Metrological traceability of Polycyclic Aromatic Hydrocarbons (PAHs) measurements in green tea and mate

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Abstract:

The development of suitable analytical methods to obtain metrologically traceable results in the determination of toxicants in food matrices is an important issue, as food represents the main way of assumption of many contaminants, among which the Polycyclic Aromatic Hydrocarbons (PAHs). The present work deals with the set up and internal validation of an analytical method carried out at INRiM for the quantification by gascromatography coupled with mass spectrometry (GC-MS) of some priority PAHs in green tea (*Camellia sinensis*) and yerba mate (*Ilex Paraguaiensis*), in order to obtain metrologically traceable results. Two approaches for the quantification were applied: an external calibration, for determining the GC-MS calibration curves by means of standard reference solutions and an internal calibration by using perdeuterated standards. For the external calibration, Weighted (WLS) and Weighted Total (WTLS) Least Squares fitting procedures were applied. The measurement uncertainty evaluation was carried out by applying the Law of Propagation of Uncertainty.

Keywords: metrological traceability, measurement uncertainty, polycyclic aromatic hydrocarbons, food, green tea, yerba mate

1. Introduction

1.1. Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic Aromatic Hydrocarbons (PAHs) are organic micropollutants having an aromatic planar structure. They are present in all the environmental compartments and in food. PAHs are formed from incomplete combustion of organic substances, generated by anthropogenic sources (vehicular and industrial emissions, domestic heating, power plants, food cooking, tobacco) or natural sources to a less extent (bushfires, agricultural combustion, volcanic eruptions) [1] The formation mechanism of PAHs has not been completely clarified but the more accredited hypothesis is based on three formation steps: pyrolisis, pyrosynthesis and, finally, polymerisation.

PAHs are characterised by high stability and low reactivity, and for this reason they can be detected even at great distance from the emission sources. Their vapour pressure is generally low and reduces with increasing molecular weight. This trend influences the different percentages of PAHs distributed in the gaseous phase in atmosphere and adsorbed on soils, particulate matter and vegetation. PAHs having from 2 to 4 aromatic rings tend to stay in the gaseous form and have short half-life. PAHs with 5 or more benzene rings, which are the compounds with greater toxicological interest, do not persist for long periods in the atmosphere as gaseous molecules [2].

There is great concern for PAHs, due to their fallouts on human health. Scientific evidence showed that some PAHs may have carcinogenic effects. The most harmful of them is benzo[a]pyrene (BaP), which was classified as carcinogenic agent to humans (Group 1) by the International Agency for Research on Cancer (IARC) [3] and assumed as the marker molecule for the carcinogenic risk of the entire PAH class. Other PAHs are classified as non carcinogenic (group 3), possible carcinogenic (group 2B) or probable carcinogenic (group 2A).

The mutagenic and carcinogenic actions are due to the formation of PAHs derivatives by means of metabolic reactions occurring in the living organisms, meant to facilitate the expulsion of these compounds. The PAHs toxicity will depend on the balance between the metabolite formation rate and the rate of its removal. These metabolites, generally diol epoxides, can bind to the DNA chains, damaging their structures and thus leading to mutations and eventually to carcinogenesis [4].

The more validated hypothesis relates the PAHs reactivity to the number and relative position of the condensed rings. Experimental observations proved that a necessary condition for the PAH carcinogenic action to occur, is the presence in the structure of more than 4 benzene condensed rings. Indeed, the condensation reduces the aromatic properties facilitating the metabolic reactions and the consequent formation of carcinogenic metabolites. The presence of the so called “bay region”,

which can be formed from a particular ramification of the atoms in the benzene rings, can bestow a major reactivity upon the molecules [5].
Several studies were carried out to highlight the contributions of each environmental compartment to the exposure to PAHs of human beings [6]. The United States Environmental Protection Agency (US EPA) defined a list of 16 priority pollutants [7]. At the European level, PAHs are listed among the atmospheric pollutants to be taken into consideration in the assessment and management of ambient air quality [8,9].
The major source of exposure is food consumption. The exchange of PAHs between the atmosphere and the vegetation is a process which determines the passage of these pollutants in the food chain and has been studied for a long time [10-12]. Plants play a key role in the removal process of particulate matter and their associated PAHs [13] and the use of different plant species for biomonitoring was highlighted [14,15].
The most diffused opinion is that the principal route of contamination of the plant leaves is the atmospheric deposition and the consequent absorption from the leaf surface. Indeed, the plant leaves are covered by a thin structure named "cuticle", which avoids the loss of water and the attack from pathogen agents [16]. PAHs in solid phase (i.e. particulate matter) can deposit on the leaf surface and, due to the affinity with the low polarity of the cuticle, they can accumulate and absorb on the epicuticolar waxes. PAHs in the gaseous phase can also penetrate directly into the leaves through the stoma [15].
Following this process, fruit, vegetables, and sugars are the major sources of PAHs intake, but also meat, fish, milk and other beverages can give a considerable contribution. In an analysis of PAHs in the UK diet, a total daily consumption of 1.46 kg of food and beverages, led to a total daily dietary load of PAHs of 3.70 µg. A similar study of the Dutch diet estimated an average daily intake of PAHs between 5 and 17 µg/day. The major contributions were related to cereals and vegetable oils. In Italy, the dietary exposure to PAHs is equivalent to 3µg/day and is significantly higher than the respiratory intake from polluted urban air [17].
Several methods have been developed in this challenging field for different food matrices, among which liquid smoke condensates [18], edible oils [19], various kind of meat [20] and tea [21].

1.2 PAHs in food legislation

Concerning the legislation about PAHs and other contaminants in food matrices, since 2001 a series of regulations and directives of the European Union have been issued, which define the maximum limits of tolerance of the more significant contaminants in different food matrices.
The Regulation EC n. 1881/06 defines nine categories of food products and for each of them, the maximum level of BaP is fixed, expressed in µg/kg of fresh weight [22]. The Regulation EC n. 333/07 [23] states the performance criteria of the analytical methods for BaP determination in food matrices in accordance with [22].
Intense research and monitoring activity have been carried out on PAHs, due to their presence in food and to their assessed carcinogenic and mutagenic action. In 2002, the Scientific Commission on Food (SCF) identified a group of 15 PAHs, having genotoxic and carcinogenic properties, considered of major relevance in food analysis. Eight of them are also included in the 16 priority PAHs for US EPA. The Joint FAO/WHO Expert Group on Food Additives (JECFA) added to this list also benzo[c]fluoranthene, thus obtaining the 15+1 priority PAHs for food [24].
The Recommendation 2005/108/EC of the European Commission [25] collects data from the Member States concerning the presence of carcinogenic substances in different foodstuffs. The European Food Safety Authority (EFSA) reviewed the PAHs occurrence in various food items in the CONTAM panel [26] and dried tea was identified as one of the food categories with consistently high content of PAHs. The EFSA was invited to re-examine the opinion of the SCF of the European Commission taking into account the new available data on the presence of contaminants in food and to evaluate the significance of using BaP as the toxicity marker of the PAHs class. The EFSA suggested that the use of a system of four specific molecules could be more suitable to evaluate the presence and toxicity of PAHs in food and that the BaP alone was not a good marker for the whole class. In the Commission Regulation EU n. 835/2011, the maximum level of the sum of four PAHs (BaP, chrysene, benzo[a]anthracene and benzo[b]fluoranthene), is added to the level set for BaP [27].

