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Validation of the analytical method using the energy dispersive X-ray fluorescence technique (EDXRF) for application in pharmaceutical sciences

Paulino[a] T.H., Oliveira Junior[a,*] J.M., Baldo[a] D.A., Aranha[b] N.,

Gonçalves[c] D.B., Vila[d] M.M.D.C., Balcão[d,e] V.M.

[a] *LAFINAU- Laboratory of Applied Nuclear Physics of the University of Sorocaba, University of Sorocaba, 18023-000 Sorocaba/SP, Brazil.*

b LabMat – Laboratory of Materials, University of Sorocaba, 18023-000 Sorocaba/SP, Brazil.

[c] *Post-Graduate Program in Technological and Environmental Processes, University of Sorocaba, 18023-000 Sorocaba/SP, Brazil.*

[d] *PhageLab - Laboratory of Biofilms and Bacteriophages, University of Sorocaba, 18023-000 Sorocaba/SP, Brazil.*

[e] *Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, P-3810-193 Aveiro, Portugal.*

** Corresponding author; e-mail: jose.oliveira@prof.uniso.br*

ABSTRACT

The determination of impurities in raw materials intended for the production of pharmaceutical products is important to guarantee the quality of the final product, as well as to avoid damage to health. Metallic impurities can exhibit toxic effects even at low concentrations and so permissible levels are defined by the regulatory agencies and pharmacopeias. However, few methods are presented in official compendia in Brazil. In this sense, fast, sensitive, and precise techniques such as the energy dispersive X-ray fluorescence technique (EDXRF) must be evaluated for the analysis of metals in materials for pharmaceutical use. This way, therefore, there is the need to investigate the presence of contaminants and their concentration levels. The major goal of this research work was to validate a method for using the Energy Dispersive X-Ray Fluorescence (EDXRF) technique to identify and quantify the chemical composition of raw materials and pharmaceutical products. The methodology used was based on the selection of a microcrystalline cellulose matrix, which was spiked with two classes of contaminant elements, Class 1 (Cd, Pb, As, Hg) and Class 2A (Co, V, Ni) as defined by ICH guideline Q3D. The qualitative and quantitative analyses were carried out using the EDXRF technique, which proved to be quite effective and met all the validation parameters required in the mandatory official compendia (Resolution of the Collegiate Board (RDC) of Brazilian Health Regulatory Agency (Anvisa) nº 166, July 24, 2017), such as selectivity, linearity, precision, detection limit, quantification limit and robustness. This study showed that EDXRF can be used as a technique for detection and quantification of elemental impurities belonging to Class 1 and Class 2A.

Keywords: **Validation; Energy Dispersive X-Ray Fluorescence (EDXRF) Technique; Heavy metals; Elemental impurities of Classes 1 (Cd, Pb, As, Hg) and 2A (Co, V, Ni).**

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1. INTRODUCTION

The presence of impurities may affect the quality, safety, and efficacy of pharmaceutical products. Therefore, the levels in the pharmaceutical products must be controlled within acceptable limits by observing the level of impurities within the permitted daily exposure (PDE), as established by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and Guideline for Elemental Impurities (Q3D) [1]. The "Guideline for Elemental Impurities (Q3D)" has been most widely accepted for assessment of metal contamination [2] and, this way, was chosen for metals limits. The EDXRF technique for identifying and quantifying elemental impurities (metals and non-metals) is not indicated as a valid technique by the Brazilian Pharmacopeia (BP) [3], but the United States Pharmacopeia (USP) [4] provides for the use of this technique. According to the BP, the approved techniques for the quantification of heavy metals are limit test and determination by atomic spectrometry (e.g., Inductively Coupled Plasma Atomic-Emission Spectroscopy - ICP-AES or Atomic Absorption Spectroscopy - AAS). The limit test consists of the formation of solid particles of heavy metal sulfides, in suspension, and subsequent visual comparison of the color intensity in the sample and standard preparations in a Nessler tube. The test is semi-quantitative and makes it possible to infer whether or not the sample passes the test, representing the sum of the concentration of contaminants in the sample. The atomic spectrometry method makes it possible to quantify each contaminating element in the sample and different limits are established for each element according to its toxicity, pharmaceutical form, and route of administration. AAS is a quantitative method of metals analysis which was suitable for the determination of 70 elements by three option accessories (flame, furnace, hydride generation) [5] however, many times, needs multiple sample preconcentration processing and expensive instruments [6].

The energy dispersive X-ray fluorescence spectroscopy can measure different elemental impurities, at different concentration levels in various matrices [7]. X-ray fluorescence can be used to quantify practically all elements of interest to pharmaceutical industry, such as heavy metals, within the Maximum Permitted Limits (MPL) as established by the Pharmacopeias assessing the safety of ingestion of each of these elements. More recently, Sauer et al. [8] published a new approach focused on the development of a methodology for screening elemental impurities in solid oral pharmaceutical products using EDXRF methodology with very promising results.

