Forensic Chemistry 7 (2018) 65–74

Contents lists available at ScienceDirect

Forensic Chemistry

journal homepage: www.elsevier.com/locate/forc

Hair elemental analysis for forensic science using nuclear and related analytical methods

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ARTICLE INFO

Article history:
Received 29 May 2017
Received in revised form 2 December 2017
Accepted 2 December 2017
Available online 5 December 2017

Keywords:
Hair
Forensic analysis
Neutron activation analysis
Particle induced X-ray emission

ABSTRACT

In this review, we explore determination of element contents in hair with neutron activation analysis (NAA) and particle induced X-ray emission (PIXE). Here we discuss factors that influence the accuracy and credibility of results, and conclusions drawn for forensic science. Hair structure, growth phase, deposition of trace elements in hair, sampling and washing procedures are important factors before analysis, whereas the availability of reference values or ranges of hair elemental composition for non-exposed populations, and toxicological considerations are vitally important for results interpretation. We present here selected applications of NAA and PIXE for testing hypothesis of criminal poisoning with toxic elements. In conclusion, we would like to emphasize the importance of future NAA and PIXE applications for accurate determination of toxic elements and for spatially resolved element contents in hair, respectively.

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1. Introduction

Hair is a tissue that provides a record of an exposure of organisms to elements and compounds, e.g., by inhalation, ingestion, and deposition of air pollutants, which may diffuse into the hair structure. The composition of human hair is markedly influenced by the health and nutritional status, the ingestion or an exposure to a variety of elements and chemical compounds from the natural or occupational environments. Moreover, there are several other factors that influence element contents in hair as discussed below. Since hair samples can be easily collected, transported, stored, and analyzed, their analysis has frequently been used as an indicator of exposure to elements in individuals and various populations [1–5]. The analytes mostly involve toxic trace elements, such as Al, As, Cd, Hg, Pb, Tl, their compounds, e.g., methyl Hg (MeHg), but also drugs and their metabolites [6,7]. Possibilities of the use of hair analysis for the assessment of nutritional status, namely of Zn, have also been reviewed. It has been concluded that the research is incomplete, and the assessment of nutritional status on the basis of trace elements in human scalp hair has its pitfalls [1,4], especially if data of doubtful quality obtained in commercial laboratories are utilized. Two groups of authors [8,9] stated: "Hair mineral analysis from these laboratories is unreliable, and we recommend to refrain from using such analyses to assess individual nutritional status or suspected environmental exposure". Correlations between the trace element concentrations in hair and specific diseases, other than those associated with dietary deficiency or systemic intoxication, have been found between cystic fibrosis and both elevated sodium and depressed calcium concentrations, between hypoglycaemia and both elevated calcium and depressed potassium concentrations, and between Keshan and Kashin-Beck diseases and depressed Se in hair [1,10]. Attempts to show an association of other diseased states, such as various types of cancer, myocardial infarction, cardiovascular ailments, and mental disorders with concentration of various elements in hair turned out to be rather inconclusive, and have been accepted by the medical community with scepticism due to the complexity of factors influencing the deposition of elements in hair [1,4,11]. Thus, besides environmental and occupational health monitoring, the identification of systemic intoxication remains the main domain of hair forensic analysis. The use of hair analysis to identify systemic intoxication has become very popular after Forshufvud et al. [12,13] examined hair of Napoleon Bonaparte with neutron activation analysis (NAA) in the beginning of the 1960s, and found allegedly elevated As levels. A historical detective story "The Murder of Napoleon" was subsequently published by Weider and Hapgood [14]. Later, more analyses of Napoleon's hair were carried out with controversial results. After summarizing the various view points of clinicians, epidemiologists, pathologists, toxicologists, nuclear physicists, and forensic pathologists, it has been concluded that

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https://doi.org/10.1016/j.forc.2017.12.002
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Napoleon Bonaparte had died of a natural cause, from cancer of the stomach [15,16]. These efforts triggered the interest in the use of NAA for forensics hair analysis. Nowadays, more analytical techniques, such as various modes of atomic absorption spectrometry (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) can be used for elemental analysis of hair. However, the use of NAA and other nuclear analytical techniques, namely particle induced X-ray emission (PIXE), especially in the micro-beam mode (μ-PIXE or proton microprobe) provide many favourable features for determination of trace elements in hair as discussed in this review.

2. Advantages and pitfalls of hair elemental analysis

2.1. Hair structure, deposition of trace elements in hair and hair growth

As publications on the hair structure, growth, and deposition (incorporation) of trace elements in hair are rather numerous [1–6 and Refs. therein] only a brief review of the above topics is given here to allow for general understanding of hair elemental analysis and interpretation of the results obtained for forensic purposes. Hair is the smallest excretory tissue that can provide a chronological record of bioavailable elements in the body. During the hair growth (extrusion) from the hair follicle, the metabolic organ of hair synthesis, elements from the blood stream, lymph, and extracellular fluids are incorporated into its structure. Since the hair growth rate is reasonably well known (the most frequently cited value is 10 mm per month [1]), the longitudinal hair scan or analysis of hair segments provides a time resolved record of exposure to trace elements or their intake. Several other mechanisms of trace elements incorporation into hair were proposed by Hopps [17], namely from sebum, eccrine sweat, apocrine sweat, and from the external environment after the hair has been extruded through the skin.

The human hair is composed of three building blocks, i.e., the cuticle, the cortex regarded as the main component of the hair, and the medulla. Each block contains various components responsible for trace element bonds. The cortex, which contains most of α-keratin, a polypeptide chain, typically high in alanine, leucine, arginine, and cysteine with disulfide bridges, is the basic building block of the hair. The disulfide bridges exert high affinity for some metals, especially for those forming insoluble sulfides. The medulla contains a citrulline-rich protein with isodipeptide bridges and very little cysteine. Finally, the hair cuticle as the outermost part of the hair shaft, is formed from dead cells, with overlapping layers that form scales. This configuration gives the hair shaft strength and provide protection. Some peptide groups and mainly cell membranes can bind ions and charged groups of atoms, causing that the hair also act as an ion exchanger. The morphological structure of hair and its chemical composition thus make it unlikely that trace elements will be evenly distributed in it [18]. Hair colour, which is determined by the amount of brownish-black melanin pigments in the cortex, is another variable that may be responsible for variation in the trace element concentrations of hair from different subgroups of the population [1], because the hair pigments play some role in the incorporation of trace elements [19]. Chemical treatment of hair, such as colouring, causes irreversible changes to hair physicochemical properties. Therefore, treated hair is not suited for trace element analysis [4,11]. There are several other confounding variables, such as UV degradation, hair shape (curly or straight), age, gender, ethnicity, personal habits and seasonal fluctuations [4,5], which may also influence the hair elemental composition.

