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Microanalytical method development for Fe, Cu and Zn determination in colorectal cancer cells

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ABSTRACT

Microanalytical methods suitable for the determination of Fe, Cu in HT-29 (human colon adenocarcinoma) cells treated with different iron compounds (Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin) and cultured in medium supplemented or not with 10% (v/v) fetal calf serum (FCS) by total reflection X-ray fluorescence spectrometry (TXRF) and simultaneous graphite furnace atomic absorption spectrometry (GF-AAS) were developed. The developed TXRF method was also suitable for Zn determination in the samples. The main advantage of the proposed methods is the execution of all sample preparation steps following incubation and prior to the elemental analysis in the same Eppendorf tubes. Sample preparation was performed at microscale (115 μL sample volume) with 65% nitric acid and 30% hydrogen peroxide. According to scanning electron microscopic measurements, the organic matrix of the cell samples could be eliminated to the extent that accurate results were obtained for Cu and Fe by analyzing the same samples by TXRF and GF-AAS. Concerning the iron uptake, HT-29 cells incubated in FCS-free medium contained Fe in cca. 5–50 times higher amounts compared to cells cultured in FCS supplemented medium. Pronounced differences in the iron uptake compared to the iron supply (inorganic vs. organic chelated as well as iron(II) vs. iron(III)) were observed in the case of cell lines incubated in FCS-free medium.

1. Introduction

The determination of trace metals in clinical studies is an important and, in many cases, a challenging task. In the case of metal-based therapies, the accurate determination of the metal ions to which patients had been subjected (i.e., platinum and rhodium originating from complexes used in metal-based cancer therapies) does not represent a real challenge, these samples being not susceptible to cross-contamination caused by sample preparation involving microwave-assisted digestion accomplished with acids and subsequent dilution with water [1,2].

Problems can arise with the determination of metal ions in biological systems when the possibility of cross-contamination during sample preparation cannot be avoided. This is the case of iron, copper and zinc whose concentrations in human colon adenocarcinoma cells (HT-29) are rather low: 10–20 ng/10^6 cells, 4–8 ng/10^6 cells and 50–80 ng/10^6 cells [3]. By diluting the cellular sample with distilled water, the dilution factor as well as the blank values for these elements in distilled water and acids, used for further sample preparation, renders impossible their microanalytical analysis. Due to the low sample demand and high sensitivity, three techniques can be successfully employed for the elemental analysis of these types of minute samples: flow-injection inductively coupled plasma mass spectrometry (FI-ICP-MS), graphite furnace atomic absorption spectrometry (GF-AAS) and total reflection X-ray fluorescence (TXRF), the feasibility of the latter for environmental and life sciences has being recently reviewed [2]. The advances achieved by these techniques in the elemental determination of cellular samples mainly in the last two decades are compiled in Table 1 [4–29].

Among the above-mentioned ubiquitous elements in clinical samples, iron determination requires special attention, as this element plays an essential role in biological processes such as electron

Abbreviations: FCS, fetal calf serum; HT-29, human colon adenocarcinoma cell line; PBS, phosphate buffered saline; RPMI-1640, Roswell Park Memorial Institute medium.

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Table 1
Literature survey on determination of elements in cell lines.