This work is focused on the study of elemental impurities in pharmaceutical products using

EDXRF methodology. The elementary impurities used were those belonging to Class 1 (As, Cd, Hg and Pb) and Class 2A (Co, Ni and V), according to the ICH Q3D classification [1]. These two classes (1 and 2) were chosen because are highly toxic to humans. Class 2 impurities are divided into two groups, 2A (Co, Ni, and V) and 2B (Ag, Au, Ir, Os, Pd, Pt, Rh, Ru, Se, and Tl). Group 2A was chosen for this study because having a higher probability of occurrence in the pharmaceutical products. Table 1 shows the MPL of the elemental impurity concentration by the BP [3] and USP <232> [9] for oral intake. The ICH Q3D [1] defines the default values of maximum Permitted Daily Exposure (PDE) for oral pharmaceutical products of Class 1 and Class 2A in units of µg/day, and therefore it is necessary to define the Daily Intake (DI) of the pharmaceutical product to convert to Concentration Limits (CL) in (µg/g). Using Equation 1 below, and considering a daily intake of 10 grams of drug product, is calculated a common permissible target elemental concentration for each component in the drug [1].

$$Concentration\left(^{\mu g}/_{g}\right) = \frac{Permitted\ daily\ exposure\left(^{\mu g}/_{day}\right)}{Daily\ amount\ of\ drug\ product\left(^{g}/_{day}\right)} \quad (1)$$

This study was conducted considering: Option (1) DI = 10 g/day (maximum daily intake (amount) of the drug product, according to ICH Q3D) and Option (2A) DI = 2.5 g/day (this option is similar to Option 1, except that the drug daily intake is assumed to be less than 10 g) [1]. Considering the value for PDE and DI in **Options (1)** and **(2A)**, the CL in (µg/g) are displayed in Table 1. In pharmaceutical products, it must be taken into account that the level of impurities cannot exceed the value defined for PDE.

Table 1. Elements to be considered in risk assessment, maximum permitted limit (MPL) for oral exposure to metals. BP and USP, permitted daily exposure (PDE) and concentration limits (CL) for 1) and 2A) options of daily intake (DI).

Element	Class	MPL-BP (µg/g)	MPL-USP (µg/g)	PDE (µg/day)	CL (µg/g) DI = 2.5 g/day	CL (µg/g) DI = 10 g/day
Arsenic (As)	1	1.5	1.5	15	6	1.5
Cadmium (Cd)	1	0.5	0.5	5	2	0.5
Lead (Pb)	1	1	0.5	5	2	0.5
Mercury (Hg)	1	1.5	3	30	12	3
Cobalt (Co)	2A	---	5	50	20	5
Vanadium (V)	2A	25	10	100	40	10
Nickel (Ni)	2A	25	20	200	80	20

Legend: MPL-BP - Maximum Permitted Limit by Brazilian Pharmacopoeia [3]; MPL-USP - Maximum Permitted Limit by United States Pharmacopoeia <232> [9]; PDE - Permitted Daily Exposure from ICH Q3D [1]; CL - Concentration Limit [1].

This work brings the validation of the EDXRF method using a matrix based on microcrystalline cellulose and Option 2A for daily intake, focusing on the elements of Class 1 (Cd, Pb, As, Hg) and Class 2A (Co, V, Ni) for elemental impurity control in pharmaceutical products.

2. MATERIALS AND METHODS

2.1. Materials

To perform the analysis via X-ray fluorescence (XRF), pellets were made based on microcrystalline cellulose (brand: Valdequímica, lot nº 023488 and Labsynth, lot nº 227833) spiked with the elements of interest belonging to Class 1 and Class 2A. In some pellets one also used the light elements Na, Mg, Si, Cl, K, Ca, Fe and Ti together with microcrystalline cellulose, in the concentrations needed for the study. The analytes used in this study (light elements and elements of classes 1 and 2A) as reference materials [10] to manufacture the tablets were purchased from Chemplex Industries Inc. (SpectroStandards® XRF reference material preparation kit number 6700). The kit contains 50 different chemical elements, most of which are oxide-based.

In addition to these two groups described above, one also used in some tests a third group of pills purchased in the Brazilian market, containing the following active ingredients: (a) Metformin 500 mg (batch: BR124143, manufacture: 08/2020, expiration date: 07/2023), (b) Simvastatin 20 mg (batch: LD851, manufacture: 01/2021, expiration date: 12/2022) and (c) Glibenclamide 5 mg (batch: ARA06380, manufacture: 11/2020, expiration date: 10/2022). Metformin, Glibenclamide (for the treatment of patients with type 2 diabetes), and Simvastatin (used to treat and prevent hypercholesterolemia) were chosen because they belong to the list of essential drugs and are widely consumed since they are actives for the treatment of diseases that affect a large number of people in the world [11]. Class 1 elements were added because they are the most toxic and thus the most interesting. This third group was spiked with Class 1 elements (Cd, As, Hg, Pb), crushed, homogenized and pressed to form 4 g tablets, similar to the other tablets used in this study. These drugs were purchased in local pharmacies.