The human hair does not grow continuously, because the hair follicle has three activity phases: anagen, catagen and telogen. The anagen growth phase with the full metabolic activity of the follicle usually lasts for 2–6 years. The developing hair becomes fully keratinized, and it is extruded through the skin as a permanent hair. In the catagen phase the metabolic activity slows down and ceases within 1–2 weeks. Finally, in the telogen phase the hair follicle becomes metabolically inactive; the dead hair remains in the scalp for another 1–6 months and then falls out. Usually, about 85%, 1%, and 14% of the hair are in the anagen, catagen and telogen phases, respectively [4,18]. After some time, the metabolic activity of the hair follicle is renewed and the whole growth cycle is repeated according to the "Phoenix from the ashes" scenario.

2.2. Sampling

The hair sampling procedure depends on the purpose of analysis. Whenever hair is collected for the assessment of environmental, occupational or nutritional exposure, there is usually no problem to obtain several hundred milligrams of hair for a bulk analysis. Two sampling protocols have been proposed [1] to overcome or minimize the biological variability of the elemental composition of hair grown in various parts of the head: (i) to collect hair specimens from one region, namely the nape of the neck or the occipital region; (ii) to collect at least 100 individual hairs taken from different sites on the scalp. The hair strands should be clipped as close to the scalp as possible and should be limited to the first 5 cm of recent growth. Longer hairs should be cut into 5-cm sections to be analyzed separately. The orientation of proximal and distal parts of the hair specimen should be recorded, especially if there is a need for subsequent analysis of hair segments to determine the chronology of the exposure. To avoid contamination of hair during sampling, the use of plastic scissors, quartz instruments, etc., has been recommended [1] but it is sometimes difficult to be accomplished in practice. Thus, the use of cutting instruments made of high-quality, surgical-grade stainless steel should be considered. Alternatively, hair specimens may be plucked using non-contaminating gloves. In the absence of scalp hair, other body hair such as beard hair, axillary hair, or pubic hair, can be used for analyses [1]. Although pubic and axillary hair is less exposed to direct environmental contamination, it is more affected by body excretions [11]. On the other hand, it has been suggested that hair from areas other than the scalp may be useful in distinguishing between exogenous contamination of scalp hair as opposed to the internal absorption of toxic elements [1].

Contrary to the assessment of environmental, occupational or nutritional exposure, a completely different situation usually occurs in case of hair sampling for forensic purposes, because individual hairs or only a their small amount may be available at the scene of crime or from victims or suspected persons.

2.3. Washing

Several different washing procedures for removal of exogenous contamination containing trace elements have been proposed, which have different efficacy of removal of external contamination for various elements. At the same time, endogenously bound elements can be removed, as well [1 and Refs. therein]. Results of various cleansing procedures suggest that there is no washing procedure, which would completely remove external contamination without influencing the endogenous element contents. The problem of different element removal using various washing procedures can thus be circumvented only by the use of a standardized washing procedure. First was proposed by the International Atomic Energy Agency (IAEA) for the use of hair as an indicator of contamination of man by environmental pollutants already in 1978 [21]. Since then, this procedure consisting in consecutive washing in acetone – deionized water (three washes) – acetone

became widely used for hair cleaning prior to the elemental analysis. Another one, which involves washing in a sequential order with acetone, deionized water and 0.5% Triton X-100 solution in an ultra-sonic bath, allowing a few minutes for each stage [22] has been claimed to be commonly used [4]. Obviously, hair specimens washed by the same procedure should be used for any kind of assessment of environmental, occupational, nutritional, etc., exposure and for forensic purposes as well, to be able to arrive at valid conclusions. Similarly, reference values or ranges of hair elemental composition should be derived from the analysis results obtained after a particular washing procedure.

2.4. Reference values or concentration ranges of hair elemental composition

Reference values or concentration ranges are needed for evaluation whether the hair elemental composition is in line with normal, physiological values or whether the element contents are elevated or depressed as a consequence of environmental or occupational exposure, and nutritional status. Elevated levels of some elements are congruent with accidental or deliberate poisoning. Several attempts have been made to compile the reference values or ranges from the literature data [23,24] or to establish such values from specifically designed studies [25–27], or just to publish a summary of elemental concentrations determined in hair [4,28]. As an example, Table 1 contains a review of As and Hg hair concentrations and reference ranges (if evaluated), because these elements are frequently encountered as poisons in criminal investigations. Certainly not all already published values are given in the present review; only selected papers mostly aimed at establishing reference values or ranges are included here. Nevertheless, the reference values and concentration ranges of hair As and Hg given in Table 1 vary widely, over one order of magnitude, for two main reasons.

(1) There are many factors, which influence the elemental composition of human hair, such as the biological variation due to gender, age, hair colour, geographic origin, dietary habits and different sampling and washing procedures.
(2) There might be insufficient (or sometimes none) analytical quality control measures employed in the analytical step and inappropriate statistical treatment procedures employed in the evaluation of the data sets obtained. For instance, the differences of arithmetic means and medians, and the high values of standard deviation suggest that the arithmetic means and standard deviations were calculated without providing evidence of normally distributed data sets.

For the former reason it seems unlikely that any universally valid reference values and ranges of hair elemental composition can be established or the reference concentration ranges would be impractically wide. Therefore, the existing literature data should be scrutinized and stratified according to the above influencing factors to arrive at reference values and concentration ranges best applicable for a given case/study. Alternatively, a laboratory or institution involved in hair analysis for environmental, occupational, nutritional monitoring or criminal investigation should establish its own reference values or concentration ranges based on the analyses of hair specimens from a well designed control group. Such a control group should match most of the factors that may influence element concentration in hair in studied subjects and should be sufficient in size to allow proper statistical evaluation, such as that recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [38].

2.5. Interpretation of analysis results

Hair analysis to be used for the identification of a particular person that was based on the presumption of an individual unique-

Table 1
Reference values and concentration ranges of As and Hg concentrations in hair (μg g^{-1}).