1.3 Scope of the work

The use of reliable analytical methods for the determination of PAHs is necessary in order to monitor accurately the levels of these toxicants in food and in the environment in general. Metrological traceability of measurement results is a fundamental feature in every measurement field but is crucial when dealing with the analytical chemistry where the application of metrological concepts is not straightforward, as the analytes are usually present at very low concentrations, even in trace, and the sample matrix can generate interferences during the instrumental identification and quantification of the analytes of interest.
The present work deals with the development and internal validation carried out at INRiM of a metrologically traceable procedure for the quantification of 8 priority PAHs in two matrices, i.e. green tea (*Camellia Sinensis*) and yerba mate (*Ilex Paraguaiensis*). Part of the work was devoted to the application of two approaches for the quantification and to highlight advantages and drawbacks of each method, comparing the results obtained. A proper uncertainty evaluation was carried out by applying the Law of Propagation of Uncertainty, taking into account all the relevant sources, as prescribed in the GUM uncertainty framework [28].

The choice of green tea and mate is due to the fact that they are infusions largely consumed worldwide, in particular in Eastern and Southern countries. Recently, the attention of the international metrology organisations has been directed to such food matrices by organising and promoting international Key Comparisons in the framework of the International Committee for Weights and Measurements (CIPM). The final goal is the assurance of the equivalence of the measurements carried out by the Countries adherent to the Meter Convention [29] and the establishment of metrological traceability chains in new fields, such as food metrology. One instrument to reach such goal is the identification of possible matrix reference materials, and the International Comparisons heavily contribute to this activity.

2. INRiM method for the analysis of priority PAHs in vegetable matrices

2.1. Choice of the analytes

The first problem when dealing with the analysis of PAHs in food, is the variety of matrices that can be contaminated by PAHs. In this work, the choice was directed to two common infusion beverages, green tea and yerba mate. The matrices were purchased in local markets in Torino, Italy.
The PAHs analysed in this work are the ones that are classified both among the 15+1 priority PAHs for food by the European Union and among the 16 priority PAHs for the US EPA.
The 8 PAHs considered are listed in table 1.

IUPAC Name	Abbreviation	Molecolar Mass [u.m.a.]
Benzo[*a*]antracene	BaA	228.30
Benzo[*a*]pyrene	BaP	252.32
Benzo[*b*]fluoranthene	BbF	252.32
Benzo[*k*]fluoranthene	BkF	252.32
Benzo[*ghi*]perylene	BgP	276.34
Chrysene	CHR	228.30
Dibenzo[*a*,*h*]antracene	DhA	278.35
Indeno[1,2,3-*cd*]pyrene	IcP	276.34

Tab. 1: list of the 8 PAHs chosen for the quantification

2.2 Materials and methods

The experimental procedure was articulated in the following stages:

- sample preparation;
- extraction of PAHs from the samples;
- quantification of the various PAHs by means of gaschromatography coupled with mass spectrometry (GC-MS);
- evaluation of the measurement uncertainty associated to the final concentrations of the analytes, taking into account all the significant uncertainty sources in the procedure.

PAHs extraction from the matrices was carried out by means of Soxhlet extractors, following the guidelines of EPA given in method 3540c, which deals with the extraction of non volatile or semi-volatile organic compounds from solid matrices [30].
This choice of some priority PAHs allowed the use, for the quantification, of a Certified Reference Material, NIST SRM 1647e containing the 16 US EPA priority PAHs.
The solvents used were acetone grade ≥ 99.5 % (Sigma Aldrich, Switzerland) and *n*-hexane for pesticide residue analysis (Fluka, Switzerland).
The quantification was carried out by applying different approaches:

- external calibrations of the GC-MS by using metrologically traceable reference solutions, prepared by diluting the NIST SRM 1647e and by applying different regression procedures;
- internal calibration, by using perdeuterated internal standards, where the unknown measurand is expressed as a function of the internal standard amount and response obtained in each chromatographic run. This last approach was used only for the PAHs for which a suitable labelled internal standard was available.

Since a standard method for the quantification of PAHs in the studied matrices is not available, the developed method had to be validated in all its steps, including linearity, repeatability, recovery efficiency, limit of detection (LOD) and limit of quantification (LOQ). The aim of the present work was the establishment of metrological traceability for each analytical step.

2.3. Sample preparation

For the different matrices, samples of about 1 g of dried and ground leaves were used. The samples to be extracted and the calibration solutions were gravimetrically prepared thus assuring metrological traceability by using calibrated mass standards (see Section 3.1). The same weighing procedure was also used for the determination of the final volumes of the extracts, which were calculated from the weighed masses of each extract, taking into account the density of the extraction solvent [31].
The extraction of PAHs from the samples was carried out by means of Soxhlet extraction using cellulose fibre thimbles. The extractors had a nominal volume of 30 ml and were equipped with 100 ml round-bottomed flasks. These extractors, having small dimensions, allow reducing the extraction time and the volume of solvent required. For the extraction, a mixture of acetone/*n*-hexane (1:1 *v*/*v*) was used, as prescribed in [30]. The extractions lasted 8 hours, with an average of 13-15 extraction cycles/h. At the end of the extraction, the extracts were collected and filtered on paper filters. The solvent mixture allows obtaining sufficiently clean extracts, giving well resolved chromatographic peaks and low signal to noise ratio, thus avoiding the clean-up step and the pre-concentration of the samples.
In figure 1, the method for the quantification of 8 PAHs in green tea and mate is summarised.

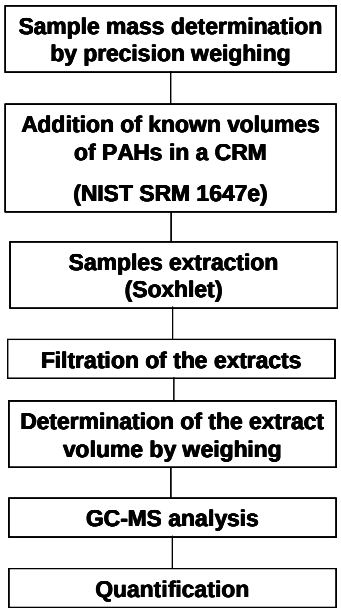

Fig. 1: scheme of the method developed for the quantification of 8 PAHs in green tea and mate

2.4. Method of analysis

The identification and quantification of PAHs were carried out by using a GC-MS Focus DSQ II Thermofisher Scientific, equipped with a gas-chromatographic column Thermo Scientific TR-5ms with the following characteristics:

- internal film thickness: 0.25 μm,
- internal diameter: 0.25 mm,
- length: 30 m
- stationary phase: 5 % phenyl polysilphenylene-siloxane

The operational parameters of the analytical method are reported in table 2.