2.2. Methods

2.2.1. Pellets preparation

The samples were homogenized using an agate mortar and rotary mixer containing steel balls inside; then, the mixture was separated in portions of 4 g and compacted using a compression machine (pressure of 10 tons and pressing time of 30 s) (brand: Amef, model AP 25 T, electric press), according to the methodology of Marguí et al. [12] with modifications. The final shape of each pellet with 4 g of material (analyte + cellulose) was 30 mm of diameter by 4 mm in height, approximately.

2.2.2. Drug samples

As a way of evaluating how this validation methodology would behave in a real situation, one prepared a recovery test using pharmaceutical tablets produced by Brazilian pharmaceutical companies, which were contaminated with elemental impurities of class 1A (Cd, As, Hg, Pb) only, in high concentration (~35 μg/g) and, in the sequence, the recovery test was performed. The tablets used in this test were **(a)** Metformin 500 mg, **(b)** Simvastatin 20 mg, and **(c)** Glibenclamide 5 mg. The samples were prepared in the same manner as the pellets.

2.2.3. XRF set up

The measurements were carried out using a Malvern Panalytical Epsilon 1, benchtop EDXRF spectrometer, equipped with a 5W, 10kV to 50kV Silver anode X-Ray tube, with energy resolution around 135 eV, having available the following filters for the X-ray beam: Ag, Cu, Ti, and Al. The spectrometer uses a high-resolution SDD (Silicon Drift Detector) detector and operates under atmospheric pressure. The evaluation of elemental concentration of interest elements was performed using the spectrometer configuration displayed in Table 2. In addition to elements belonging to Class 1 and Class 2A, some light elements such as Na, Mg, Ca, K, among others, were also measured in some situations.

Table 2. XRF spectrometer configuration.

Element	Line	Condition	Measured time (s)	Voltage (kV)	Current (µA)	Filter Thickness (µm)	Detector
As	Kα	Ni-Mo	1500	50	100	Ag	normal
Ca	Kα	K-V	900	12	300	Al-50	normal
Cd	Kα	Rh-Sb	1800	50	100	Cu-500	normal
Cl	Kα	P-Cl	600	10	150	Ti	high resolution
Co	Kα	Cr-Co	600	20	200	Al-200	normal
Fe	Kα	Cr-Co	600	20	200	Al-200	normal
Hg	Lα	Ni-Mo	1500	50	100	Ag	normal
K	Kα	K-V	900	12	300	Al-50	normal
Mg	Kα	Na-Si	600	10	150	NF	high resolution
Na	Kα	Na-Si	600	10	150	NF	high resolution
Ni	Kα	Ni-Mo	1500	50	100	Ag	normal
Pb	Lα	Ni-Mo	1500	50	100	Ag	normal
Si	Kα	Na-Si	600	10	150	NF	high resolution
Ti	Kα	K-V	900	12	300	Al-50	normal
V	Kα	K-V	900	12	300	Al-50	normal

Legend: NF - No filter.

2.2.4. Validation

To validate the method for using the EDXRF technique to investigate the elemental impurities of Class 1 and Class 2A, one analyzed selectivity, linearity, precision, accuracy, detection and quantification limits, robustness and interval according to parameters required in the mandatory official compendia [13].

Accuracy

The accuracy was evaluated using certified material with matrix fortification[14]. The accuracy was based on the recovery rate, which is the percentage of the calculated concentration in comparison to the prepared sample concentration (Equation 2), viz.

$$R_{rec} = \frac{C_m}{C_{nom}} \times 100 \quad (2)$$

where R_{rec} = recovery (%); C_m = mean concentration; C_{nom} = nominal (true) concentration.

Two groups of samples were prepared. The first group of pellets was prepared using microcrystalline cellulose spiked with elements belonging to class 1 (Cd, Pb, As, Hg) and class 2A (Co, V, Ni), in three different concentrations, viz. **i)** high (~35 µg/g), **ii)** intermediate (~15 µg/g) and **iii)** low (~2 µg/g). The second group of pellets was prepared with the same elements and

concentrations, but with the addition of a mix with several light elements (Na, Mg, Si, Cl, K, Ca, Fe and Ti). The light elements were added as a way of modifying the original matrix, creating a "possible" perturbation in the samples. The light elements, that is, elements with a low atomic number are very difficult to measure reliably via EDXRF, but can interfere in the system [15]. So they were added, in order to verify and would be able or not to disturb the analytical system. The light elements added and their respective (high, intermediate and low) concentrations were: Na (0.91; 0.45; 0.072)% (m/m), Mg (0.67; 0.33; 0.053)% (m/m), Si (0.53; 0.26; 0.041)% (m/m), Cl (2.6, 1.3; 0.21)% (m/m), K (1.4; 0.7; 0.11)% (m/m), Ca (0.82; 0.41; 0.064)% (m/m), Fe (0.8; 0.4; 0.063)% (m/m) and Ti (0.6; 0.3; 0.053)% (m/m).

