of PAHs.

2 Development of the method

2.1 Preliminary activities

The preliminary activities concerned the investigation of different matrices for the extraction, which can be used for the purification step directly inside the cell. Some tests to evaluate the possible losses of the analytes during the evaporation step were also carried out.
The matrices tested were: sodium sulfate (Na_2SO_4), aluminum oxide (Al_2O_3), Florisil and activated copper powder (Cu powder, size <63 μm, Merck).
For ASE extraction, cells with different size were tested and the tests were started with 11 ml cells. Each cell was completely filled with a different matrix and two aliquots of unlabelled and labelled PAHs solutions (200 μl) were added on top of the matrix. The cells were then extracted with dichloromethane (DCM), with the following method:

- Temperature: 100 °C
- Heating time: 5 min

- Pressure: 140 bar
- Static time: 6 min
- Flush volume: 70%
- Purge time: 100 sec
- Extraction: 3 cycles for two extractions (for each cell)

The extracts were evaporated after the extraction by means of a Büchi Multivapor (pressure: 800 mbar - temperature: 55 °C - stirring rate: 5) and then under a nitrogen stream (rate flow: 100 ml/min), to a final volume of 400 µl. Prior to the analysis by GC-MS, 200 µl of an internal standard (6-methylchrysene) were added to each extract. The internal standard was used to evaluate the absolute recoveries for each native PAH and labelled PAH. Relative recoveries were calculated by IDMS.

Al_2O_3 was activated prior to use, by putting it in oven at 600 °C for one night.

Good recoveries were obtained for Na_2SO_4 and for Al_2O_3, while for Florisil high recoveries were observed for some compounds and the absolute recoveries were not satisfactory.

The Cu powder was activated prior to the extraction, following the guidelines reported in [5], washing the Cu with diluted HCl (1:1), MilliQ water, methanol and DCM. The activated Cu was left in DCM until use. The recoveries obtained for Cu were satisfactory, but this matrix was not practical for use as the preparation is quite long and the extracts contained water residues at the end of the extraction.

Some results obtained with the different matrices are reported in table 1.

		Recovery efficiency (%)								
		naphtalene	anthracene	phenanthrene	fluoranthene	bbf	bkf	bap	indeno	bghip
ASE FLORISIL	IDMS	213	97	408	126	102	99	70	106	85
	HAP/6MC	145	116	461	201	76	74	10	65	55
	HAP*/6MC	68	123	113	159	76	74	15	62	66
ASE ALUMINUM OXIDE	IDMS	109	97	103	98	94	97	93	95	104
	HAP/6MC	88	89	93	120	73	74	71	76	77
	HAP*/6MC	81	93	90	123	78	75	77	80	75
ASE Na_2SO_4	IDMS	24	96	101	96	96	93	95	93	102
	HAP/6MC	32	79	80	86	93	96	95	92	85
	HAP*/6MC	131	83	79	89	97	102	100	99	83
ASE Cu powder	IDMS	36	94	102	98	100	82	93	95	99
	HAP/6MC	50	102	113	87	84	76	82	82	107
	HAP*/6MC	138	111	111	89	84	93	88	85	106

Table 1: relative (IDMS) and absolute recoveries for 9 PAHs using different matrices for ASE extraction.

For the evaporation tests, we prepared three samples by adding 200 µl of the unlabelled and labelled PAHs solutions in about 20 ml of DCM and evaporating them as the extracted samples. We obtained good recoveries both for the labelled and unlabelled PAHs, thus proving that the evaporation is similar for both compounds.

2.2 Extraction tests with LGC 6188 Reference Material

Various extractions were carried out using a Reference Material produced by the Laboratory of the Government Chemist (LGC), namely LGC 6188, which consists of contaminated river sediment containing 15 PAHs at different concentrations. Samples with different matrices (Na_2SO_4, Al_2O_3, Florisil, Cu) were prepared and extracted with DCM. The amount of sample tested was 250 mg for each cell. Good recoveries (72-127%) were obtained for Na_2SO_4 and for Al_2O_3 (68-127 %) except for naphthalene. After these tests, it was decided to decrease the evaporation temperature to 45 °C, in order to reduce the evaporation of the more volatile compounds.

Toluene was also tested as extraction solvent, but it was decided to use DCM for further analysis as the evaporation time of this solvent is quite long and no significant improvement of the recovery efficiencies were observed.

Tests with an increased amount of sample extracted (500 mg) were carried out obtaining good recoveries, thus it was decided to use this amount of sample for the subsequent tests (data not shown).

2.3 Extraction tests with NIST SRM 1941b

Other tests were carried out on a Certified Reference Material from the National Institute of Standards and Technology (NIST), the SRM 1941b "Organics in marine sediment". The SRM 1941b is intended for use in evaluating analytical methods for the determination of selected PAHs, polychlorinated biphenyl (PCB) congeners, and chlorinated pesticides in marine sediment and similar matrices. Certified mass fraction values are given for 24 PAHs, 29 PCB congeners, and 7 chlorinated pesticides. Reference and information values are give for other compounds. All of the constituents for which certified, reference, and information values are provided in SRM 1941b were naturally present in the sediment.