Element	Mean	SD[a]	Range	Reference range[b] (Lower percentile–Upper percentile)	N	Literature
As	0.26[c]	–	0.085–0.500	–	17	[23]
	<0.15	–	–	–	1019	[25]
	0.83 [d]	0.1	0.73–0.94	–	150	[4,28]
	0.085[d], 0.067[c]	0.054	0.034–0.319	–	114	[4,22]
	0.11[d], 0.11[c]	0.05	0.01–0.91	–	568	[4,29]
	0.05[c]	–		0.03–0.08 (5–95)	45	[4,26]
	0.09[d], 0.06[c]	0.11	–	0.14–0.24 (5–95)	263	[30,31]
	0.834[d], 0.760[c]	0.325	0.651–3.959	0.686–1.025	117	[27,30]
	0.00[d], 0.00[c]	0.01	–	0.0003–0.03 (2.5–97.5)	130	[30,32]
	0.09[d], 0.08[c]	0.06	0.02–0.37	0.05–0.20	87[e]	[29,30]
	0.11[d], 0.11[c]	0.05	0.01–0.91	0.05–0.20	568[f]	[29,30]
	0.007[d], 0.006[c]	0.005	0.001–0.02	0.0011–0.016 (10–90)	167	[30,33]
Hg	3.25[c]		0.5–12.2		24	[23]
	4.10[c]		1.4–15.0			[24]
	1.73[d]	2.12	0.02–16.34		263	[4,34]
	3.4[d], 1.9[c]	2.8	0.69–10		19	[4,35]
	1.51[d]	0.91		0.49–3.60 (5–95)	59	[4,36]
	1.03[d]	0.82	0.2–4.8		83	[4,37]
	0.261[d], 0.249[c]	0.145	0.053–0.927		114	[22]
	0.66[d], 0.60[c]	0.32	0.15–1.83	0–1	87[e]	[29,30]
	0.47[d], 0.41[c]	0.25	0.05–1.77	0–1	568[f]	[29,30]
	0.66[c]		0.31–1.66		45	[26,30]
	0.14[d], 0.07[c]	0.18	0.004–0.873	0.009–0.42 (10–90)	167	[30,33]
	0.208[d], 0.164[c]	0.160	0.032–0.800	0.063–0.437 (10–90)	117	[27]

[a] Standard deviation.
[b] Defined by International Federation of Clinical Chemistry (IFCC) [38].
[c] Median.
[d] Arithmetic mean.
[e] Metropolitan population.
[f] Small-city population.

ness of hair composition, like in the case of fingerprints, was shown to be unreliable by Cornelis [39] more than 40 years ago. Her unique study of determining the elements Mn, Cu, Zn, As, Sb, Au and Hg by NAA in hair samples of two brothers collected regularly over the period of 25 and 26 years showed that only Zn concentrations remained reasonably constant (within 15 per cent), whereas concentration of the other elements varied widely, up to 110 per cent. Thus, disregarding the purpose of study (environmental, occupational, nutritional monitoring or criminal investigation), hair analysis results of an "exposed" person or a group are to be compared with the reference values or ranges (if such reliable parameters exist) or with a control group (locally and purposely established reference values or ranges). This latter approach works very well in large epidemiological studies, where a large number of subjects and sufficient amount of hair from one subject (more than 30 mg) can be made available.

As already mentioned in para 2.2, somewhat different situation may be encountered in forensic investigation of samples that require the interpretation of analysis results from a small number or even single hairs. Frequently, the time course of exposure (element intake) determined by analysis of hair segments cut in various distances from the scalp (hair follicle) is of considerable importance to be able to distinguish a permanent elevation of element contents along the hair length (chronic exposure or poisoning in case of toxic elements) or the elevation in a certain period of time (acute exposure or poisoning). In such cases, the growth phase of each hair to be analyzed should be determined according to the hair follicle shape by optical microscopy, from histological or scanning transmission ion microscopy (STIM) images or from elemental composition, e.g., from elemental maps obtained by μ–PIXE analysis [20]. Hairs whose follicles are in the catagen or telogen phase (i.e., metabolically less active or inactive, respectively) may remain in the skin for a couple of months before they fall out. If such a hair is analyzed, the association of a recorded exposure with the time scale (the distance of a hair segment from the follicle) becomes uncertain, because it is not known how long the "dead hair" was retained in the skin, and interpretation of the results obtained may become inconclusive.

In analysis of other types of hair than the scalp hair (beard hair, axillary hair, pubic hair), somewhat different growth rates of these types of hair (Cf. Table 2) should be considered in determining the time resolved exposure. Obviously, the published growth rates of beard hair differ by a factor of almost two and the growth rates and duration of follicular activity of other types of hair may also be variable and dependent upon several factors, namely age, race, gender, season, etc. [1]. There is very little or no information on correlations between the element composition of various types of hair. To advance the knowledge in this field, we compared the element contents in scalp and beard hairs, determined by instrumental neutron activation analysis (INAA), in a pilot study of specimens taken from 7 subjects. Fig. 1 shows the results obtained by analysis of hair washed with the above mentioned IAEA method [21]. The study indicated that the content of some elements in beard hair match well the data obtained for scalp hair (e.g., S, Cl, Zn, and Hg). For some other elements, such as Cr, La, Ce, Au, however, we observed significant differences. Unfortunately, the subject size was too small to judge whether the variations reflected the differences in scalp and beard hair structures and metabolisms of their respective follicles. Therefore, no statistical evaluation of the differences observed has been carried out; just a hypothetical regression line $y = x$ has been drawn in Fig. 1.

An elevation of toxic elements in hair in forensic applications should not be evaluated only from comparison with reference values or concentration ranges, but rather according to the toxicological significance of the elevation. Thus taking again the toxic elements As and Hg as examples, moderate intoxication (but not necessarily deadly) results in As hair contents, which are from several times to over 100 times higher compared to normal levels [41]. For Hg in hair, levels up to 200 times higher compared to the normal values may be observed on moderate intoxication, i.e., in the range of 200–800 μg g^{-1} [42].

Table 2
Hair growth rates.

Tissue	Monthly growth rate (mm) [1]	Monthly growth rate (mm) [40]
Scalp hair	7–12	7.7–10.5
Beard hair	6	11.4
Pubic hair	6	6
Axillary hair	9–12	9

3. Advantages of nuclear and related analytical techniques for hair analysis

Analytical techniques intended for determination of trace element levels in hair, similarly to other analytical tasks, should satisfy several criteria. These are as follows: accuracy, low blank values and limits of detection are of major significance. Furthermore a multielemental capability, speed, simplicity, dynamic range and reasonable cost are also important. Many of the criteria can be satisfied if we use nuclear and related analytical techniques, namely NAA and PIXE.