Cell lines	Elements	Sample preparation	Standardization	Technique	LOD	Ref.
Cultured skin fibroblasts	Cu	Sonication	External	GF-AAS	n.a.	[4]
K562	Cr	Lysis with 20 mL L^{-1} Nonidet P40	Matrix matching + 20 mL L^{-1} detergent	GF-AAS	0.175 μg L^{-1}	[5]
MCF-7, GM3189, IARC 1104	Cu, Fe, Zn	Freezing (liq. N_2)/thawing (5×)	n.a.	GF-AAS	n.a.	[6]
V79 Chinese hamster lung cells	Cr	Acidic dogerstion; 6 h		GF-AAS	n.a.	[7]
Caco-2	Zn	Acidic digestion; 80 °C	n.a.	GF-AAS		[8]
KB	Ru	Tetramethyl ammonium hydroxide	External	GF-AAS	10 ng mL^{-1}	[9]
Caco-2	Fe	Dissolution in DMSO	Standard addition	GF-AAS	<1.3 μg L^{-1} (3.3 μg g^{-1})	[10,11]
HT-29, MCF-7	Au	Lysis with sonotrode	Standard addition	GF-AAS	1.7 μg L^{-1}	[12,13]
Cultured retinal pigment epithelium cells	Cd	0.1 M NaOH; room temperature; overnight; neutralization with 0.1 M HCl; centrifugation (10,000 × g; 5 min)	External	GF-AAS	n.a.	[14]
MCF-7 (cellular, nuclei, DNA)	Pt	Triton X-100 solution (1%, w/w)	External	GF-AAS	0.005 mg L^{-1}	[15]
A549, PC-3, RERF-LC–MS, RERF-LC-OK, VMRC-LCD, EBC-1, LK-2, PC-10	Pt, Tl	+0.9% NaCl; vacuum oven (120 °C; 2 h)	External	ICP-MS	n.a.	[16]
LL 24 HLF (DNA)	Cr	Direct after DNA separation	n.a.	μFI-ICP-MS	29 fmol 20 μL^{-1}	[17]
MCF-7	Pt	Acidic digestion; 120 °C	In (IS)	ICP-MS	5 ng L^{-1}	[18,19]
2008, T289 (exosomes, DNA)	Pt	Direct after separation of exosomes and DNA	External	FI-ICP-MS	26 pg g^{-1}	[20]
Rabbit skin cells	Ag, Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Zn	MW-digestion	External	FI-ICP-MS	Cu: 0.199 ng g^{-1}; Fe: 3.36 ng g^{-1}; Zn: 1.64 ng g^{-1}	[21]
MeT-5A, MSTO-211H, NCI-H226, NCI-H2052, NCI-H2452, ACC-MESO-1	Cu, Mn, Zn	Acidic digestion; 130 °C; 5–7 h	Rh (IS)	ICP-MS	n.a.	[22]
HeLa (DNA)	Pt	DNA extraction	V (IS)	TXRF	<5 pg	[23,24]
HepG2, Caco-2, HeLa, NIH 3T3, N2A, B12	Cu, Fe, Zn, Ca, S	Acidic digestion; room temperature; 48 h	Se (IS)	TXRF	0.006 μg mL^{-1} (S), 0.001 μg mL^{-1} (Ca, Cu, Zn, Fe)	[25]
Caco-2	Cu, Fe, Zn	Acidic digestion; room temperature; 48 h	Se (IS)	TXRF	<5 pg	[26]
A2780/A2780cisR, CH1/CH1cisR, and 41 M/41McisR (cellular, DNA)	Pt	DNA extraction	V (IS)	SR-TXRF	<5 pg	[27]
HepG2, Caco-2, HeLa, NIH 3T3, N2A, B12, F805, NRK	Cu, Fe, Zn	Acidic digestion; room temperature; 48 h	Se (IS)	TXRF	n.a.	[28]
HepG2	Cu, Fe, Zn	Acidic digestion; room temperature; 48 h	Se (IS)	TXRF	n.a.	[29]
HT-29, HCA-7	Cu, Fe, Zn	Microwave assisted direct digestion	Ga (IS)	TXRF	n.a.	[3]

Abbreviations: (μFI-)ICP-MS = (micro flow-injection) inductively coupled plasma mass spectrometry; GF-AAS = graphite furnace atomic absorption spectrometry; IS = internal standard; MW-digestion = microwave-assisted digestion; n.a. = not available; and TXRF = total reflection X-ray fluorescence spectrometry.

transport, erythropoiesis, DNA synthesis, etc. In biological systems, iron exists in two oxidation states: the ferrous form, Fe(II) and the ferric form, Fe(III). The Fe(III)/Fe(II) conversion confers iron a pivotal importance in many biological processes. With this respect, iron has a fundamental role in oxygen transport, but interaction between free Fe and O_2 can result in reactive oxygen species (ROS) [30–32]. ROS formation is detrimental as they can interact with DNA, proteins, lipids inducing mutations and cellular damage [34]. Positive correlation between Fe stores in the body and risk of the development of colorectal, liver, kidney, lung, stomach cancer has been found in many studies [35]. Rapidly dividing cancer cells require higher amounts of Fe than the normal cells, therefore cancer cells are more sensitive to iron depletion [36]. Iron chelation therapy can be a new way of cancer treatment. Thus, numerous studies and clinical trials were carried out investigating the effectiveness of novel iron chelators as anticancer drugs [31,32].

Iron uptake and depletion can be investigated by the above-mentioned powerful analytical techniques and also by using ^{59}Fe radiolabelled isotopes [33]. Considering that other chelatable metals coexist with Fe in cells (i.e. Cu, Zn), the aim of this study was to develop methods suitable for the simultaneous determination of Fe, Cu and Zn in HT-29 human colorectal cancer cells treated with different iron compounds (Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin) by total reflection X-ray fluorescence spectrometry (TXRF), keeping as low as possible the possibility of cross-contamination by reducing the sample preparation steps and consequently, the reagent usage and sample preparation time. In the lack of certified reference materials (SRMs), another aim was to check the accuracy of the results by performing measurements with two different techniques. Therefore, graphite furnace atomic absorption spectrometric (GF-AAS) method development for these biological samples was also aimed. At last but not least, our scope was to develop an analytical method suitable for Fe determination having a wide dynamic concentration range as the metal-based clinical studies involves exposure to metal ions in increasing concentrations as well as Fe assessment in Fe-depleted samples is also important.