Different tests were carried out with aliquots of 500 mg of SRM. We used both 11 ml and 22 ml extraction cells and we observed that with the smaller cells the recoveries of some

PAHs were around 125% (i.e. fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[ghi]perylene).
For anthracene, benzo[a]pyrene and indeno[123-cd]pyrene the recoveries were good (97-101%). Using bigger cells, the situation was the opposite and the recoveries for fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[ghi]perylene were between 103-108% while for anthracene, benzo[a]pyrene and indeno[123-cd]pyrene were between 85-89%. For naphthalene e phenathrene the recoveries were higher than 100% in both cases. The results of the analysis of some blank samples (prepared at the same time of the sediment samples) allowed excluding the pollution of the samples, so the high values obtained for some PAHs could be related to a loss of labelled PAHs during the extraction process.
Further tests were carried out to improve the recoveries for the latter compounds, using the 22 ml cells, changing different parameters:

- new solvent: n-hexane:DCM (1:1 v/v);
- increased number of extraction cycles: 4 cycles instead of 3;
- increasing amounts of sample: 250 mg, 500 mg, 3 g (the latter amount was suggested in the SRM certificate).

As no significant improvements of the recovery efficiencies were observed, it was decided to keep DCM as extraction solvent, 500 mg as sample amount and 3 cycles of extraction.
A different labelled compound for naphthalene quantification was tested (C^{13}-naphtalene instead of deuterated naphtalene), as for this compound the recoveries were higher than 100% but we observed that the recoveries were similar with the two labelled compounds.
A test was also carried out adding the labelled solution on the matrix (and not directly on the sample, as previously done), in order to investigate the effect of the matrix on labelled compounds retention, but lower recoveries were obtained.

2.4 Extraction of filtered sediment

Aliquots of 500 mg of the SRM 1941b were added to 1 l of Evian water and the samples were left to agitate overnight. The water was then filtered on glass fibre filters, which were extracted with ASE using the parameters tested before. Two filters were used for the

filtration of 1 l of water and they were extracted in the same cell. The recoveries were very low, probably because DCM is not able to extract the PAHs from a partially wet matrix. For this reason it was decided to try a new solvent, both for the extraction of the dry sediment and for the filtered sediment.

A mixture of n-hexane:acetone (1:1 v/v) was used and we obtained good results for the sediment while, for the filters, the recoveries were better than with DCM, but not yet satisfactory. The results are reported in tables 2 and 3.

	Mean recovery (%)	**Standard deviation**
Naphtalene	94	4
Phenanthrene	126	19
Anthracene	89	22
Fluoranthene	124	18
Benzo(b)fluoranthene	97	13
Benzo(k)fluoranthene	90	14
Benzo(a)pyrene	79	15
Indeno(123-cd)pyrene	72	7
Benzo(ghi)perylene	112	18

Table 2: mean recoveries for PAHs extracted from SRM 1941b with n-hexane: acetone.

	Mean recovery (%)	**Standard deviation**
Naphtalene	69	1
Phenanthrene	68	3
Anthracene	47	2
Fluoranthene	66	1
Benzo(b)fluoranthene	46	6
Benzo(k)fluoranthene	43	8
Benzo(a)pyrene	35	0
Indeno(123-cd)pyrene	23	5
Benzo(ghi)perylene	44	9

Table 3: mean recoveries for PAHs extracted from SRM 1941b in water (filters) with n-hexane:acetone.

2.5 Quechers tests

Finally, some samples were prepared and extracted with the Quechers (Quick Easy Cheap Effective Rugged Safe) technique, using both DCM and acetonitrile (ACN) as extraction solvents. This technique is widely used for the extraction of pesticide residues in particular

from food matrices (fruit, vegetables, cereals…). The procedure for the extraction used is the following:

1. Weighing of the sample (500 mg)
2. Addition of 5 ml of solvent
3. Equilibration: 1 min
4. Agitation: 2 min
5. Centrifugation (4000 rpm - 20 °C): 5 min
6. Filtration with 0,2 µl PTFE filters

Similar results were obtained for the two solvents used, which are reported in table 4 and 5. The recoveries were below 50%.

	Mean recovery (%)	**Standard deviation**
Naphtalene	29	1
Naphtalene*	32	3
Phenanthrene	52	1
Anthracene	30	1
Fluoranthene	51	1
Benzo(b)fluoranthene	51	1
Benzo(k)fluoranthene	47	1
Benzo(a)pyrene	38	1
Indeno(123-cd)pyrene	33	1
Benzo(ghi)perylene	44	3

Table 4: mean recoveries for PAHs extracted from SRM 1941b with DCM (Quechers)

	Mean recovery (%)	**Standard deviation**
Naphtalene	17	2
Phenanthrene	34	11
Anthracene	13	5
Fluoranthene	50	8
Benzo(b)fluoranthene	48	7
Benzo(k)fluoranthene	47	2
Benzo(a)pyrene	34	7
Indeno(123-cd)pyrene	34	4
Benzo(ghi)perylene	42	7

Table 5: mean recoveries for PAHs extracted from SRM 1941b with ACN (Quechers)

3 Conclusions

The activity presented in this report was carried out at LNE with the aim of developing a method for the extraction of 8 PAHs from particulate in water by means of ASE technique, in compliance with the European Water Framework Directive (WFD) [1].
The method has proved to be effective for the extraction of dry sediments, but it needs to be improved for the extraction of sediment filtered from water.
Further tests could include the extraction of the filters with different solvent like acetone, acetone:DCM mixture, or an optimized ratio of the mixture n-hexane:acetone, previously tested. Other tests could be carried out by adding low amount of sample in water, extracting one filter for each cell or drying the filters prior to the extraction.
Concerning the Quechers technique, other tests have to be done to improve the extraction procedure, also using different kinds of CRMs.

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