3.1. Neutron activation analysis (NAA)

The principle, methodology and applications of NAA are described in monographs, e.g., [43,44], and in a comprehensive review [45]. Briefly, NAA is carried out by sample irradiation in a high flux of neutrons generated in a nuclear reactor and subsequent spectrometric measurement of gamma-rays emitted by the induced radionuclides. A favourable position of NAA is given by the simplicity of sample preparation for analysis (weighing and encapsulation only), which results in very low blank values. Other remarkable features of NAA involve the multielemental capability with very low limits of detection (LOD) down to the sub-μg g^{-1} level for up to 30 elements. Further advantageous features of the method include the possibility to perform analysis non-destructively, in the so-called instrumental neutron activation analysis (INAA) mode and a high potential for accuracy. The primary source of accuracy is the well-understood nuclear based principle that offers advantages compared to the electronic nature of many other analytical methods. For this reason, and because all sources of uncertainties can be experimentally evaluated or modelled, NAA has recently been recognized as a primary measurement technique, i.e., the techniques with the highest metrological properties [45]. Several NAA protocols have been developed consisting usually of two irradiation-decay-counting cycles for multielemental analysis. The irradiation, decay and measurements times can be tuned to achieve the lowest uncertainties of results for a group of elements. The gamma-ray spectra of short-lived radionuclides are measured after a decay of few minutes using counting times of few minutes. The spectra of medium-lived radionuclides are measured after a decay of several days using counting times that usually do not exceed 1 h. The most time-consuming part of analysis is the measurement of long-lived radionuclides, which is carried out after several weeks of decay using counting times of several hours. Hair bulk analysis can be performed for assays of a large number of samples, which is important for large population studies. Instances of suspected exposure or poisoning can be studied by longitudinal hair analysis that can be implemented by assaying sectioned hair

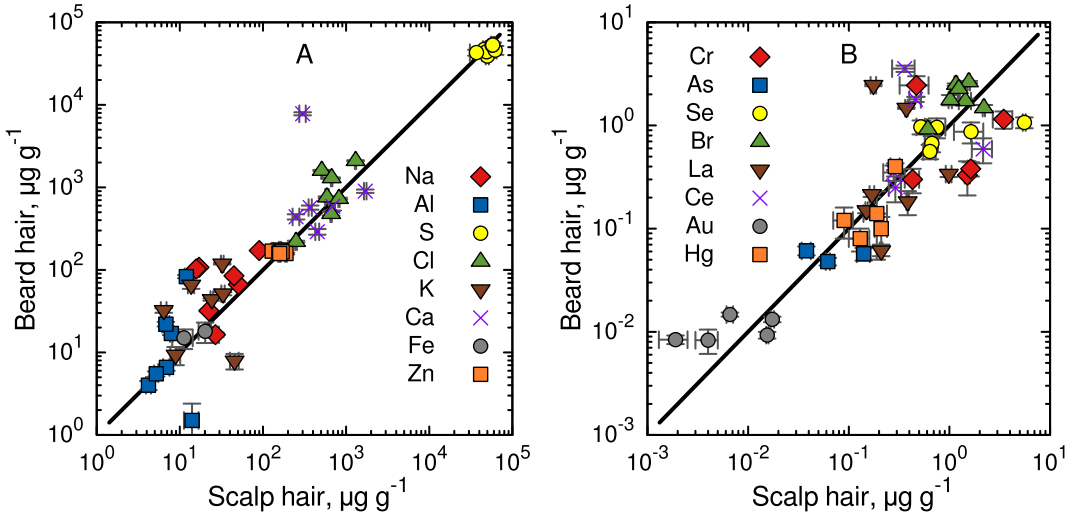

Fig. 1. Scatter plots of element contents in scalp and beard hair of the same individuals (N = 7). The elements are separated into two groups with higher and lower contents in plots A and B, respectively. The diagonal line in both plots represents a hypothetical regression line $y = x$.

samples. The time resolution of exposure can be set as a function of the length of hair segments. For instance, 5 mm long sections will provide information on the exposure to particular elements within about 14 days of person's life, considering the most frequently cited growth rate of 10 mm per months. If LOD of an INAA procedure is not sufficient, radiochemical neutron activation analysis (RNAA) can be performed to separate one or a group of elements (their radionuclides). This procedure usually yields lower LOD by two to three orders of magnitude compared to INAA.

The disadvantages of NAA is the necessity of an easy access to a nuclear reactor that provides the highest neutron flux available and the long turn-around time of analysis, which may last up to 4–5 weeks. The latter does not apply for elements forming short-lived radionuclides where the analysis time can be much shorter, i.e., such assay can be completed within several hours.

3.2. PIXE analysis

The principle and main applications of PIXE are described in a monograph [46]. More technical details of nuclear microprobe techniques, including μ-PIXE, can be found elsewhere [47]. PIXE measurements entail sample bombardment with charged particles, most frequently protons, having energy of several MeV. The accelerated particles are produced by Van de Graaff generators or tandem accelerators. The ensuing X-rays, emitted as a consequence of inner shell ionization of atoms in a specimen that is followed by drop down of outer shell electrons to replace inner shell vacancies, are then measured with suitable detectors. Thus, strictly speaking, PIXE is not a nuclear based technique, because it is based on the properties of electron shells of atoms, but it is frequently counted among nuclear analytical techniques. This attribution may results from the similarity of measuring equipment to that used in other nuclear analytical techniques. Similarly to INAA, PIXE is mostly performed non-destructively and has multi-element capability allowing the determination of up to 15–20 elements in a sample. In general, LODs are somewhat higher than in INAA. On the other hand PIXE can be used for low-level determination of P and Pb, which cannot be assayed by INAA. LODs for several elements, such as Si, S, Ni and Cu, are lower when compared with INAA. Unlike the long measuring times of INAA methods, PIXE measurement can be performed within a relatively short time. Depending on the concentration of desired elements, the measurement may take less than one hour. Matrix effects, that occur in PIXE, result from various interactions of bombarding particles with different materials (penetration depth, energy losses) and the absorption of low-energy X-rays emitted from different sample depth. Therefore, careful calibration and matrix corrections are necessary for obtaining accurate results. The most appreciated feature of PIXE for hair assay is the possibility to perform spatially-resolved analysis using proton beams focused to a small spot (about 1 × 1 μm or less) in so-called μ-PIXE (proton microprobe) mode. Longitudinal scans of individual hairs using μ-PIXE allow to distinguish between chronic and acute exposure. These scans provide relatively constant signal emitted by any particular element which makes it easy to detect any sudden increase along the analyzed hair filament. The excellent lateral resolution of μ-PIXE also allows distinguishing between the endogenous element contents in hair and external contamination on the surface of hair. Thus, no washing of hair is needed for μ-PIXE analysis, since the endogenous element content can be measured directly. Transverse line scans, on the other hand, are useful for fundamental studies of elemental association with individual components of the hair matrix and for understanding incorporation mechanisms of various elements. Hair analysis using μ-PIXE is a challenging task, because one needs specialized equipment and sample preparation procedures, because fixation of a hair specimen in the vacuum target chamber of a μ-PIXE device is a rather complicated matter. Finally, long measurement times are needed to perform scans over the distance of a few mm of hair. This may take up several hours.