The accuracy acceptance criteria for elemental recovery according to the working range were 70-150% [7].

Linearity

The linearity was expressed by a linear regression calculated by the method of least squares and by residual analysis. The correlation coefficient (R) is the correlation between the predicted and observed values. This will have a value between 0 and 1; the closer the value is to 1, the better the correlation [16]. The linearity test was conducted using tablets of microcrystalline cellulose spiked with the elements of interest (class 1 and class 2A) at seven concentrations: 70 μg/g, 35 μg/g, 17.5 μg/g, 8 μg/g, 4 μg/g, 2 μg/g and 1 μg/g, and for each concentration the measurements were carried out in triplicate.

Precision

The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision in this study was evaluated by the dispersion of the results, calculating the Relative Standard Deviation (RSD) of the measurement series, as shown in Equation (3), with 9 (nine) determinations being performed, considering the linear range of the analytical method and 3 (three) levels of concentrations: **(i)** high (~35 μg/g), **(ii)** intermediate (~15 μg/g) and **(iii)** low (~2 μg/g), with 3 (three) replicates on each level, for all elemental impurities studied, namely Cd, As, Pb, Hg, Co, V, Ni, viz.

$$RSD = \frac{SD}{C_m} \times 100 \quad (3)$$

where SD = standard deviation; C_m = mean concentration.

The acceptable relative standard deviation (RSD) for precision parameters considered in this work was: **a)** concentrations between 1 µg/g to 10 µg/g, RSD < 11%, **b)** concentrations between 11 µg/g to 100 µg/g, RSD < 7.3% [17].

Detection and Quantification Limits

The detection limit (DL) and quantification limit (QL) are usually calculated using the following set of equations (4):

$$DL = \frac{3.3 \times SD}{m} \quad and \quad QL = \frac{10 \times SD}{m} \quad (4)$$

where DL = detection limit; m = slope of the calibration curve; SD = standard deviation; QL = quantification limit.

However, in quantitative X-ray fluorescence analysis, the DL and QL limits are determined somewhat differently. It is generally accepted that the minimum detectable intensity of a spectral line should exceed by a factor of 3.3 the standard deviation of the integrated background under the spectral line [18]. According to this definition, DL and QL can be expressed in terms of the ratio between the intensity of the background counts (I_b) and the intensity of the spectral line of interest (I_0) (peak counts), multiplied by the initial mass (m_o) used in the analysis, as shown in Equation (5). DL and QL limits were obtained by analyzing a tablet spiked with a mass (m_0) of approximately 2 µg/g of class 1 and class 2A contaminants, in the matrix of cellulose microcrystalline plus light elements. DL and QL depend only on the ratio of background to signal count rates [19, 20].

$$DL = \left(\frac{I_b}{I_0}\right) m_0 \quad and \quad QL = \frac{10}{3.3} DL \quad (5)$$

where DL = detection limit; m_o = mass concentration (µg/g) of class 1 and class 2A contaminants; I_b = intensity of the background line; I_o = intensity of the spectral line of interest (peak counts); QL = quantification limit.

Robustness

Robustness may be determined during development of the analytical procedure [4] under a variety of test conditions, such as different laboratories, analyses, instruments, batches of reagents, elapsed test times, temperature, days, etc. In this experiment, the robustness was determined by using an excipient from another supplier (Labsynth, lot nº 227833) used in the preparation of the pellets.

Interval

The interval must be established from the linearity studies, along with the results from precision and accuracy, depending on the intended application [21]. For determination of impurities, the interval is defined as from the quantification limit up to 120% of the concentration at the specification limit of each individual impurity [22]. In this work, the linear working range was established from the linearity studies, together with the precision and accuracy results for the analyzed values, from 2 µg/g to 35 µg/g.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) or relative standard deviation (RSD), using Microsoft Excel (Microsoft, Redmond WA, USA). The linear regression parameters, fits, statistical calculations such as t and ANOVA tests and graphs shown in this study were performed using Origin Pro 8.5.0 SR1software (www.OriginLab.com).

3. RESULTS AND DISCUSSION

The final goal of the validation of an analytical method is to ensure that every future measurement in the routine analysis will be close enough to the unknown true value for the content of the analyte in the sample [23]. In this way, for evaluation of the methodology the main tests performed were carried out as described before and the results obtained are displayed next.

3.1. Accuracy

The results shown in Tables 3 and 4 indicate that the EDXRF method is very accurate, since the recovery rate for both matrices was within the acceptable range (70-150%) for XRF methods [14].