Operational parameters	Experimental conditions
Column temperature	70 °C (hold 2 min) gradient 25 °C/min at 180 °C gradient 5 °C/min at 300 °C (hold 4 min)
Injector temperature	300 °C
Injection mode	Splitless (1 min)
Carrier gas	Helium
Carrier gas flow	1.2 ml/min
Transfer line temperature	270 °C
Analysis mode	SIM (228 – 252 – 264 – 276 – 278 – 288 m/z)
Start time	7 min
Ion source temperature	250 °C
Injected volume	2 µl

Tab. 2: operational parameters for the GC-MS method

In figure 2 a typical chromatogram of the analysis of a solution at 10 ng/ml shows the elution order of the analysed PAHs.

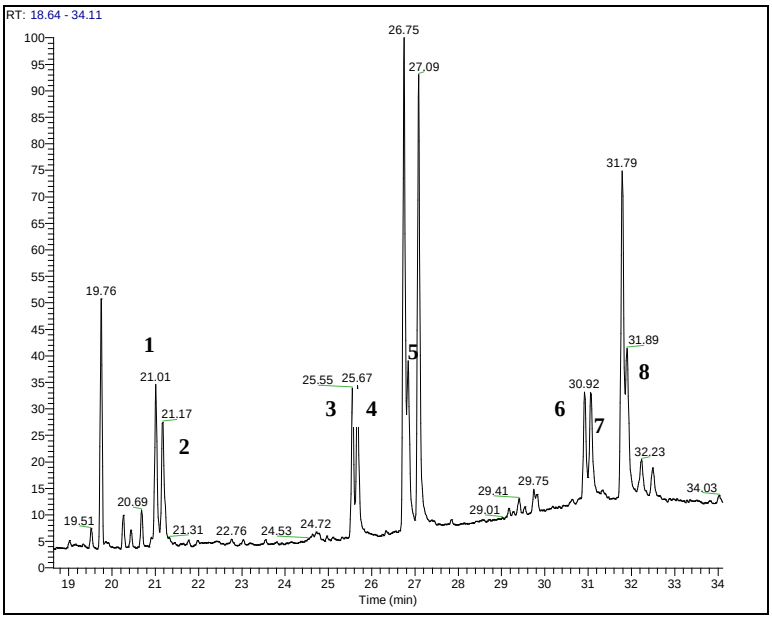

Fig. 2: chromatogram of a solution at 10 ng/ml of the analysed PAHs. The elution order is: 1) benzo[a]anthracene (21.01min); 2) chrysene (21.17 min);3) benzo[b]fluoranthene (25.55 min); 4) benzo[k]fluoranthene (25.67 min); 5) benzo[a]pyrene (26.84 min); 6) indeno[1,2,3-cd]pyrene (30.92 min); 7) dibenzo[a,h]anthracene (31.07 min); 8) benzo[ghi]perylene (31.89 min).

The quantification of PAHs has to be carried out by using some isotopically labelled internal standards; in particular two perdeuterated compounds having the same chemico-physical properties of the analyte but different molecular weight were used. In figure 2, the chromatographic peaks at 26.75 min (benzo[a]pyrene-d_{12}; 264 m/z), and 31.79 min (benzo[ghi]perylene-d_{12}; 288 m/z) correspond to the perdeuterated PAHs used for the quantification with the internal standard method while the peak at 27.09 min (perylene-d_{12}; 264 m/z) was used for the normalisation of the instrumental response in the external quantification.

The extracts of green tea and mate were injected and preliminary analysed in full scan mode. The chromatogram of the tea extract is reported, as an example, in figure 3. The chromatographic peaks of the PAHs correspond to the elution order reported in figure 2.

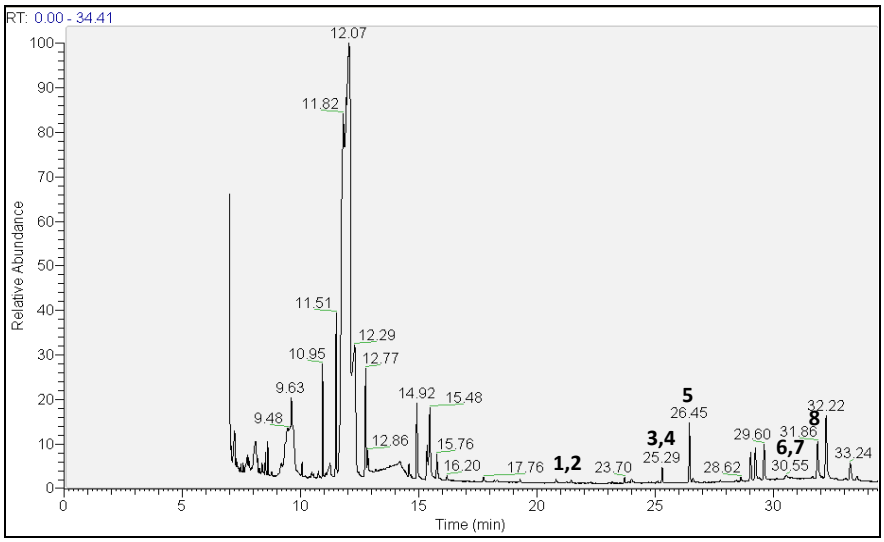

Fig. 3: chromatogram of the green tea extract analysed in full scan mode.

The extracts of the two beverages are very similar, except for the presence of a very intense peak at the retention time around 12 min, which corresponds to the theine and is clearly visible in the green tea extract. The same peak is present in mate, but the intensity is considerably lower.
Since the theine peak is particulary intense, but it does not overlap with the PAHs peaks, it was decided to analyse the extracts without purifying them prior to the analysis, by using Selected Ion Monitoring (SIM) mode. The ions chosen for the quantification of the analytes are reported in table n. 2. Figure 4 shows, as an example, the SIM chromatogram of the analysis of a green tea extract.

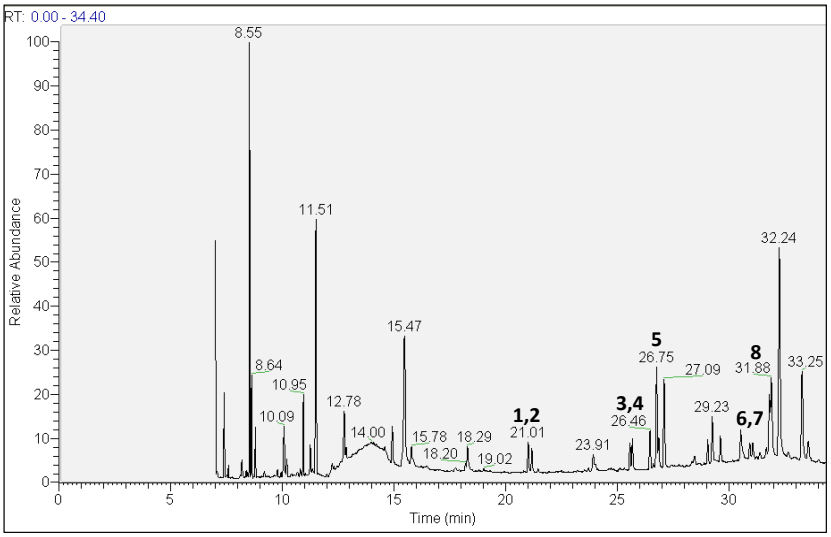

Fig. 4: chromatogram in SIM mode of the analysis of a green tea extract (masses: 228 – 252 – 276 – 278 m/z).