3.3. When should NAA or PIXE be used?

Both techniques have their merits and demerits. NAA is more suited for hair analysis of bulk samples weighing from several mg to several hundred mg. The use of bulk samples is the approach most appreciated in studies aimed at detection of environmental, occupational or nutritional exposure. For forensic applications, determination of time resolved exposure to elements can be achieved by analysis of sectioned hair samples taken at different distances from the identified hair bulb (follicle). NAA of single hairs weighing several hundred μg is also feasible thanks to very low LODs for many elements. In all these applications, the hair specimens should be cleansed prior to analysis to remove external contamination using a selected washing procedure, preferably one of those mentioned in para 2.3 [21,22]. PIXE, especially in the μ–PIXE mode, is most frequently used method for analysis of single hair fil-

aments. Thanks to the excellent spatial resolution of μ–PIXE, in the order of 1 μm or less, longitudinal scans can provide information about time resolved exposure to elements, whereas transversal scans can yield data about elemental composition of the hair structure. There is no need to wash hair specimens prior to PIXE analysis, because external contamination can be revealed from 2D element maps constructed from μ–PIXE measurements as shown in Fig. 2. The detected external contamination can be subtracted from analysis results by integration of the μ–PIXE signal over a noncontaminated part of the hair analyzed or by using a PIXE-tomographic arrangement, which can produce 3D quantitative maps [48].

Bulk hair samples can be analyzed by PIXE too, provided that the hair specimen is decomposed by an acid digestion procedure and a small aliquot (a few μL) of the resulting solution is deposited onto a thin, usually polycarbonate foil that provides a holder for the target substance [49,50]. However, such a procedure is not employed in PIXE laboratories very frequently.

NAA and PIXE should not be considered as competitive techniques, rather they are complementary, because they allow the assay of different sets of elements that can be determined with different LODs (cf. paras 3.1 and 3.2) by either technique. The former technique provides highly accurate results of elemental composition of representative bulk hair samples, whereas the latter technique (in the μ-PIXE mode) is most appreciated for obtaining spatially-resolved element data from small hair spots. Such data, however, may not necessarily be representative of the bulk hair composition as demonstrated in Fig. 3, which shows a comparison

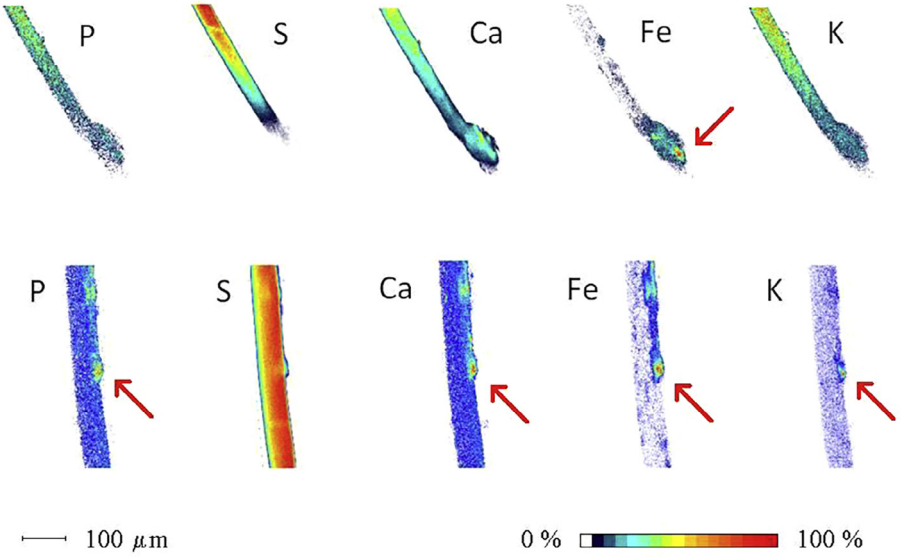

Fig. 2. μ-PIXE elemental maps in longitudinal scans of two different Brahe's hairs. The element contents increase from blue to red colour as shown by a scale bar, in which the colours indicate relative element contents only. The hot spot of Fe content in the upper row is probably due to blood remainder at the hair follicle. The hot spots of P, Ca, Fe, and K contents in the lower raw are due to non-removed external contamination of the hair filament. The hot spots are denoted with arrows.

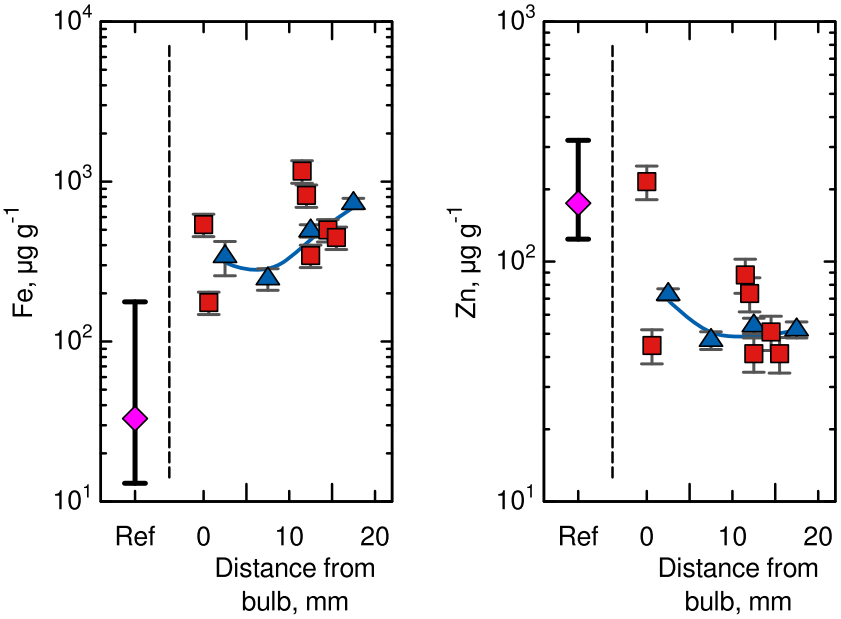

Fig. 3. Comparison of Fe and Zn results in Tycho Brahe's hair determined by NAA (triangles) and μ-PIXE (squares). Reference values (diamonds) and ranges (thick error bar) for the contemporary population (see references in [52]) are depicted next to the abscissa.