2. Experimental

2.1. Chemicals and reagents

Throughout the experiments, deionized Milli-Q (Millipore) water with a resistivity of 18.2 MΩ cm was used. Otherwise not indicated, all chemicals and reagents were of analytical grade. The 1000 mg L^{-1} Ga, Cu, Fe and Zn stock solutions were purchased from Merck (Darmstadt, Germany). Concentrated (65%) nitric acid and 30% hydrogen peroxide of Suprapur quality were purchased from Sigma Aldrich (Hungary). Iron(II) sulfate, Fe(III) citrate, Fe(III) chloride, Fe(III) transferrin as well as fetal calf serum (FCS) and trypsin-EDTA were purchased from Sigma Aldrich (Hungary). The HT-29 human colon adenocarcinoma cell line, obtained from ECACC (European Collection of Cell Cultures, UK) was cultured in an RPMI-1640 (Roswell Park Memorial Institute) medium (Sigma Aldrich, Hungary). Phosphate buffered saline (PBS) was freshly prepared in the laboratory.

2.2. Instrumentation and analytical procedures

2.2.1. Total reflection X-ray fluorescence spectrometry (TXRF)

The TXRF analyses were performed by using an TXRF 8030C spectrometer (Atomika Instruments GmbH, Oberschleissheim, Germany), equipped with a 2.5 kW X-ray tube made of a Mo/W alloy anode and a double-W/C multilayer monochromator, adjusted to obtain an excitation energy of 17.4 keV (Mo K_α). In this equipment, the characteristic radiation emitted by the elements present in the sample is detected by a Si(Li) detector with an active area of 80 mm^2 and a resolution of 150 eV at 5.9 keV. The measurements were performed working at 50 kV and the current was adjusted automatically as a trade-off between the detector dead time and total analysis time. The acquisition time was 500 s. Gallium was used as internal standard. Ten microlitres from the samples were pipetted onto quartz reflectors and dried at 80 °C on a ceramic coated heater (Cole Palmer, USA) prior to analyses. The K_α lines used for determination of Cu, Fe and Zn were 8.047 keV, 6.403 keV, 8.638 keV, respectively.

Recovery for these elements was checked by spiking the samples with Cu, Fe and Zn solutions at 5 concentration levels, the first spike aiming at doubling the initial concentration of the investigated element in the sample. Thus, samples were spiked with 2, 5, 10, 50 and 100 ng Fe or Zn, meanwhile for Cu, the spiked amounts were 10 times less.

2.2.2. Graphite furnace atomic absorption spectrometry (GF-AAS)

The GF-AAS measurements for the simultaneous determination of Fe and Cu were performed on a Perkin-Elmer Model SIMAA 6000 atomic absorption spectrometer equipped with a transversely heated graphite atomizer (THGA), a longitudinal Zeeman-effect background (BG) corrector and an AS-72 (Perkin-Elmer, Überlingen, Germany) autosampler providing stabilized temperature platform furnace conditions. Integrated L'vov-platforms were placed in the end-capped graphite tubes (Perkin-Elmer, Part No. B3000653) of the THGA. Perkin-Elmer hollow-cathode lamps of Cu and Fe were operated at 15 mA current.

The GF-AAS measurements were carried out with the application of $Pd(NO_3)_2$ as a chemical modifier. Amounts of 5 μg of $Pd(NO_3)_2$ were added to 10 μL of samples and then mixed prior to be dispensed onto the integrated L'vov-platforms of the end-capped graphite tubes. The spectral lines of Cu 324.8 nm and Fe 305.9 nm were selected for the measurements. The Zeeman-effect BG corrected integrated absorbance signals of Cu and Fe were simultaneously measured with signal integration times of 5 s. Each data presented in the tables and in the figures corresponds to an average value of three replicate determinations. For quantitative determination, external standardization was used. The concentration range of the Cu and Fe calibrants were between 10 and 100 ng mL^{-1} and 100 and 1000 ng mL^{-1}, respectively. The optimized temperature program of the developed GF-AAS method is shown in Table 2.

Recovery was checked by spiking selected samples with 5 μL of a standard solution containing Fe and Cu in a concentration of 500 ng L^{-1} and 50 ng L^{-1}, respectively.

