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Analysis of human tissues by total reflection X-ray fluorescence. Application of chemometrics for diagnostic cancer recognition[1]

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Abstract

For the determination of trace element distributions of more than 20 elements in malignant and normal tissues of the human colon, tissue samples (approx. 400 mg wet weight) were digested with 3 ml of nitric acid (sub-boiled quality) by use of an autoclave system. The accuracy of measurements has been investigated by using certified materials. The analytical results were evaluated by using a spreadsheet program to give an overview of the element distribution in cancerous samples and in normal colon tissues. A further application, cluster analysis of the analytical results, was introduced to demonstrate the possibility of classification for cancer diagnosis. To confirm the results of cluster analysis, multivariate three-way principal component analysis was performed. Additionally, microtome frozen sections (10 μm) were prepared from the same tissue samples to compare the analytical results, i.e. the mass fractions of elements, according to the preparation method and to exclude systematic errors depending on the inhomogeneity of the tissues. © 1997 Elsevier Science B.V.

Keywords: Carcinoma; colon; Cluster analysis; Pressure digestion; Three-way principal component analysis; Total reflection X-ray fluorescence analysis

1. Introduction

Until now, the role of essential elements, possibly in combination with toxic elements, on carcinogenesis has not been wholly elucidate. It is known only that many trace elements can have an important influence on a large number of biological processes by activating, inhibiting or promoting enzymatic reactions. The possible function of trace elements in relation to the structure of nucleic acids and nucleoproteins is also poorly understood. The comparison of trace element profiles in cancerous samples with those in normal tissues from cancer patients can give indications of interchangeable element reactions and possibly an indication of a change in cell metabolism. Previous investigations into the Zn and Cd concentrations in the human prostate gland suggest a distinct biological antagonistic effect between these two elements [1]. Further papers have been published in which it is shown that concentrations of several trace elements are altered in malignant tissues of the human urinary bladder and kidney compared with normal tissues [2,3]. Drake and Sky-Peck show that the element patterns of breast, colon and lung tissue of cancer patients can be used in cancer diagnosis as a methodology for making malignant/normal and tissue type classifications [4].

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In view of this background, it seems desirable to analyse the trace element distribution in malignant and normal tissues of different human organs. This study requires analytical multi-element techniques appropriate to the large numbers of samples and measurements.

Every two years, a review of atomic spectrometric methods in the field of clinical and biological application is published in the Journal of Analytical Atomic Spectrometry [5]. Summing up, it may be said that ICP-MS, XRF, NAA and PIXE have become established as representative multi-element techniques during the past few years. Comparative studies of the extent of sample preparation, the instrumental equipment and operating costs have been carried out by application to biological reference materials [6,7]. It has become apparent that total reflection X-ray fluorescence analysis (TXRF) is a suitable method for biological and clinical chemistry, and in particular is practicable for the screening and biomonitoring of biological specimens [8–10]. The analysis of microtome sections of biomaterials and the procedure for quantification are also demonstrated, showing that TXRF is a simple method applicable to direct elemental analysis [11].

The purpose of this paper is to determine the ratio of element concentrations in neoplastic tissues versus those in normal tissues of cancer patients by TXRF in combination with high pressure wet digestion. In addition, the resulting patterns of element distribution in eight patients with colon carcinoma (catchment area Viersen, Germany), determined by using microtome sections or pressure wet digestion are compared, and questions on the role of nutritional factors need to be answered.

2. Experimental

2.1. Sampling and sample preparation

Histologically normal and neoplastic human colon tissue were taken from the same individual at the time of surgery. The tissues were excised into small pieces ($2 \times 4 \times 1$ cm) by the surgeon, and the remaining cancerous tissue was sent to the pathologist for diagnosis. Portions of the samples were immediately transferred to the analytical laboratory in iced polyethylene foils and were stored at −15°C for no longer than 7 days.

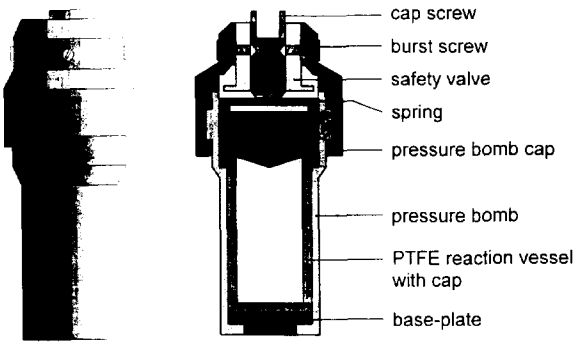


Fig. 1. Autoclave for pressure wet digestion of biological materials.