of NAA and μ-PIXE results for Fe and Zn in Tycho Brahe's hairs in our study of his remains aimed at the elucidation of possible reasons for his death [51,52]. The NAA results were obtained by assaying 5 mm long sections from 20 to 25 individual hairs washed according to the IAEA procedure [21], whereas the μ-PIXE results were accomplished by analysis of a single, unwashed hair sample using multiple scans over 500 μm sections with a proton beam focused to an area of about 1.5 × 1.5 μm. Fig. 3 shows that both techniques found that Fe values were elevated and Zn values lower in comparison to reference values. Similar time trends of exposure/intake (distance from the hair bulb) can be inferred from the results of both techniques. However, the individual NAA and μ-PIXE data points for both elements differ significantly, once we account for the combined uncertainties of results obtained by either technique. This is simply because NAA and μ-PIXE analyzed different objects, i.e., a representative bulk sample and a region of interest of the single hair, respectively.

The decision whether to use NAA or PIXE also depends on the availability of the experimental facilities for the individual techniques. Nowadays, the facilities for PIXE, including microbeams, are becoming more easily available [53] than those for NAA, because several experimental nuclear reactors have been shut down world-wide without a replacement [54]. Obviously, if both techniques are available for a given study, both of them should be used, because each of them offers somewhat different outcome of the analysis, as discussed above. However, the availability of the facilities for both techniques in one institution, like in our institute [55], is the exception rather than the rule.

4. Examples of forensic applications of hair analysis using NAA and PIXE

4.1. Historical cases of American personalities and Sir Isaac Newton

Several exemplary studies have been published about the use of NAA and PIXE for hair analysis to elucidate possible criminal poisoning with toxic elements or to distinguish between acute or chronic poisoning that may indicate murder or suicide. The cases examined therein include analytical studies of remains of historical personalities, criminal evidence and records of trial proceedings.

Of the "historical forensic" cases, in addition to the already mentioned story of Napoleon Bonaparte, the case of Charles F. Hall, an American explorer in command of the U.S. North Polar Expedition (the Polaris Expedition), has also attracted attention because of an alleged arsenic poisoning. On November 8, 1871, he died in his cabin on the S.S. Polaris anchored to the shore of northwest Greenland. C. F. Hall had suffered from gastrointestinal and central nervous system symptoms that recurred over a two-week period until his death. He accused many of the ship's company of poisoning him, because of a difference of opinion over mission strategy. In August 1968, an autopsy was performed on the body at the Greenland burial site and his hair, fingernail and bone samples were analyzed by INAA. The hair and fingernail samples grown during the last two weeks of Hall's life showed markedly increased levels of arsenic, indicating acute poisoning, in accordance with his clinical symptoms [10,56].

The 7th U.S. president Andrew Jackson was treated with calomel and sugar of lead (lead acetate). He also had two lead pellets in his body from an 1806 duel and an 1813 gunfight. Jackson had many symptoms compatible with mercury poisoning and plumbism [10]. Samples of his hair taken in 1815 and 1839 were analyzed for mercury and lead. Mercury levels of 6.0 μg g^{-1} and 5.6 μg g^{-1} found in the respective 1815 and 1839 hair specimens were unspectacular. The lead levels, on the other hand, were significantly elevated, exhibiting values of 130.5 μg g^{-1} and 44 μg g^{-1}, respectively. Hence, Jackson's death was probably not the result of acute lead poisoning, but it was chronic toxicity that probably contributed to his death from chronic renal failure [10]. Other authors [57] assumed that Andrew Jackson might have had mercury poisoning instead of lead poisoning. However, Deppisch and Gemmel [58] stated their disbelief in the theory of mercury poisoning.

The 12th U.S. president Zachary Taylor was also considered to be poisoned with arsenic. Shortly after breaking grounds for the Washington Monument on July 4, 1850, he fell ill and died a few days later. The cause of death was listed as gastroenteritis, one of symptoms of arsenic poisoning. After exhumation of his remains in 1991, arsenic was determined in his hair by NAA, but the results obtained indicated insufficient arsenic levels to cause poisoning [10].

Another historical personality, whose hair specimens were examined for suspected poisoning, was Sir Isaac Newton. He was engaged not only in mathematics, astronomy, and physics, but he was also an ardent alchemist spending in certain periods of his life a lot of time with experiments in his alchemical laboratory [59,60]. In 1692 and 1693 Newton fell ill and underwent a period of severe emotional and mental disturbance, also denoted as "Derangement of his intellect" [60]. In 1979, four hair specimens, said to have been Newton's, were analyzed for As, Sb, Ag, Au, Hg among other elements by NAA, and for Pb by AAS. The element levels found were 3.0 μg g^{-1} for As, 3.8 μg g^{-1} for Sb, 5.2 μg g^{-1} for Ag, 0.14–1 6.0 μg g^{-1} for Au, 7.2–197 μg g^{-1} for Hg, and 19–191 μg g^{-1} for Pb [60]. Obviously, the values of Hg and Pb exceeded "normal levels" and are of toxicological significance. Elevated Hg and Pb could have possibly contributed to the causes of Newton's illness, from which he recovered and died aged 84 in 1727.