2.3. Cell culture and sample preparation

The HT-29 human colorectal cancer cell line samples were cultured at the Department of Clinical Research of the National Institute of Oncology, Budapest, Hungary. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in antibiotic-free medium supplemented with 10% (v/v) fetal calf serum (FCS). The

Table 2
Temperature program of GF-AAS method suitable for the simultaneous determination of Cu and Fe applying 5 μg Pd in form of $Pd(NO_3)_2$ as a chemical modifier.

Step	Temperature (°C)	Ramp (s)	Hold (s)
Drying	110	1	30
Drying	130	15	40
Ashing	400	10	20
Pyrolysis	1400	10	20
Atomization	2300	0	5
Cleaning	2450	1	3

FCS contains several growth factors, cytokines, steroid and peptide hormones, enzymes and adhesion molecules which are essential for cell proliferation. Samples were cultured to 80% confluency in 6-well plates (10^6 cells/well). Cells were incubated for 4 h with increasing concentrations (10, 20, 50 and 100 μmol L^{-1} expressed as Fe in medium) of different iron compounds: Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin. Simultaneously, cells were also cultured without iron treatment. Similar treatments were also carried out in FCS-free medium. After incubation, the cells were harvested with a trypsin-EDTA solution (5.0 g L^{-1} porcine trypsin and 2.0 g L^{-1} EDTA·4Na in 0.9% (v/v) sodium chloride solution). Trypsinization was stopped by dilution with phosphate buffered saline (PBS) and then, the cells were washed two times with 1 mL PBS. The cell number was counted with a haemocytometer using trypan blue. The cells were centrifuged (J2-21 Beckman centrifuge) at 20,000 × *g* for 15 min at 4 °C in Eppendorf tubes. After centrifugation, 20 μL of 30% H_2O_2, 80 μL of 65% HNO_3 and 15 μL of 10 μg mL^{-1} Ga were added to the cells and digested for 24 h at room temperature. From the resulting solutions, 10 μL was pipetted on the quartz reflectors used for TXRF analysis. For GF-AAS analysis, 10 μL was directly dispensed from the sample together with 5 μL chemical modifier and 5 μL distilled water into the graphite tube by the autosampler. For the recovery measurements, the aliquots of distilled water were replaced with the spiking solution. The reflectors were dried on a hot plate at 80 °C for 10 min.

The surface of the dried samples placed on reflectors was checked by scanning electron microscopy (SEM), model AMRAY 1830, in vacuum ($p = 10^{-4}$ mbar) at 20 kV accelerating potential and 0.1 nA current intensity.

2.4. Statistical analysis

The statistical calculation of data with paired-samples *t*-test was done by a statistical analysis software purchased from NCSS (Kaysville, Utah, USA). *p* Values < 0.05 were considered as statistically significant.

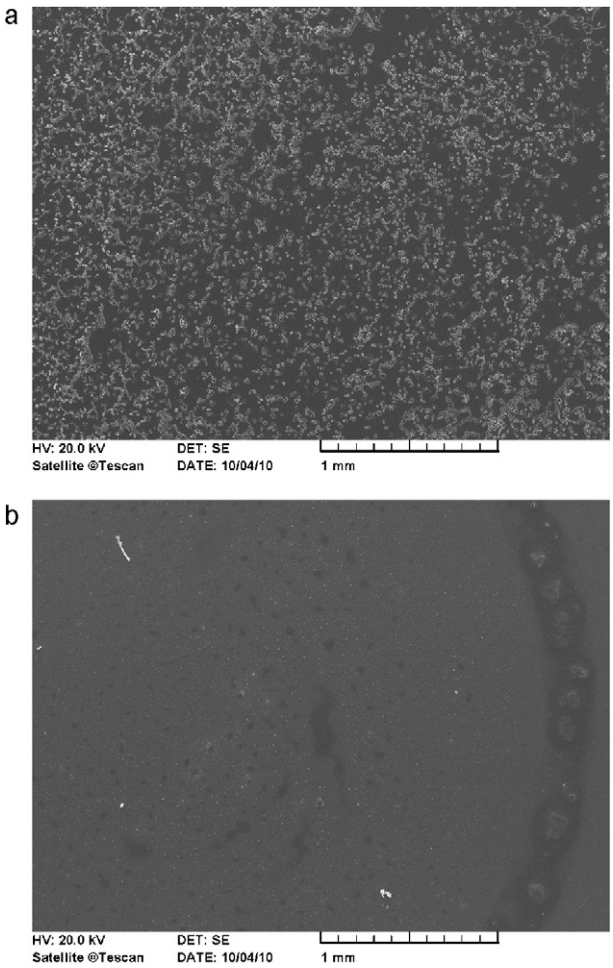

Fig. 1. SEM images for undigested HT-29 cell samples (a) and digested ones for 24 h (b). Operating conditions of the instrument: 10^{-4} mbar pressure; 20 kV accelerating potential and 0.1 nA current intensity.