2.5. Internal validation of the method

On each matrix, blank analyses were carried out by applying the extraction method on 1 g aliquots of non-spiked samples.
The Limit of Detection (LOD) of the quantified PAHs was determined from the standard deviation S_b of n = 10 repeated injections of a blank sample, following the guidelines given in [32]:

From the value of S_b, the minimum detectable concentration D_M of the single analyte can be obtained, by applying equation 1:

$$D_M = t_{n-1;0.95} \cdot S_b \quad (1)$$

where:

D_M = minimum detectable concentration of the analyte in ng/ml
$t_{n\text{-}1;0.95}$ = Student's *t* value for *n* measurements and a 95 % level of confidence
S_b = standard deviation of the sample blanks expressed in ng/ml.

The limit of quantification (LOQ) was calculated as 10 times the standard deviation of the blanks, as found in literature [33]. Table 3 reports the values for LOD and LOQ for green tea matrix, chosen as the more challenging one. Similar values were obtained also for mate.

PAH	Limit of detection [µg/kg]	Limit of quantification [µg/kg]
Benzo[*a*]anthracene	0.6	2.5
Chrysene	0.7	3.1
Benzo[*b*]fluoranthene	0.3	1.3
Benzo[*k*]fluoranthene	0.3	1.3
Benzo[*a*]pyrene	0.7	3.1
Indeno[1,2,3-*cd*]pyrene	0.7	3.1
Benzo[*ghi*]perylene	0.4	1.8
Dibenzo[*a,h*]anthracene	0.7	3.1

Tab. 3: LOD and LOQ for the eight quantified PAHs in green tea

It has to be noted that the performance criteria reported in the EC Regulation [23] use a different approach for the calculation of LOD and LOQ, with respect to the criteria chosen in this work [32, 33]. In particular, the approach followed here takes into account the standard deviation of repeated measurements, instead of the standard deviation of the mean. The LOD and LOQ for BaP, calculated in accordance with [23] are 0.3 µg/kg and 0.6 µg/kg, respectively, thus in agreement with the above mentioned EC Regulation.

The recovery efficiency was evaluated by spiking a known volume of the NIST SRM 1647e (100 µl) on 1 g subsamples of green tea and mate. The spiking level varied from 350 µg/kg to 500 µg/kg, depending on the mass fraction of each PAH in the SRM. The spiked samples were then extracted and the recovery efficiency was therefore evaluated by dividing the PAHs concentrations determined in the spiked samples by the theoretical concentrations of each PAH. The quantification approach used for the recovery efficiency calculation was the external calibration (WLS). In table 4 the recovery efficiencies obtained for the PAHs are reported.

PAH	Green tea Recovery (*R*%)	Mate Recovery (*R*%)
Benzo[*a*]anthracene	91	89
Chrysene	87	105
Benzo[*b*]fluoranthene	76	77
Benzo[*k*]fluoranthene	71	73
Benzo[*a*]pyrene	77	87
Indeno[1,2,3-*cd*]pyrene	48	60
Benzo[*ghi*]perylene	37	45
Dibenzo[*a,h*]antracene	81	50

Tab. 5: values of the recovery efficiencies *R* obtained for quantified the PAHs.

As for the smaller PAHs, having 4 or 5 benzene rings, the recovery efficiencies were generally satisfactory and in compliance with [23], which prescribes recoveries between 50 % and 120 %. For the heavier PAHs, the recoveries were considerably lower, in some cases even below 50 %. These results might be due to the low analyte polarities, which may lead to a limited affinity with the solvent mixture that is not sufficient for a quantitative extraction.

3 External calibration based on Weighted Least Squares (WLS) and Weighted Total Least Squares (WTLS) approaches

3.1 Preparation of the calibration solutions and quantification procedure

The metrological traceability of the quantification step was assured by calibrating the GC-MS with a set of reference standard solutions prepared by gravimetric dilution of NIST SRM 1647e.
The solutions were prepared in three subsequent steps, weighing the empty volumetric flasks and weighing the flask after the addition of the aliquots of solution and solvent. The solutions were prepared in *n*-hexane (Fluka Analytical for pesticide residue analysis). The weighings were carried out on a Mettler-Toledo H51AR balance using calibrated mass standards (class E2, Häfner, Germany). The weighing of the volumetric flasks followed the double substitution scheme:

$$M_C \; - \; U \; - \; M_C+m \; - \; U+m$$

where:

M_C = calibrated mass standards
U = unknown sample (volumetric flask)
$M_C + m$ = calibrated mass standards + sensitivity masses
$U + m$ = unknown sample + sensitivity masses.

This cycle was repeated 3 times for each preparation step of the solutions. From the masses calculated in each weighing step, the mass fractions (ng/g) of the PAHs in the three calibration solutions were calculated, with the associated combined standard uncertainties. The mass fractions were then converted into mass/volume concentrations (ng/ml), by multiplying each mass fraction by the densities of each calibration solution.
The quantification procedure was carried out by analysing each extract in one day and by injecting the extract and the calibration solutions several times to take into account possible instrumental drift and to evaluate also the instrumental repeatability.

As internal standard the certified reference solution NIST SRM 2270 was used. It contains 6 perdeuterated PAHs in hexane/toluene mixture (96:4 *v*/*v*). A known volume of internal standard was added to the calibration solutions and to the extracts, in order to obtain a constant concentration of internal standard in each aliquot to be analysed by GC-MS. For the external calibration, the internal standard was used to normalise the instrumental response and benzo[a]pyrene–d_{12} having a 5 rings structure, was used for benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene; benzo[ghi]perylene-d_{12}, having a 6 rings structure, was used for dibenzo[a,h]anthracene, benzo[ghi]perylene and indeno[1,2,3-cd]pyrene. The SRM was diluted (1:500) to have concentrations of the internal standard compatible with the analytes concentrations.

3.2 External calibration based on Weighted Least Squares (WLS) regression and uncertainty evaluation

The calibration curves for the determination of the concentration *C* of PAHs in the extracts were obtained by means of an algorithm developed at INRiM [34], based on the Weighted Least Squares (WLS) regression which calculates a linear correction to be applied to the instrument readings according to equation 2:

$$x = y + d(y) = y + \alpha_0 + \alpha_1 \cdot y \tag{2}$$

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.