4.2. Was Tycho Brahe poisoned with mercury?

Our laboratory participated in another "historical forensic" study. Recently, we investigated the remains, namely samples of hair and bones, of Tycho Brahe, the Imperial Astronomer for Emperor Rudolph II since 1599, because of his alleged poisoning in Prague in 1601. On 13 October 1601, Tycho Brahe, after he had attended a banquet at the Count of Rosenberg, was taken seriously ill – apparently without any previous symptoms – and died on 24 October 1601 [61]. At the banquet, he supposedly held his urine longer than was his habit, allegedly due to etiquette, and later he could no longer urinate. There are three contemporary accounts of the illness and death of Tycho Brahe. These were given by Brahe's assistant Johannes Kepler, Brahe's friend and physician Johannes Jessenius, and a young German doctor, Johannes Wittich. Kepler wrote that "He endured five days and nights of agony, unable to sleep. Uninterrupted insomnia followed; intestinal fever; and little by little, delirium". Somewhat similarly, Jessenius mentioned in his account: "Urine retention and strong pain followed. Bladder inflammation, which—as is usually the case—was immediately accompanied by continuous fever and from this quite slight delirium arose". On the other hand, Wittich wrote that a stone caused Brahe to be unable to urinate and he died because of a burst bladder [51,61]. The first two accounts describe symptoms of uraemia. The third account, which has become the most popular, alleged that Brahe died from a ruptured bladder. Several conspiracy theories regarding his death have been aired shortly after his death. As acute mercury poisoning can result in uraemia and kidney failure, the burst bladder has been seen by some researchers as indirect evidence for mercury poisoning; others conclude that a rupture of the bladder must generally be considered a very rare and unlikely occurrence [51 and Refs. therein]. To test these controversial hypotheses, Tycho Brahe's grave was opened in 2010 by a Czech-Danish research consortium and samples of his bones,

hair, teeth and the textiles were procured for scientific investigation. We investigated three different hair specimens by NAA and μ-PIXE. The hair samples were cut into 5-mm sections, washed with the IAEA procedure [21], and 25–30 hair sections weighing 0.227–0.629 mg were first analyzed by INAA to determine as many elements as possible, and consequently Hg was determined by RNAA to achieve the lowest LOD possible. Several elements, including Pb, were also determined by μ-PIXE in longitudinal scans of intact hairs [51,52]. The three hair specimens, analyzed by INAA and RNAA, had hair bulbs (follicles) easily identifiable by optical microscopy. Fig. 4 shows element contents in the hair sections as a function of the increasing distance from the hair bulb. Taking into account the most frequently cited hair growth of 10 mm per month, the element contents in hair sections cut in the distance 0–5 mm from the hair bulb represent an exposure in the last 14 days of Brahe's life, etc. The most important finding was that about 2 months prior to Brahe's death the Hg contents determined by RNAA only slightly exceeded the normal range of the contemporary population. The Hg levels gradually decreased in those hair sections representing periods closer towards Brahe's death (cf. Fig. 4). Since the slightly elevated Hg content two months prior to Brahe's death is of no toxicological significance, and it further decreased towards Brahe's death, we proved that there was no acute exposure to Hg. This can be taken as evidence that Brahe was not exposed to lethal (or fatal) doses of Hg, as was previously speculated. Chronic exposure was also excluded by analysis of Brahe's bones with cold vapour atomic absorption spectroscopy (CV–AAS) [51]. Surprisingly, similar time trends of element contents as for Hg were also found for the elements Fe, As, Ag, and Au (cf. Fig. 4), which were determined by INAA. No such trends were observed for the elements Cr, Co, Zn, Br, Sb determined by INAA, and Pb determined by μ-PIXE using a Tandetron 4130 MC