3. Results and discussion

3.1. Development of TXRF method for determination of Cu, Fe and Zn in HT-29 cell lines

The starting point of our work was the employment of 1×10^6 cells based on the works of Gonzalez et al. [25,26,28,29] reporting on the determination of low intracellular concentration of several elements in mammalian cell lines prior to an acidic digestion of 48 h at room temperature. TXRF investigations were performed in order to check the relationship between analytical response and the increasing cell number in the range of 3×10^5–10^7 cells. Linearity could be obtained up to 4–6 × 10^6 cells. Above this cell number value, formation of organic debris was observed resulting in the increase of standard deviation of the obtained results. Therefore, as a compromise between low sample demand and analytical signal, cca. 10^6 cell number was used for all our further experiments. As the primary demand in the case of minute biological samples is to analyze them in the lowest possible amount without altering the reliability of the results, the only remaining way was the minimization of cross contamination and reduction of the blank values. With this respect, as Suprapur reagents and deionized water had to be used in order to achieve the lysis of the cells, the only possibility was to reduce the sample preparation steps that implies minimization of the contact time of the samples with ambient air. Thus, the feasibility of performing several sample preparation steps in the same Eppendorf tubes was investigated by executing centrifugation, washing with PBS and microdigestion (with a mixture of nitric acid and hydrogen peroxide containing simultaneously the internal standard for the TXRF measurements) of cell samples incubated with or without Fe as well as cultured in a growth medium supplemented with or without FCS. SEM analysis of an undigested sample (Fig. 1a) and a digested one for 24 h (Fig. 1b) was employed in order to check the remaining salt particles and organic debris after sample preparation. The SEM images revealed that a 24 h-long microdigestion performed at room temperature was enough in order to destroy the layered cellular structure and thus, offering the possibility of obtaining reliable results for Cu, Fe and Zn in the cell lines by TXRF. This finding was also confirmed by analyzing the same samples after rotating the quartz carriers containing the samples 4 times (each time by 90°) (Table 3). Representative figures of merit for Cu, Fe and Zn in untreated HT-29 cells cultured in a medium containing FCS are the following: 4.55 ± 0.3; 10.57 ± 1.0; 41.33 ± 3.1 ng/10^6 cells, respectively. In addition, the 5-level recovery values for Cu, Fe and Zn ranged between 87 and 105%. The best recovery values were obtained for Zn but this was expected as the Zn concentration in the samples is higher than that of Cu and Fe. For higher initial Fe concentration in the samples, excellent recovery values ranging between 95 and 105% could be also obtained (Fig. 2). Thus, in the case of cell samples where high amounts of Fe are taken up, TXRF is an ideal tool for accurate quantitative determination of Fe, meanwhile in the case of GF-AAS, excessive dilution should be applied for these types of samples.

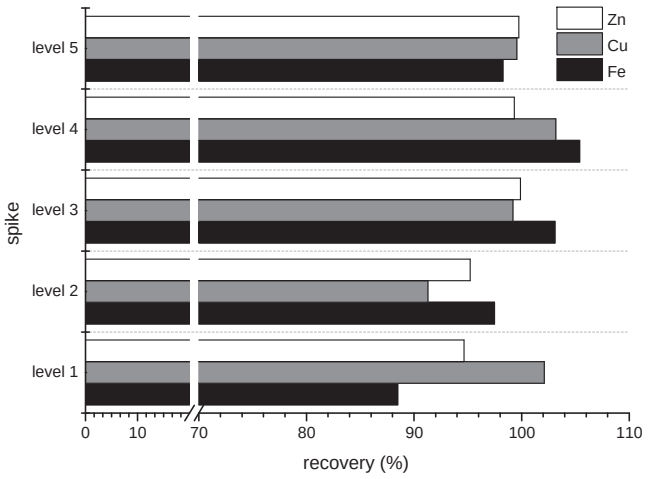

Fig. 2. Recovery rates obtained by TXRF for Cu, Fe and Zn in digested HT-29 cell samples spiked with different amounts of the elements investigated at different spike levels. Level 1: addition of 2 ng; level 2 = addition of 5 ng; level 3 = addition of 10 ng; level 4 = addition of 50 ng; and level 5 = addition of 100 ng of Fe and Zn. For Cu, the amounts added were ten times less than for Fe and Zn.