After the calibration the coefficients α_0, α_1 are estimated as α'_0, α'_1. The unknown concentration *C* of PAHs can be calculated according to equation 3:

$$C = y + d(y) = y + \alpha'_0 + \alpha'_1 \cdot y \tag{3}$$

This model allows to take into account both the uncertainty contribution due to the calibration curve and to instrument repeatability.
A fundamental contribution to the correction uncertainty derives from the uncertainty of the concentrations of the calibration solutions used within the calibration process.
The uncertainty budget for the PAHs in the calibration solutions was developed taking into account all the possible uncertainty sources, coming both 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 covariances between the mass values measured in the weighing procedure of the calibration solutions were taken into account, as described in [35].
In figure 5, the Ishikawa diagram (or "fishbone" graph) shows the uncertainty components for each input quantity in the model equation 3. The input quantities are the coefficients of the correction curve, α'_0 and α'_1 (implicitly influenced by the instrumental readings corresponding to the calibration solutions) and the instrumental readings *y* corresponding to the samples analysed.

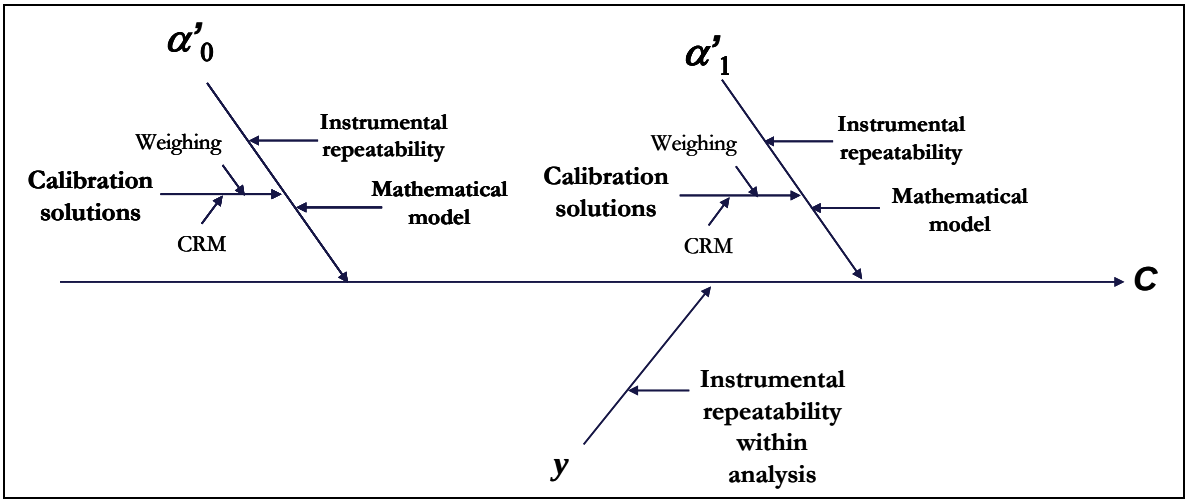

Fig. 5: Ishikawa diagram for the concentration *C* of the PAHs by WLS calibration.

3.2 External calibration based on Weighted Least Squares (WTLS) regression and uncertainty evaluation

For the external calibration, the Calibration Curves Computing (CCC) software was used, which is a MATLAB-based software recently developed at INRiM within the EMRP Project NEW04 [36]. With this software, a Weighted Total Least Squares (WTLS) fitting procedure was also applied for directly determining the straight line calibration curve:

$$x = a + by \tag{4}$$

where:
x = concentration of the PAH in the standard solutions
y = instrument output (normalised areas of the chromatographic peaks).

The WTLS method can take into account correlations and uncertainties both in the dependent and the independent variables [37].
Hence, this method is suitable for the quantification of PAHs, as both the variables x and y are subjected to uncertainty.
From the estimated coefficients a' and b' and the instrumental readings y, the uncertainty on the concentrations of PAHs $u(C)$ can be calculated, by applying the law of propagation of the uncertainty [28] to the following model equation 5:

$$C = a' + b'y \tag{5}$$

In figure 6, the Ishikawa diagram shows the uncertainty components for each input quantity in the model equation 5 of the PAHs concentration *C*.

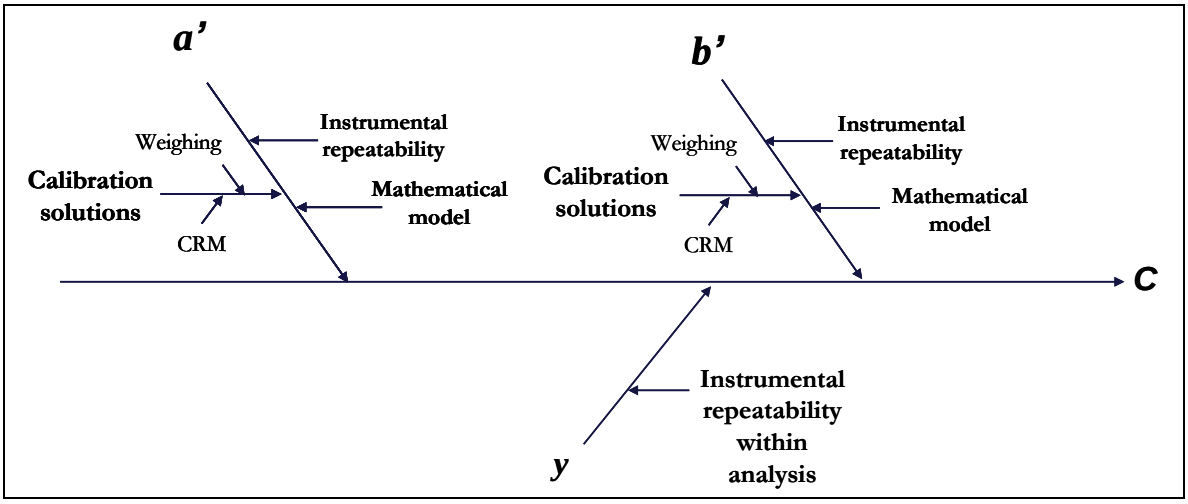

Fig. 6: Ishikawa diagram for the concentration *C* of the PAHs by WTLS calibration.

4. Quantification of the PAHs by means of internal calibration and uncertainty evaluation

The internal standard (IS) quantification is based on the assumption that the instrumental response is equal for different compounds, having similar structure and comparable concentration. An internal standard is chosen as a reference, which has chemical structure similar to the analytes, but is not present in the sample. The internal standard is added to the sample prior to the quantification. The internal standard has to be analysed in the same way of the unknown analytes, in order to obtain an instrumental response subjected to the same variability of the unknown analytes. The relationship assumed is expressed in equation 6:

$$C_{IS} : A_{IS} = C : A \tag{6}$$

where

C_{IS} = perdeuterated PAH concentration expressed in ng/ml
A_{IS} = chromatographic peak area of the perdeuterated PAH
C = PAH concentration expressed in ng/ml
A = chromatographic peak area of the PAH.

The concentration of each PAH can be calculated from equation 7:

$$C = \frac{C_{IS} \cdot A}{A_{IS}} \tag{7}$$

On the basis of the previous considerations, benzo[a]pyrene-d_{12} was used as IS for benzo[*a*]anthracene (assuming a response factor equal to 1) and for benzo[*a*]pyrene, while benzo[*ghi*]perilene-d_{12} was used for benzo[*ghi*]perylene.