For pressure wet digestion, the samples were cut into pieces of about 250–400 mg wet mass with a quartz knife and transferred to the PTFE vessels of an autoclave system, as illustrated in Fig. 1.

The autoclave equipment (ANCON-AT Ltd., Moscow), which includes an aluminum heater and a cooling system, is briefly described as follows:

Body material:	titanium
Reaction chamber material:	PTFE
Enclosure volume:	25 ml
Maximum operating pressure:	15–mpa
Number of simultaneous digestions:	10.

The weighed mass of certified reference material (bovine liver, CRM 1577B) was 100–200 mg. For the digestion of the samples, 3 ml nitric acid (sub-boiled quality) and a internal standard solution of Y and Te were added (100 μg Te and Y per g wet mass of tissue sample) and the mixture was heated at 180°C for 2 h. Four 30 μl aliquots of the digestion solution were pipetted onto a quartz glass carrier and heated at 85°C to dryness. Owing to the small volume of the real tissue sample it was possible to repeat the measurements only twice.

2.2. Apparatus

The measured elements, those of atomic number between 15 and 56 with the exception of Ga, Ge, Kr, Tc, Ru, Rh, Pd, In, I, Xe, and Cs, were either physiologically relevant or toxic in action. The excitation of these elements was carried out on an Extra II TXRF spectrometer (R. Seifert and Co.,

Ahrensburg) operated with molybdenum and tungsten fine focus tubes at 48 kV (Mo) or 50 kV (W). The current was adjusted to yield a count rate of ≈ 10 000 counts per second corresponding to a dead time of 45%. The overall measuring time was 300 s. The emitted fluorescence radiation was detected by a Si(Li) detector with an effective area of 30 mm^2. The electronic equipment, the multi-channel analyzer and the software were supplied by Link Systems Ltd., London, UK (QX 2000, System 860/500).

2.3. Data analysis

To process the data from the multi-channel analyzer, the data were transmitted via an interface to a personal computer by use of a terminal program. The first evaluation step is the application of spreadsheet program (Microsoft™ EXCEL®) to represent the different element distributions in cancerous tissue versus those in normal human tissue. The mass fractions of elements in malignant and in normal tissues were calculated by use of the average element concentrations of the two digestion solutions, corresponding to eight measurements per tissue sample, with use of the Nalimov outlier test [14]. The calculation of the mass fractions of elements is necessary because the interpatient mass fractions of elements in both tissue types differ widely. This is caused by the different absorption of trace elements according to their biological availability, nutrition status and cell metabolism. The second evaluation step was to group or classify tissue samples based solely upon their trace element concentration. This was achieved by cluster analysis and by principal component analysis (PCA), showing that the method is potentially valuable for neoplastic/normal and tissue type classification [15,16]. For this purpose a commercially available software program, Statgraphics Plus (Manugistics Inc., Rockville, USA), was utilized.

3. Results

3.1. Certified reference material

In order to demonstrate the reliability of the analytical procedure, the mass fractions of elements in the certified reference material (bovine liver, CRM1577B) were determined and the certified values were compared with the measured values. The results are given as recovery rates and are listed in Table 1.

Generally speaking, the measured values do not deviate significantly from the certified values except for P and K. These greater deviations, 50% for P and 30% for K, are caused by the extremely high mass fractions of these two elements in relation to the concentration of the internal standard. The negative result for Cl is based upon the element loss during the digestion process and is in agreement with other published results [7,12,13]. The measured mass fractions, given as recovery rates for Mn, Fe, Cu, Zn and Se, correspond to the certified values. The unsatisfactory recovery rate for Pb is attributed to measurement near to the detection limit. It is possible that contamination problems may have contributed.