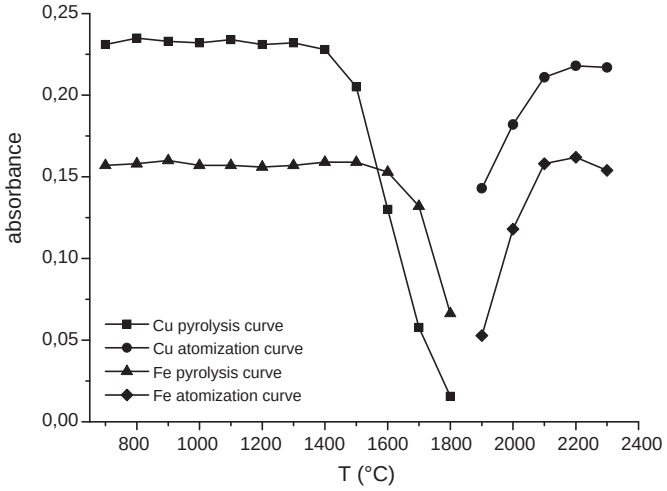

Fig. 3. Pyrolysis and atomization curves of the optimized simultaneous GF-AAS method (with the application of 5 μg Pd as $Pd(NO_3)_2$ as chemical modifier).

3.2. Development of GF-AAS method for simultaneous determination of Cu and Fe in HT-29 cell lines aiming at accuracy check

In the lack of SRMs, special attention was paid to analyze the digested samples by two independent techniques. In order to achieve this goal, GF-AAS was chosen due to its excellent detection limits and low sample demand. Moreover, the GF-AAS equipment used in this study allowed the simultaneous determination of Cu and Fe that reduces the sample volume demand. Zinc determination by GF-AAS was not considered as this element provides only a single and very sensitive wavelength that is very susceptible for blank values. Generally, the same applies to Fe; however, in this case, the selection of the less sensitive analytical line corresponding to 305.9 nm and lower blank values confer the possibility of Fe determination in the case of high Fe concentrations. In this way, the concentration range of Fe, which can be determined by GF-AAS, was between 0.1 and 1 μg mL^{-1}, meanwhile that of Cu was 10 times less. Luckily, the Cu concentration in cells is constantly low compared to that of Fe as the Fe/Cu ratio is generally regulated in these biological compartments. The critical steps for the GF-AAS method development were the pyrolysis and atomization temperatures, where beyond the attention that had to be paid due to the salinity and organic debris remnants of the sample, a compromise had to be found for the simultaneous determination of Cu and Fe as their volatility and atomization characteristics are different. Moreover, the chemical modifier can alter the vaporization and atomization behavior of the analytes. According to our results, a 1400 °C pyrolysis and 2300 °C atomization temperature were required in order to get reliable results when 5 μg of Pd in form of $Pd(NO_3)_2$ was also employed as a chemical modifier (Fig. 3). Regarding the optimized temperature program used for GF-AAS analysis (Table 2), it can be seen that duration of the second drying step is unusually high but it was required by the high nitric acid content of the samples. In addition to this, an ashing step of 20 s had to be included to in order to eliminate the organic matrix. The ramps of the drying steps were optimized in order to avoid sample sputtering. The sensitivity of the determinations, expressed as the characteristic mass, was 17 and 253 pg for Cu and Fe, respectively.

The analytical capabilities of the developed TXRF and GF-AAS methods in terms of analysis time, calibration, repeatability, reproducibility, recovery and limit of detections (LODs) can be seen in Table 4. Comparing the analytical parameters of the two methods, it can be stated that the GF-AAS method has a better repeatability, recovery, LOD than the TXRF one. However, TXRF is a more relevant multielemental technique than GF-AAS and its dynamic range is much wider; thus, samples containing Fe, Cu or Zn in high concentration could be determined only by TXRF. The most important outcome of this comparison is that the results obtained for Fe

Table 3
Repeatability of TXRF analyses expressed as relative standard deviation (RSD) after rotating the sample carriers by 4 × 90° (n = 10).

Sample no.	RSD (%)		
	Cu	Fe	Zn
1	5.6	1.5	1.0
2	5.1	3.9	1.3
3	4.1	2.4	1.6
4	6.9	4.8	1.4
5	2.4	2.2	0.9
6	3.3	2.0	1.0
7	3.2	2.9	1.3
8	2.4	2.2	0.8
9	5.4	3.7	1.5
10	4.2	3.1	1.8
Average	4.3	2.9	1.3

Table 4
Comparison of the analytical capabilities of the developed TXRF and GF-AAS methods.

Parameter	TXRF	GF-AAS
Minimal sample volume (μL)	2	5
Analysis time (s)	100–500	cca. 100
Calibration	IS (Ga)	External
Precision		
Repeatability[a]	<5%	<2%
Reproducibility[b]	<10%	<5%
Recovery (%)[c]	87–105	98–101
LODs (ng mL^{-1})		
Cu	10.30	0.69
Fe	14.50	9.70
Zn	9.80	–

Abbreviation: IS = internal standard; LOD = limit of detection; and cca. = circa.