For the evaluation of the uncertainty of the PAHs concentrations, the contributions of each input quantity in model equation 7, were considered. Due to the high variability of the instrumental signals, the areas of the analytes and of the IS were also normalised for a different perdeuterated PAH, perylene-d_{12}. The expression of the combined standard uncertainty $u(C)$, reported in equation 8, is obtained by applying the GUM approach [28]:

$$u(C) = C\sqrt{\left(\frac{u(A)}{A}\right)^2 + \left(\frac{u(C_{IS})}{C_{IS}}\right)^2 + \left(\frac{u(A_{IS})}{A_{IS}}\right)^2 + 2 \cdot 0.9 \cdot \left(\frac{u(A) \cdot u(A_{IS})}{A \cdot A_{IS}}\right)} \tag{8}$$

where:

- $u(A)/A$ = relative standard uncertainty of the instrumental response for each PAH, evaluate as standard deviation of the mean of the areas of the three repetitions carried out for each PAH.
- $u(A_{IS})/A_{IS}$ = relative standard uncertainty of the instrumental response for the IS used for the quantification;
- $u(C_{IS})/C_{IS}$ = relative standard uncertainty of the final concentration of the IS, obtained by dilution of the CRM;
- $0.9 \cdot [(u(A)\ u(A_{IS}))/(A\ A_{IS})]$ = relative covariance between the areas A e A_{IS}, which are correlated since they are generated in the same analytical run.

In figure 7, the Ishikawa diagram shows the uncertainty components for each input quantity contributing to the concentration C of the PAHs, obtained by the internal standard calibration.

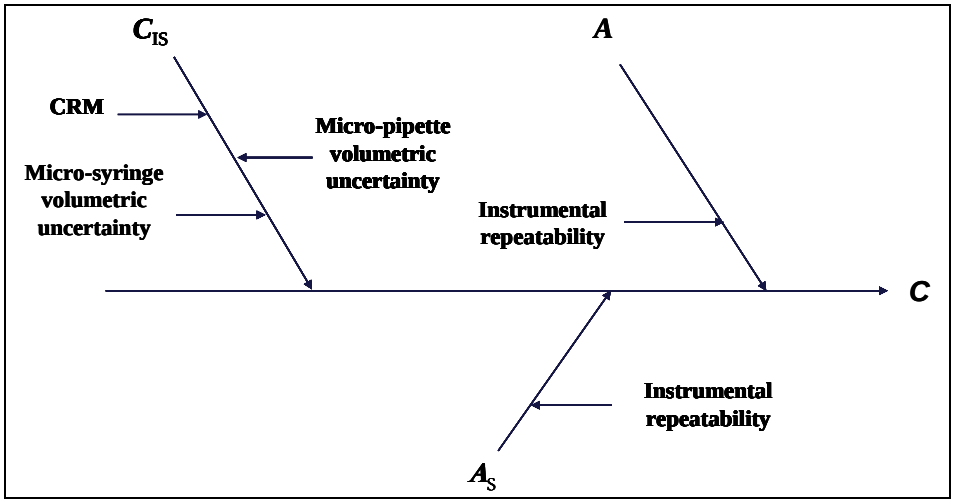

Fig. 7: Ishikawa diagram for the concentration C of the PAHs by IS calibration.

5. Results and discussion

The PAHs concentrations in the extracts of green tea and mate were calculated by means of the WLS procedure for the 8 PAHs examined. The results are reported in tables 6 and 7.

PAH	Nominal concentration [ng/ml]	Calculated concentration [ng/ml]	Standard uncertainty $u(x_i)$ [ng/ml]	Expanded uncertainty $U(x_i)$ [ng/ml]
Benzo[*a*]anthracene	15.4	14.0	2.7	5.4
Chrysene	13.6	11.8	4.0	8.0
Benzo[*b*]fluoranthene	15.8	12.1	2.9	5.8
Benzo[*k*]fluoranthene	17.7	12.5	2.7	5.4
Benzo[*a*]pyrene	18.3	14.1	3.2	6.4
Indeno[1,2,3-*cd*]pyrene	16.1	7.8	4.9	9.8
Benzo[*ghi*]perylene	13.8	5.1	6.4	12.8
Dibenzo[*a,h*]anthracene	13.1	10.6	7.3	14.6

Tab. 6: nominal and calculated concentrations for the PAHs in the green tea extract, with the corresponding combined standard uncertainties and expanded uncertainties (k = 2), obtained by applying the WLS calibration.

PAH	Nominal concentration [ng/ml]	Calculated concentration [ng/ml]	Standard uncertainty $u(x_i)$ [ng/ml]	Expanded uncertainty $U(x_i)$ [ng/ml]
Benzo[*a*]anthracene	16.9	14.9	2.5	5.0
Chryisene	14.8	15.5	3.5	7.0
Benzo[*b*]fluoranthene	17.3	13.4	2.9	5.8
Benzo[*k*]fluoranthene	19.3	14.1	2.6	5.2
Benzo[*a*]pyrene	20.1	17.4	1.8	3.6
Indeno[1,2,3-*cd*]pyrene	17.6	10.6	4.8	9.6
Benzo[*ghi*]perylene	15.1	6.8	6.3	12.6
Dibenzo[*a,h*]anthracene	14.4	7.2	7.4	14.8

Tab. 7: nominal and calculated concentrations for the PAHs in the mate extract, with the corresponding combined standard uncertainties and expanded uncertainties (k = 2), obtained by applying the WLS calibration.

The uncertainties of the heavier PAHs (indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene and dibenzo[*a,h*]anthracene) are particularly high and this is due to the mathematical model used in this approach, which is not able to give a linear response for concentrations very close to the low limit of the calibration range: the assumption of the linearity in the entire calibration range adds a significant contribution to the final combined standard uncertainty.

By applying the IS method, the concentrations of benzo[*a*]anthracene, benzo[*a*]pyrene and benzo[*ghi*]perylene were also calculated. The results are reported in table 8.

PAH in green tea	Nominal concentration [ng/ml]	Calculated concentration [ng/ml]	Standard uncertainty $u(x_i)$ [ng/ml]	Expanded uncertainty $U(x_i)$ [ng/ml]
Benzo[*a*]antracene	15.4	15.0	0.9	1.9
Benzo[*a*]pyrene	18.3	13.9	0.5	1.1
Benzo[*ghi*]perylene	13.8	9.6	0.3	0.7
PAH in mate	**Nominal concentration [ng/ml]**	**Calculated concentration [ng/ml]**	**Standard uncertainty $u(x_i)$ [ng/ml]**	**Expanded uncertainty $U(x_i)$ [ng/ml]**
Benzo[*a*]antracene	16.9	15.8	1.2	2.4
Benzo[*a*]pyrene	20.1	15.6	1.1	2.2
Benzo[*ghi*]perylene	15.1	11.5	0.4	0.9

Tab.8: nominal and calculated concentrations of some representative PAHs in green tea and mate, obtained by applying the internal standard method, with the associated combined standard uncertainties and expanded uncertainties (k = 2).