Table 3. Relative Standard Deviation (RSD) and Recovery (Rec) for matrix with cellulose microcrystalline plus class 1 and 2A impurities.

Impurity	Concentration matrix: cellulose microcrystalline + impurities class 1 and class 2A											
	High (~35 µg/g)				Intermediate (~15 µg/g)				Low (~2 µg/g)			
	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	RSD (%)	Rec (%)	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	RSD (%)	Rec. (%)	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	RSD (%)	Rec (%)
Cd	32.58	32.63	0.77	100.1	16.29	15.90	2.22	97.6	2.04	2.03	0.28	99.7
Pb	34.77	34.81	1.87	100.1	17.39	17.36	0.62	99.8	2.17	2.21	0.69	102.0
Hg	34.97	31.69	0.75	90.6	17.49	13.87	2.67	79.3	2.19	1.74	7.60	79.4
AS	32.79	30.55	2.18	93.1	16.4	14.53	3.20	88.6	2.05	1.92	1.56	93.7
Co	27.67	27.24	1.04	98.4	13.84	13.95	0.39	100.8	1.73	1.59	2.02	92.1
V	22.42	22.66	1.59	101.1	11.21	11.29	1.62	100.7	1.4	1.36	6.74	97.1
Ni	29.43	29.40	3.09	99.9	14.72	14.53	4.56	98.7	1.84	2.00	4.92	108.7

Table 4. Relative Standard Deviation (RSD) and Recovery (Rec) for matrix with cellulose microcrystalline, class 1 and 2A impurities plus light elements

Impurity	Concentration matrix: cellulose microcrystalline + impurities class 1 and class 2A + light elements											
	High (~35 µg/g)				Intermediate (~15 µg/g)				Low (~2 µg/g)			
	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	RSD (%)	Rec (%)	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	RSD (%)	Rec (%)	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	RSD (%)	Rec (%)
Cd	31.95	30.61	2.13	95.8	14.56	15.06	4.12	103.4	2.43	2.64	3.41	108.4
Pb	34.66	34.11	1.58	98.4	15.8	17.07	1.41	108.0	2.64	2.15	3.25	81.6
Hg	34.44	30.46	0.56	88.4	15.7	18.09	0.83	115.2	2.62	2.36	0.42	90.2
AS	29.72	25.98	1.54	87.4	13.98	15.54	1.16	114.7	2.26	2.51	3.19	111.2
Co	26.79	25.48	0.97	95.1	12.21	14.53	0.48	119.0	2.04	1.77	1.13	86.8
V	21.03	16.91	0.35	80.4	9.59	9.88	2.23	103.0	1.60	1.82	4.39	113.7
Ni	30.66	28.83	1.18	94.0	13.98	15.57	2.38	111.4	2.33	2.05	1.46	88.0

3.2. Linearity

Linearity should be established across the range of the analytical procedure. The chosen range (70 µg/g, 35 µg/g, 17.5 µg/g, 8 µg/g, 4 µg/g, 2 µg/g) aimed to evaluate linearity in a wide analytical range, because although strict limits of contamination of heavy metals in pharmaceutical products are established, it has been verified that products, particularly vegetable raw materials, exceed the allowed limits [24]. Attention should also be paid to some specific mined excipients, or materials used in very high quantities in a dose form [25].