Table 1
Examination of accuracy by analysis of certified reference material (Bovine liver CRM 1577B)

Element	Certified value/($\mu g\ g^{-1}$)	Measured value/($\mu g\ g^{-1}$), $n = 8$	Recovery rate/%
P	11 000 ± 300	5890 ± 1213	54
Cl	2780 ± 60	not determined	–
K	9940 ± 20	7403 ± 1082	74
Ca	116 ± 4	124 ± 15	107
Mn	10.5 ± 1.7	9.7 ± 0.7	97
Fe	184 ± 15	175 ± 6	95
Cu	160 ± 8	166 ± 5	104
Zn	127 ± 16	124 ± 3	98
Se	0.73 ± 0.06	0.73 ± 0.25	99
Rb	13.7 ± 1.1	12.29 ± 0.26	90
Pb	0.129 ± 0.004	0.594 ± 0.239	461

3.2. Malignant and normal colon tissue

To investigate the inhomogeneity of the malignant tissue and of the normal colon tissue, the repeatability of measurements was calculated as relative standard deviation for $n = 8$. The relative standard deviations are in the range 1% to a maximum of 20% for all elements and for all colon tissues. No difference between the RSD of all elements in malignant tissues in comparison with normal tissues could be recorded. However, in relation to these results, the inhomogeneity of the sample has to be taken into consideration when estimating the significant elements in neoplastic versus normal tissues without statistical evaluation programs. The concentrations of elements, such as Mo, Cd, Sn, Sb, Ba, Pb, Tl, Hg and I, which are excited by W bremsstrahlung are at or near the detection limit. An evaluation of the analytical results for these elements is not necessary.

Concentrations of the Mo-excited elements Zr and V are not far above the detection limit, and these elements could be recorded in only a few measurements, although V is physiologically present in the organism and acts according to different biological functions [17]. Cl and Br were not evaluated because of the element losses during pressure digestion. The following elements show variable mass fractions in malignant versus normal tissue: Ca, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, As and Sr. It is remarkable that Cr, Mn, Fe and Co show a very similar pattern for all samples, but it cannot be established whether these elements are accumulated significantly in malignant or normal tissue. It appears that a correlation exists between these four elements. Possibly patient data or histological diagnosis could give some indication as to this correlation.

In order to identify significant elements (definition below), two interdependent criteria have to be met. Firstly, each colon sample has to show a ratio of concentrations > 1 or < 1; secondly, if a recognition of accumulation or depletion of the elements is possible, an element is defined as significant when the ratio for the element is > 1.5 (accumulation) or > 0.6 (diminution). To confirm a difference between the mass fraction of an element in malignant tissue and that in normal tissue, the levels should differ by a factor of at least 2. The elements K, P, Rb, Se, S and Ti fulfill the first criterion, demonstrated in Fig. 2 as a histogram to give an overview of all measured samples. These elements are accumulated in each malignant tumor.

Based on the actual findings—in a few cases the ratio of element concentrations is smaller than 1.5—it can be assumed that only the elements Rb, K, P, S, Se, and Ti show a tendency to accumulation in neoplastic colon tissue. Further validation of these results needs to be performed by chemometric methods. Also, the investigation of a larger number of samples would be an advantage.

The results of the application of hierarchic cluster analysis (Wards algorithm) to the analytical measurements (evaluation of the crude data without use of outlier tests) are mainly in agreement with the results obtained by the spreadsheet program. The elements K, P, S, and Se can be grouped into two clusters corresponding to their mass fractions in cancerous tissues versus those in normal colon tissues. An optimal result of 100% classification accuracy would be a 50%/50% division of the measured samples for each element. The calculated cluster weights of 41%/59% show a satisfactory result considering the small amounts of the samples. It seems that Rb and Ti do not correlate to K, P, S, and Se, because the calculated 67%/33% division is an indication that Rb in combination with P, S, K and Se cannot be grouped significantly into one of the two clusters. Equally, none of the other elements can be grouped into the predefined clusters.

In order to reveal complex connections between the elements, the element mass fractions and the classification of malignant tissue versus normal colon tissue were performed by the three-way principal component analysis described in [18]. Some interesting reports relevant to the problem of the current work are given in [19–21]. A further number of references to different practical applications of PCA is listed in a book by Henrion and Henrion [15]. A further advantage of the application of multivariate data analysis is to achieve a clear presentation of complex relationships between a large number of data and variables.