As an example, the green tea matrix was chosen to carry out a comparison between the results obtained by applying the different described approaches, because this matrix shows a larger amount of interferents with respect to mate, and this characteristic could affect the quantification step.

In figures 8, 9 and 10, the comparisons between the results obtained with the different tested approaches are presented, and the calculated values are compared also with the nominal concentration values, obtained by sample spiking.

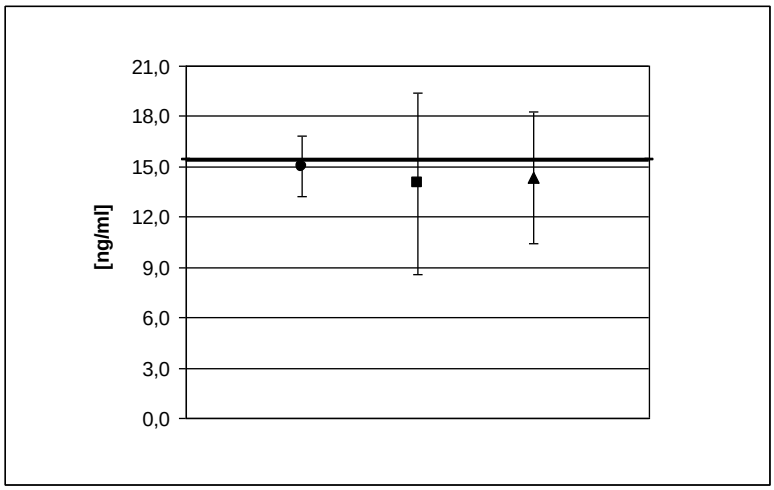

Fig. 8: comparison of the results obtained for benzo[a]anthracene in green tea, by means of IS (●), WLS (■) and WTLS (▲) approaches. The horizontal line is the nominal concentration of benzo[a]anthracene (15.4 ng/ml).

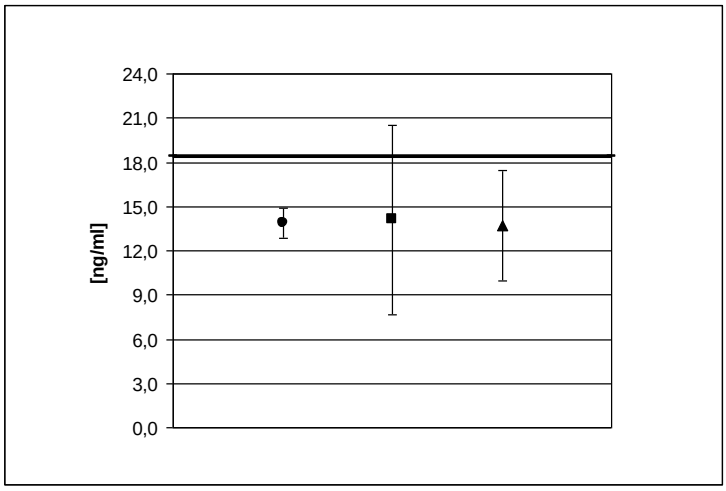

Fig. 9: comparison of the results obtained for benzo[a]pyrene in green tea, by means of IS (●), WLS (■) and WTLS (▲) approaches. The horizontal line is the nominal concentration of benzo[a]pyrene (18.3 ng/ml).

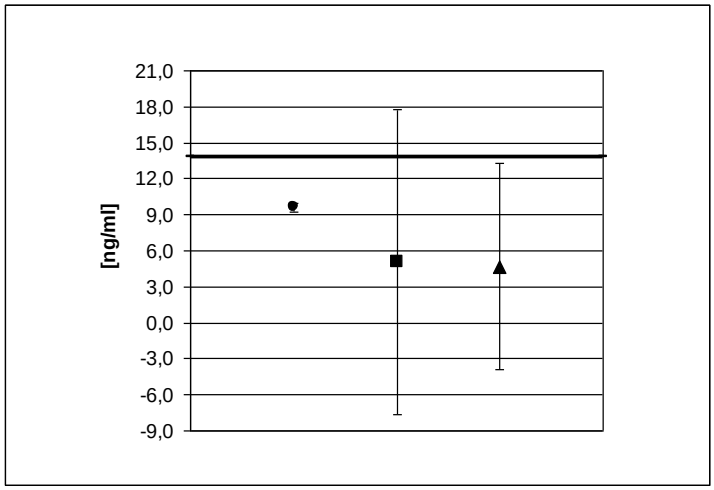

Fig. 10: comparison of the results obtained for benzo[ghi]perylene in green tea, by means of IS (●), WLS (■) and WTLS (▲) approaches. The horizontal line is the nominal concentration of benzo[ghi]perylene (13.8 ng/ml).

The application of the IS method to the quantification of some PAHs in green tea showed good estimates and uncertainties. The uncertainties with this approach are very small but this could be a drawback when the recoveries of the analytes are not satisfactory, as can be seen in figures 9 and 10. The IS method also requires the use of a suitable isotopically-labelled PAH, or the calculation of the response factors for each analyte in the case a proper IS is not available.
The external calibration avoids the use of the IS but larger uncertainties are obtained, due to the regression procedure, which generates a further uncertainty contribution. Generally, WTLS provides lower uncertainty than WLS. The external calibration by WLS gives very high uncertainty for benzo[ghi]perylene as its concentration is near to the lower limit of the calibration range and the instrumental response is not linear in the entire calibration range. Moreover, since the molecule is very heavy, the retention times are larger, the peaks not very well resolved and the variability in the areas in different runs quite large. Such large uncertainties in the peak areas lead to a poor goodness of fit.
From the comparison of the three approaches, it can be highlighted that:

- For 4 benzene rings PAHs (as benzo[a]anthracene) good consistency between the approaches and very good recovery were obtained;
- For 5 benzene rings PAHs (as benzo[a]pyrene): good consistency and good recovery were obtained;
- For 6 benzene rings PAHs (as benzo[ghi]perylene): poor consistency and unsatisfactory recovery were obtained.

6. Conclusions

The analysis of PAHs in food is a challenging field both for analytical method development and establishment of metrological traceability, to support risk assessment for human health. Food contamination with PAHs is primarily connected to cooking practices, but environmental contamination is also possible [38].
The analytical procedure developed and validated at INRiM allowed the quantification of 8 PAHs classified as priority pollutants by the US EPA and by the European Commission. Spiked samples were analysed and metrological traceability was established in each step of the analytical procedure, which can be applied to real contaminated samples.
The metrological approach followed at INRiM has very important fallouts in the field of food contaminant measurements, in particular for the significance of the considered analytes: metrological traceability of the results of these measurements is crucial also from an epidemiological point of view, to establish the correlations between human exposure to PAHs and their effects on human health. This is the starting point for the realisation of preventive actions for the reduction of negative effects deriving from the exposure of living beings to such toxicants.
Further developments are foreseen in order to implement the internal standard method for the selected PAHs, evaluating the response factors, as the internal standard method gives the best results both in terms of calculated concentrations and associated uncertainties. Finally, the recovery efficiencies will be improved, also by applying different extraction techniques and other food matrices of interest could be investigated.