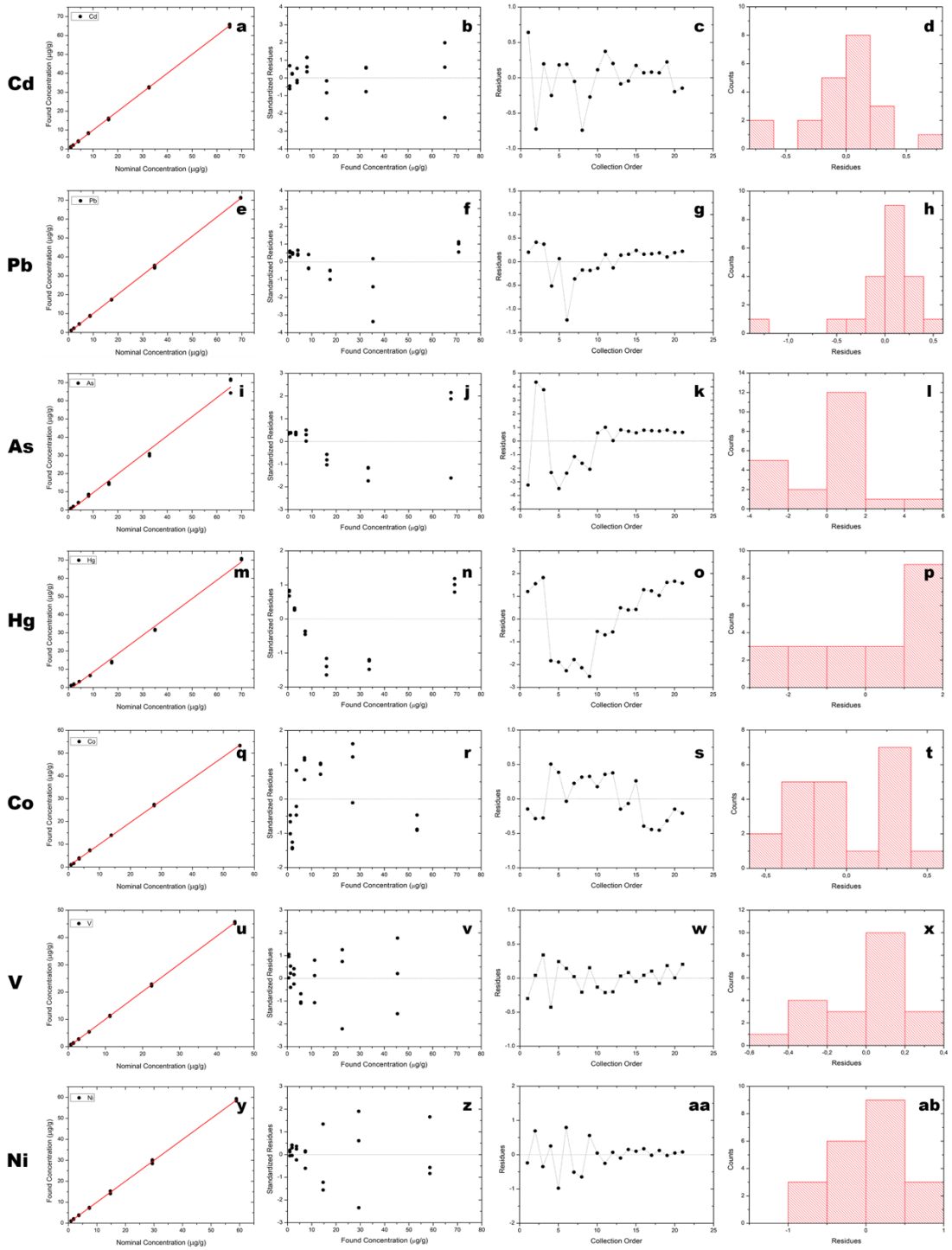
Figure 1 displays the linear fittings performed between the nominal (true) concentration of impurities and the concentration found (recovered) via EDXRF analysis, in µg/g. The main parameters obtained from the linear regression analyses performed are summarized in Table 5.

Table 5. Linear regression parameters for microcrystalline cellulose spiked with elements of classes 1 and 2A.

Parameter		Elemental impurity						
		Cd	**Pb**	**As**	**Hg**	**Co**	**V**	**Ni**
Slope (m)	**m-value**	1.0029	1.0225	1.0450	1.0109	0.9610	1.0152	0.9956
	SD	0.00335	0.00356	0.02073	0.01481	0.00383	0.0029	0.00478
	***t*-value**	299.5809	287.2890	50.411	68.2481	251.1559	350.3225	208.5073
	***p*-value**	0	0	0	0	0	0	0
	95% LCL	0.99592	1.01504	1.00158	0.97986	0.95303	1.00918	0.98564
	95% UCL	1.00993	1.02994	1.08835	1.04186	0.96905	1.02131	1.00563
Intercept (y)	**y-value**	-0.0863	-0.1752	-0.9888	-1.6567	0.3626	-0.0848	0.0738
	SD	0.09519	0.10799	0.59322	0.45201	0.09241	0.05671	0.12265
	***t*-value**	-0.90683	-1.62232	-1.66686	-3.66522	3.92417	-1.49491	0.60153
	***p*-value**	0.37585	0.12121	0.11194	0.00165	0.000911	0.15136	0.5546
	95% LCL	-0.28556	-0.40123	-2.23042	-2.60276	0.16921	-0.20348	-0.18293
	95% UCL	0.11291	0.05083	0.25281	-0.71064	0.55602	0.03392	0.33048
Regression Statistics	**Num. of points**	21	21	21	21	21	21	21
	R	0.9998	0.9998	0.9963	0.9980	0.9997	0.9998	0.9998
	Root-MSE (SD)	0.33164	0.37624	2.06668	1.57478	0.32192	0.1976	0.4273
	Norm-Residuals	1.44559	1.63997	9.00843	6.86429	1.40322	0.86131	1.86254
ANOVA	**gl**	1	1	1	1	1	1	1
	Sum of Squares	9871.082	11683.121	10854.182	11551.015	6537.092	4791.793	7937.790
	Mean Square	9871.082	11683.121	10854.182	11551.015	6537.092	4791.793	7937.790
	***F*-value**	89748.770	82534.982	2541.279	4657.814	63079.312	122725.901	43475.302
	***p*-value**	0	0	0	0	0	0	0

Notes: In the *t*-test, the *p*-value must be interpreted as: $p < 0.05$, reject H_0 (means that the slope or intercept is $\neq 0$); $p > 0.05$, accept H_0 (means that the slope or intercept is = 0); The ANOVA statistical test can be used to test the significance of the linear fitting, and hence, to test the significance of the angular coefficient of the model with the *F*-test from ANOVA, one postulate the following hypothesis: H_0, angular coefficient is = 0; H_1, angular coefficient is $\neq 0$. Since the *p*-value associated to the test is = 0, one rejects the null hypothesis at the significance level of 5%, indicating that the angular coefficient is statistically significant at the significance level of 5%. Regarding the correlation coefficient, R, the RDC-166 norm states that it must be higher than 0.990, which is indeed verified in all linear fittings performed.