[a] Expressed as the deviation of the results of the analysis carried out by the same operator on the same instrument during three different days.

[b] Expressed as the relative standard deviation of the 3 independent measurements.

[c] By spiking the samples with standard solutions of 500 ng L^{-1} Fe and 50 ng L^{-1} Cu by GF-AAS method, and five different concentration levels by TXRF method.

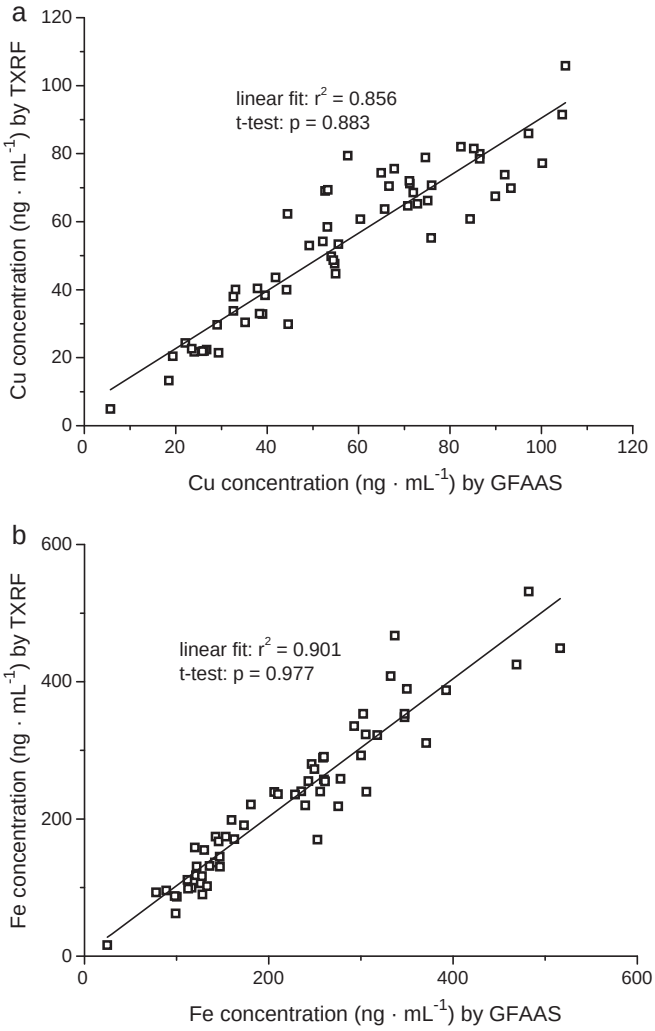

Fig. 4. Correlation between the Cu (a) and Fe (b) concentration values obtained by the developed TXRF and GF-AAS methods for untreated and treated HT-29 adenocarcinoma cells ($n = 64$).