References

[1] Tamakawa K., Kato T., Oba M., Polycyclic Aromatic Hydrocarbons in the environment, *Food Sci. Technol.*, 77(2), (1996), 1641-1663.

[2] Odabasi M. *et al*, *Sci. Tot. Environ.*, 227, (1999), 57-67.

[3] http://monographs.iarc.fr/ENG/Classification/index.php

[4] Shimada T., Fujii-Kuriyama Y., *Cancer Science*, 95 (1), (2004), 1-6.

[5] Lehr R.E., Kumar S., Levin W., Wood A.W., Chang R.L., Conney A. H., Yagi H., Sayer J.M., Jerina D.M., Polycyclic Hydrocarbons and Carcinogenesis, ed. Ronald G. Harvey, American Chemical Society, 283, (1985), 63-84

[6] Shibamoto T., Chromatographic Science Series, 77, Editor Marcel Dekker, New York, (1998).

[7] US EPA, Appendix A to 40 CFR, Part 423--126 Priority Pollutants (http://www.epa.gov).

[8] Council Directive 96/62/EC of 27 September 1996 on ambient air quality assessment and management, OJ L 296 , 21.11.1996 0055 - 0063.

[9] Directive 2004/107/EC of the European Parliament and of the Council of 15 December 2004 relating to arsenic, cadmium, mercury, nickel and polycyclic aromatic hydrocarbons in ambient air, OJ L 23, 26.1.2005, 3–16.

[10] Barber J. L., Thomas G. O., Kerstiens G., Jones K. C., Current issues and uncertainties in the measurement and modelling of air-vegetation exchange and within-plant processing of POPs, *Environ. Pollut.*, 128, (2004), 99-138.

[11] Smith K. E. C., Jones K. C., Particle and vegetation: implications for the transfer of particle-bound organic contaminants to vegetation, *Sci. Tot. Environ.*, 246, (2000), 207-236.

[12] Franzaring J., van der Eerden L. J. M., Accumulation of airborne persistent organic pollutants (POPs) in plants, *Basic Appl. Ecol.*, 1, (2000), 25-30.

[13] Terzaghi E. Wild E., Zacchello G., Cerabolini B. E. L., Jones K. C., Di Guardo A., Forest Filter Effect: Role of leaves in capturing/re leasing air particulate and its associated PAHs, *Atmos. Environ.*, 74, (2013), 378-384.

[14] Murakami M., Abe M., Kakumoto Y., Kawano H., Fukasawa H., Saha M., Takada H., Evaluation of ginko as biomonitor of airborne polycyclic aromatic hydrocarbons, *Atmos. Environ.*, 54, (2012), 9-17.

[15] Lehndorff E., Schwark L., Biomonitoring of air quality in the Cologne Conurbation using pine needles as a passive sampler-Part II: polycyclic aromatic hydrocarbons (PAH). *Atmos. Environ.*, 38, (2004), 3793-3808.

[16] Salisbury F. B., Ross C., Plant Physiology, (1992) 4th ed. Wadsworth Publishing, Belmont, California.

[17] Phillips D. H., Polycyclic aromatic hydrocarbons in the diet, *Mutat. Res.*, 443, (1999), 139-147.

[18] Simon R., Gómez-Ruiz J. Á., Wenzl T., Results of an European inter-laboratory comparison study on the determination of the 15 + 1 EU priority polycyclic aromatic hydrocarbons (PAHs) in liquid smoke condensates, *Food Chem.*, 123, (2010), 819-826.

[19] Hollosi L., Wenzl T., Development and optimisation of a dopant assisted liquid chromatographic-atmospheric pressure photo ionisation-tandem mass spectrometric method for the determination of 15 + 1 EU priority PAHs in edible oils, *Journal Chrom. A*, 1218, (2011), 23-31.

[20] Gómez-Ruiz J. Á., Cordeiro F., López P., Wenzl T., Optimisation and validation of programmed temperature vaporization (PTV) injection in solvent vent mode for the analysis of the 15 + 1 EU-priority PAHs by GC-MS, *Talanta*, 80, (2009), 643-650.

[21] Drabova L., Pulkrabova J., Kalachova K., Tomaniova M., Kocourek V., Hajslova J., Rapid determination of polycyclic aromatic hycrocarbons (PAHs) in tea using two-dimensional gas chromatography coupled with time of flight mass spectrometry, *Talanta*, 100, (2012), 207-216.

[22] Commission Regulation (EC) N. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, OJ L 364, 20.12.2006, 5–24

[23] Commission regulation (EC) N. 333/2007 of 28 March 2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs (text with EEA relevance), OJ L 88, 29.3.2007, 29-38

[24] Evaluation of certain food contaminants. In WHO (Ed.), WHO, technical report series 930. Geneva: JECFA (2006)

[25] Commission Recommendation 2005/108/EC of 4 February 2005 on the further investigation into the levels of polycyclic aromatic hydrocarbons in certain foods (notified under document number C(2005) 256), OJ L 34, 8.2.2005, 43–45

[26] http://www.efsa.europa.eu/en/panels/contam.htm

[27] Commission Regulation (EU) N. 835/2011 of 19 August 2011 amending Regulation (EC) N. 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs, OJ L 215, 20.8.2011, 4–8

[28] JCGM 100:2008 "GUM 1995 with minor corrections – Evaluation of measurement data – Guide to the expression of uncertainty in measurements"

[29] http://www.bipm.org/en/worldwide-metrology/metre-convention/

[30] EPA method 3540c – Soxhlet extraction

[31] Rolle F., Maurino V., Sega M., Metrological traceability for benzo[a]pyrene quantification in airborne particulate matter, *Accred. Qual. Assur.*, 17(2), (2012), 191-197 (DOI: 10.1007/s00769-011-0862-2)

[32] EN 15549: 2008 Air quality—standard method for the measurement of the concentration of benzo[a]pyrene in ambient air

[33] Smith R. K., Handbook of Environmental Analysis, Genium, New York, (1999).

[34] Plassa M., Mosca M., Sega M. in: Proceed. of the 16th international conference IMEKO TC3/APMF '98, Chung MS (ed), Taejon, Korea, (1998), 183–191.

[35] Sega M., Amico di Meane E., Rolle F. Metrological traceability chain for PCBs: I.N.Ri.M. activity, *Accred. Qual. Assur*., 14(11), (2009), 601-605 (DOI 10.1007/s00769-009-0579-7).

[36] Malengo A., Pennecchi F., Spazzini P. G., Calibration Curves Computing – CCC software, Release 1.2, software for

the evaluation of instrument calibration curves, http://www.inrim.it/download/CCC/index.html

[37] Malengo A., Pennecchi F., A weighted total least-squares algorithm for any fitting model with correlated variables, *Metrologia* 50, (2013), 654-662.

[38] Wenzl T., Simon R., Anklam E., Kleiner J. Analytical methods for polycyclic aromatic hydrocarbons (PAHs) in food and the environment needed for new food legislation in the European Union, *Trends Anal. Chem.*, 25(7), (2006), 716-725.

Supplementary Material

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