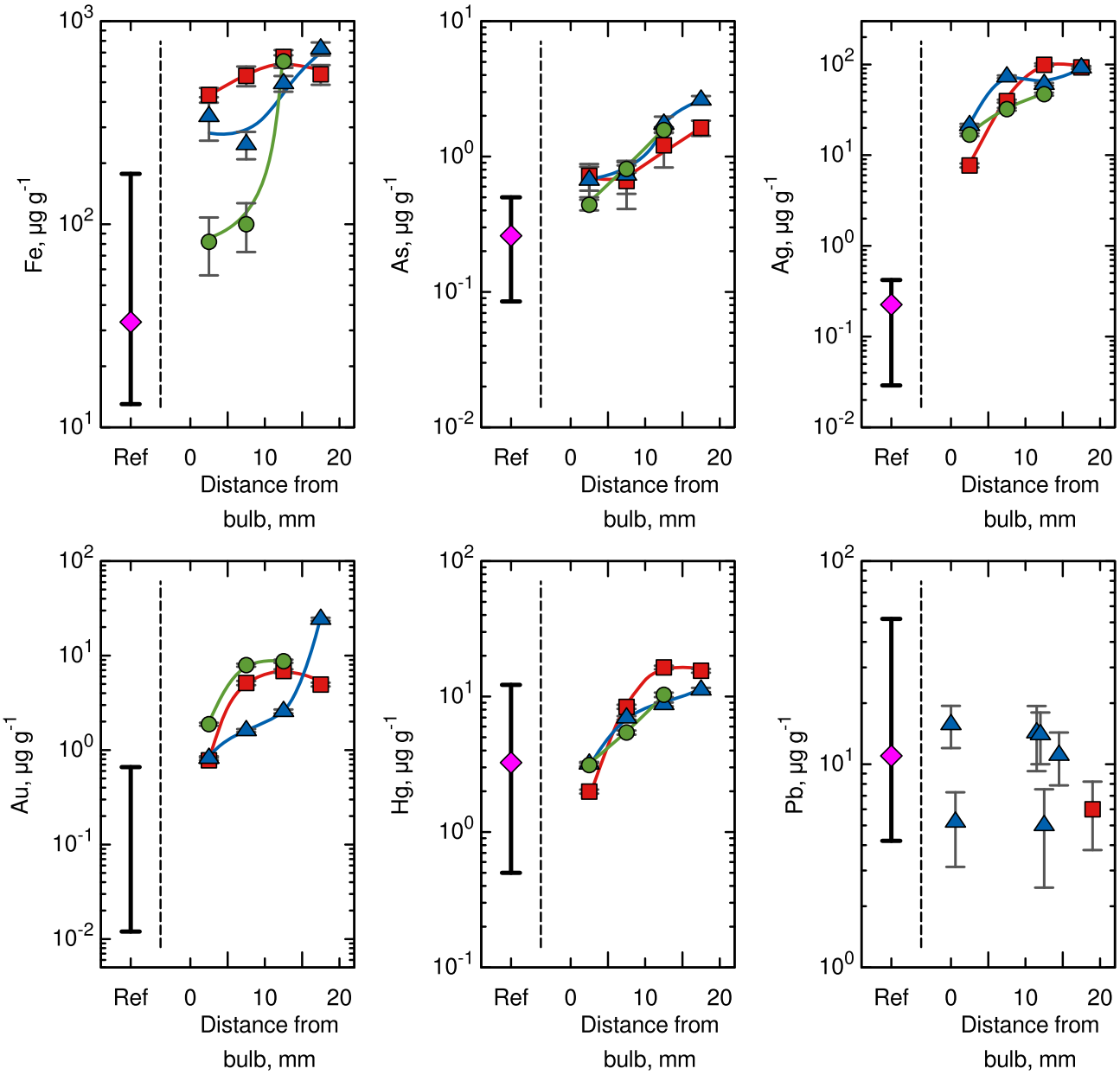

Fig. 4. Time course of element contents in three sectioned hair specimens of Tycho Brahe denoted in symbols (squares, triangles, circles) and their comparison with reference values (diamonds) or concentration ranges (thick error bars) next to the abscissa. The distance of the 0–5 mm section from hair bulb (follicle) corresponds to the last 14 days of life, the distance of 5–10 mm corresponds to 15–30 days of life, etc., presuming the hair growth rate of 10 mm per month. For reference values or concentration ranges see references in [52].

accelerator with a 2.6 MeV proton beam, focused to an area of 1.5 μm [52]. We suggested that the decreasing exposure of Tycho Brahe to the elements Fe, As, Ag, Au, and Hg might be associated with his alchemical activities. Similarly to several Renaissance scientists, Brahe was also involved in alchemy, namely in the preparation of Paracelsian medicines containing inorganic constituents. It is possible that Tycho Brahe could have tested or self-administered Elixir Tychonis [62], which is the most famous of these preparations. Had this been the case, then he had to discontinue the intake of the medicine 2 months prior to his death, or some time before [51,52].

In addition to the determination of Pb and confirmatory determination of several other elements in two intact Brahe's hairs, μ-PIXE appeared quite useful for detection of other important features of the hair specimens analyzed, which are depicted in Fig. 2. One of the hairs that originates from the first opening of Brahe's tomb in 1901 was deposited in the City of Prague Museum since then (upper row of elemental maps), the other one was secured on the second opening of the tomb in 2010 (lower row of elemental maps). Depleted contents of the elements P and S (the major constituents of the hair matrix) in the hair bulb seem to suggest that the follicle was in the resting stage (catagen or telogen, Cf. [20]). The lower row shows an exogenous particle deposited on the hair surface, which indicates an external contamination, because the hair specimen was only mechanically cleaned without any additional washing.

4.3. More recent cases

In forensic applications, hair analysis has been used several times to determine whether arsenic poisoning was acute or chronic (indicating possible suicide or murder) [63] or whether chronic exposure to As resulted from drug-intoxication or criminal poisoning [64]. Two cases submitted to the court of justice have been reported in the literature. In the U.S., measurement of As in sectioned hair samples by INAA were used in two murder trials as an evidence for the first degree murder and the defendants were sentenced to death [65], and in the other case to life imprisonment [66]. In our laboratory, the use of INAA helped to resolve discrepancies between clinical symptoms and pathological findings of a deceased person. The INAA results of sectioned hair samples allowed the Office of Criminal Police and Investigations in Prague to conclude that the deceased person committed suicide by ingestion of a single large dose of As_2O_3 rather than by chronic self-administration of sub-lethal doses of As or by administration of the poison by someone else over long period before her death [67].

The experiments in our laboratory described in paras 4.2. and 4.3. were carried out within the CANAM infrastructure supported by the Ministry of Education, Youth and Sports of the Czech Republic, project LM 2015056.

5. Conclusions

Analysis of hair specimens used for forensic purposes is a complex issue, in which many factors such as the mode of sampling and sample preparation, the use of suitable analytical technique and result interpretation are to be considered, was reviewed here at length. A comparison of the element elevated levels found with the reference values or concentration ranges for occupationally non-exposed, healthy populations should be taken only as an indication that criminal poisoning could have been committed. The reason for this assertion is that the adequate reference values or concentration ranges are frequently missing or only wide reference ranges are available, which may make any comparison inconclusive. Thus, the proof of criminal activities should be based on the evaluation of the elevated levels with respect to those of toxicological significance. Obviously, analytical techniques providing accurate results are of utmost importance for this purpose. NAA with its favourably low LODs for many elements, the high potential for accuracy, high metrological and traceability properties perfectly satisfy the above criterion. Time resolved exposure to, or an intake of particular elements can be achieved by analysis of hair sectioned in selected distances from the hair bulb. The hair bulb is a metabolic organ that should always be identified to know in what growth stage the hair was. At least, the distance of the hair section from the scalp must be known.

PIXE, especially in the μ-PIXE mode, has also several advantages in hair analysis for forensic purposes. Several elements can be measured obtaining LODs that are lower than in NAA. Some elements like P and Pb that cannot be assayed using NAA, can be determined with PIXE. The most appreciable feature of μ-PIXE is its capability for determination of spatial-, especially longitudinal-distribution of elements in hair. On the other hand, it should be realized that the spatially-resolved element contents, if obtained in analysis of a single hair, are not representative of the bulk hair composition. This should always be taken into account in the interpretation of the results thus obtained. Hence, it would always be advantageous if both NAA and μ-PIXE are used for testing the same sample whenever possible, because these techniques are not competitive, but rather complementary.

There are also several analytical techniques capable of hair analysis for forensic purposes, which are rapidly developing, such as Secondary Ion Mass Spectrometry (SIMS), synchrotron based X-ray Fluorescence (XRF) and Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). These techniques can also provide information on contents of many elements with very low LOD and sometimes offer an excellent spatial resolution. However, most of these techniques are difficult to calibrate to produce accurate values of element contents traceable to the SI units of mass or mass fraction. The achievement of accurate and traceable results using these techniques is also hampered by the lack of adequate certified reference materials. For this reason, NAA and PIXE will maintain their very important position for hair analysis in forensic science, because they can help in elucidating some crimes committed by poisoning with toxic elements.

Conflict of interest

None.

Acknowledgement

This work was supported by the Czech Science Foundation within grant P108/12/G108. We also thank to anonymous reviewers for their suggestions to improve the manuscript and K. Kranda for linguistic corrections.

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