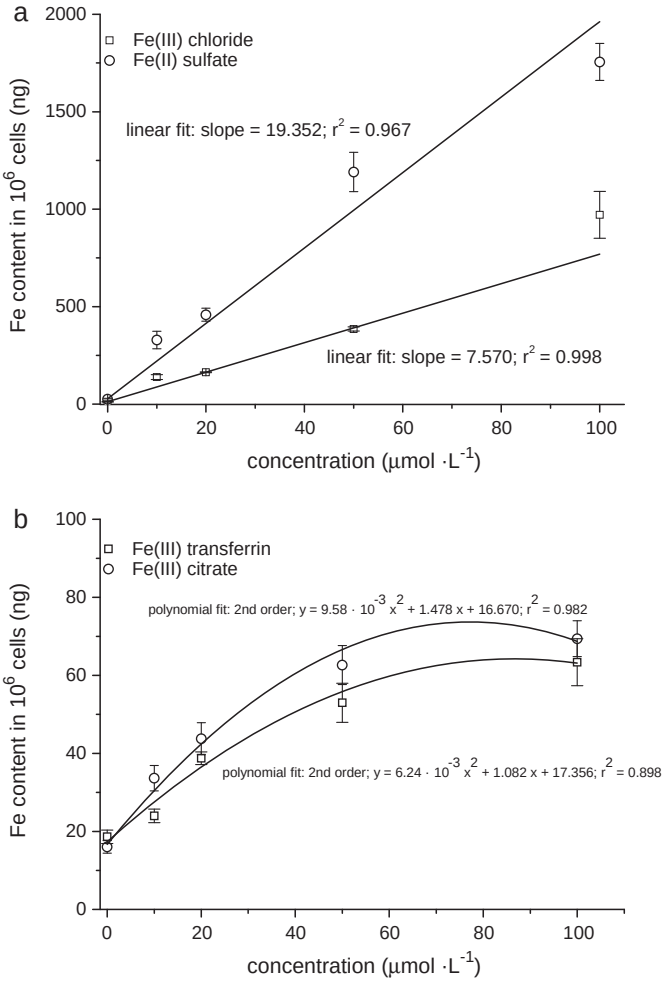

Fig. 5. Results for Fe uptake studies; iron content of HT-29 cells cultured in a fetal calf serum (FCS)-free medium determined by TXRF after a 4-h treatment when the Fe supply was Fe(II) sulfate and Fe(III) chloride (a); Fe(III) citrate and Fe(III) transferrin (b).

and Cu concentration data originating from samples whose Fe concentration fitted in the calibration range of GF-AAS were in good agreement. Therefore, the plot of the Fe and Cu concentration data pairs obtained by GF-AAS and TXRF in the same samples (Fig. 4) could be characterized with an adequate linear fit ($r^2 = 0.856$ for Cu; $r^2 = 0.901$ for Fe). Moreover, the same data sets analyzed by paired samples *t*-test were in good agreement with the linear fitting, which was proved by the two-tailed significance levels $p = 0.883$ obtained for Cu and $p = 0.977$ for Fe ($n = 64$).

3.3. Iron uptake study

A 4 h-long iron uptake of HT-29 adenocarcinoma cell line was investigated from different aspects: employment of inorganic ferro ($FeSO_4$) and ferri ($FeCl_3$) salts vs. chelated iron(III) forms, namely Fe(III) citrate and Fe(III) transferrin.

FCS was chosen as it is the most widely used serum for cell culture, it is low in antibodies and it contains more growths factors. However, data interpretation may be difficult due to complexation of Fe in this pH = 7.4 medium by other matrix constituents. In the case of inorganic Fe supplies, Fe uptake by HT-29 cell lines could be characterized as linear over the whole concentration range (10–100 μmol L^{-1}) of the Fe treatment of cells cultured in FCS-free medium (Fig. 5a). The highest Fe uptake was observed in the case of Fe(II) sulfate for the 4 h-long treatments indicating that Fe is taken up in the divalent form.

When the cells cultured in FCS-free medium were treated with chelated Fe(III) forms, the Fe uptake was modest and it could be characterized with a polynomial fitting of second order (Fig. 5b). The reasons for this behavior could be attributed to different Fe uptake mechanisms. However, the chemical form of Fe practically did not influence the amounts of Fe taken up by adenocarcinoma cells cultured in the FCS supplemented medium. This was proved by the similar slope values obtained for Fe(II) sulfate, Fe(III) chloride and Fe(III) citrate being 0.145, 0.204 and 0.107, respectively, by performing linear fitting. The lowest Fe uptake in the case of cells cultured in FCS-containing medium was observed in the case of Fe(III) transferrin (slope value for linear fitting being 0.04). Comparing the iron uptake from different Fe(III) supplies in FCS-free and FCS containing medium, it can be stated that independently of the iron supply, the HT-29 cells cultured in FCS-free medium contained between cca. 5 and 50 times higher amounts of Fe than the cells cultured in FCS supplemented medium in the case of 4 h incubation.

4. Conclusions

The TXRF and simultaneous GF-AAS methods developed in the present study are suitable for simultaneous determination of Fe,

Cu in HT-29 human colorectal cancer cells treated with different iron compounds (Fe(II) sulfate, Fe(III) chloride, Fe(III) citrate and Fe(III) transferrin) after a relatively simple sample preparation protocol consisting of treatment of cell sample with a mixture of 65% nitric acid and 30% hydrogen peroxide for 24 h in the same vessels used for centrifugation. Although complete digestion of the samples could not be achieved, the selected analytical methods (TXRF and simultaneous GF-AAS) allowed an accurate determination of these types of biological matrices minimizing their contamination. Thus, results obtained for Cu and Fe by TXRF and GF-AAS method were in good agreement. Determination of Fe in high concentration and Zn were possible only by TXRF.

According to the Fe uptake studies, HT-29 cells incubated in FCS-free medium contained cca. 5–50 times higher Fe amount comparing to cells cultured in FCS supplemented medium and notable differences could be observed in the iron uptake according to the different Fe containing chemicals used in the uptake-experiments. Further prospective for this study is to carry out experiments with other different iron chelators aiming at the investigation of their effect on Fe, Cu, and Zn homeostasis in neoplastic cells.

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