Figure 1. Linear fittings between the nominal (true) concentration of impurities and the concentration found (recovered) via EDXRF analysis (μg/g), Standardized residues, Collection order and Histograms of residues, respectively, for **(a,b,c,d)** cadmium, **(e,f,g,h)** lead, **(i,j,k,l)** arsenic, **(m,n,o,p)** mercury, **(q,r,s,t)** cobalt, **(u,v,w,x)** vanadium, and **(y,z,aa,ab)** nickel.



The results of the correlation coefficients (R) for all contaminants (Table 5) exhibit a strong linear dependence, since the regression coefficient found for all elements is greater than 0.9963, in compliance with the RDC-166 regulation [13] which states that the correlation coefficient must be above 0.990.

The linear fittings performed (see Figure 1, a,e,i,m,q,u,y) produced correlation coefficients R > 0.990 for all impurities, with this minimum limit being imposed by the RDC-166 norm for the correlation coefficient. According to the results of the statistical analysis of variance (ANOVA) displayed in Table 5, the linear fitting is statistically significant at the significance level of 5%. In addition, the *t*-test performed allows to conclude that the angular coefficients are ≠ 0 at this level of significance. Moreover, one can easily observe from inspection of Figure 1 that the linear fittings performed were great.

From observation of the data displayed in Figure 1, namely the Standardized Residues *versus* Found Concentrations (see Figure 1, b,f,j,n,r,v,z), one can clearly see that no possible outliers were found, meaning that none of the data points exhibits a high residue value, with the vast majority of the standardized residues being less than 4. From inspection of the data displayed in Figure 1 (b,f,j,n,r,v,z), one can clearly observe that the data points appear to be randomly distributed, meaning that no clear trends in data distribution such as smile or cone were observed, which is a clear indication that the variance of the experimental errors is homoscedastic. Hence, Figure 1 (b,f,j,n,r,v,z) allows to observe a random distribution in the standardized residue graphs, indicating homoscedasticity, thus confirming the linearity of the method.

Regarding the plots of residues *versus* collection (sampling) order (see Figure 1, c,g,k,o,s,w,aa), no trends whatsoever could be observed in the data points, meaning that no increasing or decreasing sequences in the data points could be observed and, hence, this is a clear indication that no dependences existed in the observations performed.

In relation to the histograms of the data residues (see Figure 1, d,h,l,p,t,x,ab), a Gaussian distribution centered around zero was expected, without significative deviations to either left or right, which appears to occur with the experimental data presented herein.

The plots exhibited in Figure 1, added to the statistical analysis presented in Table 5, allows to conclude that the experimental errors are independent with normal distribution, and that the variance of the errors is homoscedastic.

3.3. Precision

As shown in Table 6, the dispersion of results for all impurities studied, calculated via RSD, is well within the national norm [13].

Table 6. Concentrations used to obtain the RSD for the evaluation of the parameter precision.

Impurity	Concentration matrix: cellulose microcrystalline + class 1 and 2A impurities											
	High (~35 µg/g)				Intermediate (~15 µg/g)				Low (~2 µg/g)			
	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	SD	RSD (%)	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	SD	RSD (%)	$C_{nominal}$ (µg/g)	C_{mean} (µg/g)	SD	RSD (%)
Cd	32.58	32.63	0.25	0.77	16.29	15.90	0.35	2.22	2.04	2.03	0.01	0.28
Pb	34.77	34.81	0.65	1.87	17.39	17.36	0.11	0.62	2.17	2.21	0.02	0.69
Hg	34.97	31.69	0.24	0.75	17.49	13.87	0.37	2.67	2.19	1.74	0.13	7.60
AS	32.79	30.55	0.67	2.18	16.4	14.53	0.47	3.20	2.05	1.92	0.03	1.56
Co	27.67	27.24	0.28	1.04	13.84	13.95	0.06	0.39	1.73	1.59	0.03	2.02
V	22.42	22.66	0.36	1.59	11.21	11.29	0.18	1.62	1.4	1.36	0.09	6.74
Ni	29.43	29.40	0.91	3.09	14.72	14.53	0.66	4.56	1.84	2.00	0.10	4.92

Legend: SD - Standard deviation; RSD - Relative Standard Deviation.

3.4. Detection Limit (DL) and Quantification (QL) Limit

The value for maximum permitted limit (MPL) (Table 1) for all impurities of class 1 and class 2A is larger than 2 µg/g. The values obtained for DL and QL (Table 7), indicated that the method can identify and quantify the elements studied in concentrations below the limits stablished in this study and recommended by regulatory agencies. If Option 2A is considered (daily intake of 10 g), the results indicate that only the limit of quantification of cadmium would not be reached.

Table 7. Detection Limits (DL) and Quantification Limits (QL), in cellulose microcrystalline matrix plus light elements, spiked with 2 µg/g of class 1 and class 2A contaminants.

Matrix: cellulose microcrystalline + class 1 and 2A contaminants + light elements							
Limits	**Contaminant elements**						
	Cd	Pb	Hg	As	Co	V	Ni
DL (µg/g)	0.40	0.03	0.20	0.05	0.02	0.03	0.20
QL (µg/g)	1.21	0.09	0.61	0.15	0.061	0.09	0.61

3.5. Robustness

The robustness of the method was evaluated by measuring the concentration of impurities of elements belonging to class 1 and class 2A, present in the microcrystalline cellulose matrix, acquired from different suppliers and analyzed on different dates. The results obtained are displayed in Table 8.

Table 8. Results obtained in the identification of impurities present in different batches containing only microcrystalline cellulose.

Product: Cellulose Microcrystalline			**Class 1 and 2A impurities (µg/g)**						
Supplier	**Date**	**Lot**	**Cd**	**Pb**	**Hg**	**As**	**Co**	**V**	**Ni**
Valdequímica	04/13/2021	023488	ND*	ND*	0.33	ND*	ND*	ND*	ND*
Labsynth	04/14/2021	227833	ND*	ND*	0.20	ND*	ND*	ND*	ND*

Legend: ND* - not detected

The results displayed in Table 8 confirm that the method is robust, since it was used cellulose purchased from two different manufacturers and the results obtained for the concentration of impurities are identical for both.

3.6. Interval

In this work, the established linear working range (2 µg/g to 35 µg/g) presented adequate results considering linearity, precision and accuracy.

3.7. Drug samples

The results obtained in the recovery tests can be found in Table 9. Some elements, such as As, presented higher variable recovery, probability due to the analytical process itself, since the X-ray hits on a very small surface. The accuracy of the method was good, however, a deviation of ± 20% can be deemed acceptable [26].

Table 9. Recovery test in tablets produced by Brazilian industry.

Impurity	Metformin 500 mg			Simvastatin 20 mg			Glibenclamide 5 mg		
	$C_{Nom.}$ (µg/g)	$C_{Rec.}$ (µg/g)	Rec. (%)	$C_{Nom.}$ (µg/g)	$C_{Rec.}$ (µg/g)	Rec. (%)	$C_{Nom.}$ (µg/g)	$C_{Rec.}$ (µg/g)	Rec. (%)
Cd	35.49	35.68	100.53	35.88	42.90	119.56	35.11	41.35	117.77
Pb	39.53	30.39	76.87	39.96	47.60	119.11	39.10	44.53	113.88
Hg	37.24	30.66	82.33	37.64	39.45	104.81	36.84	39.67	107.68
As	35.42	32.25	91.05	35.81	44.14	123.26	35.04	28.04	80.02

Legend: $C_{Nom.}$ - Nominal concentration (true); $C_{Rec.}$ - Recovery concentration (found); Rec. – Recovery.

In view of the accepted criteria for the recovery tests (70% up to 150%) [7], the contaminants intentionally placed in the tablet samples were recovered, with the only exception being Simvastatin, with Arsenic (As) slightly exceeding the specification limit for recovery. This result was not expected, since these tablets use in their formulations excipients that are not exactly the same as those used in the test performed and in concentrations that are not known, however, considering the accepted recovery range, one can safely claim that the test results were a success.

4. CONCLUSIONS

In this research, it was demonstrated that Energy Dispersion X-Ray Fluorescence (EDXRF) technique can be used for detection of elemental impurities of class 1 and class 2A elements in oral tablets. The method proved to be simple, selective, precise, accurate, robust, presenting linear responses for working ranges of interest and low detection and quantification limits (for the group of elements object of this study, LD < 0.4 µg/g and LQ < 1.2 µg/g). It was shown that all validation parameters are satisfied when the daily intake is limited to 2.5 µg/day (Option 1) and remain valid for the maximum daily intake of 10 µg/day (Option 2A), except for the cadmium element, since the

limit of quantification for this intake dose should be ≤ 0.5 μg/g and the limit of quantification obtained in this work was 1.2 μg/g.

The EDXRF technique greatly simplifies the chemical analysis of elemental impurities in pharmaceutical products, when compared to the techniques currently accepted by regulatory agencies, such as ICP-MS, AAS, among others, especially in relation to sample preparation.

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