By using principal component analysis, linear combinations (principal components) are constructed of the original variables, so that the new synthetic variables are not correlated one with another. A few of the synthetic variables describe a major part of the data variance. A calculated small number of the synthetic variables is an indication that a strong correlation

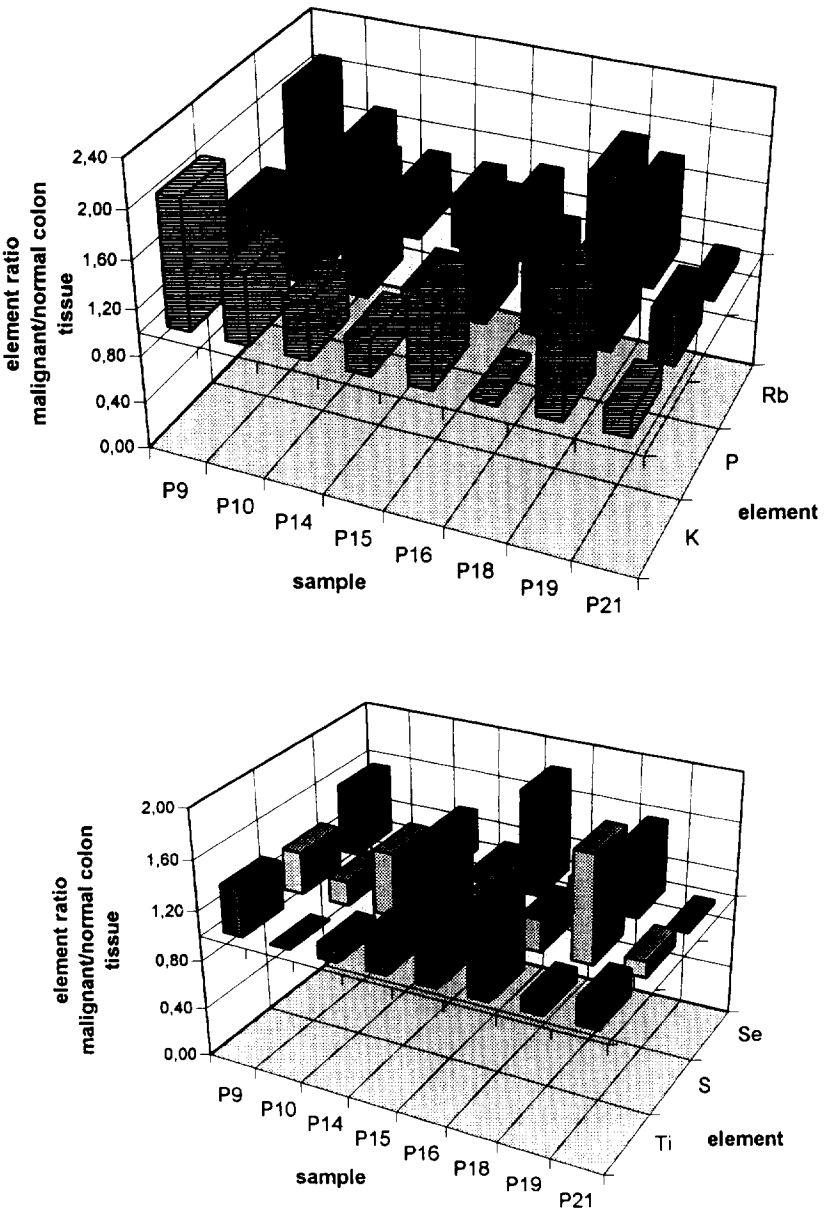
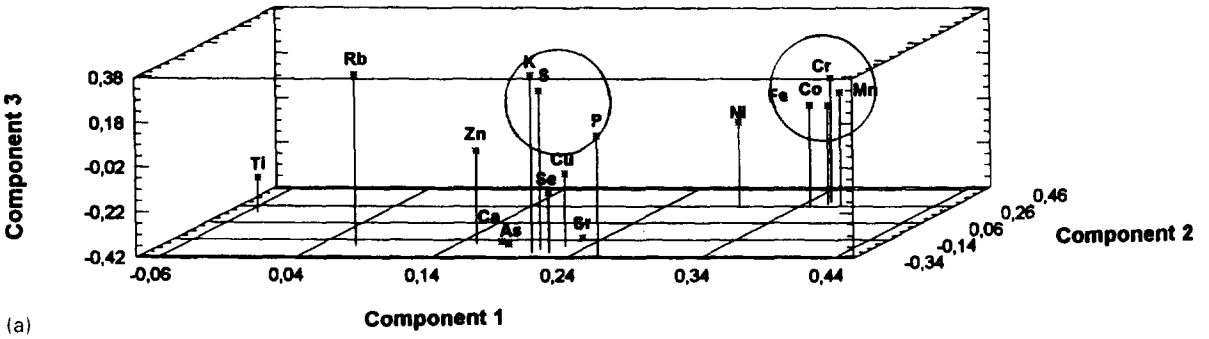


Fig. 2. Elements showing a tendency to accumulation in malignant colon tissue.

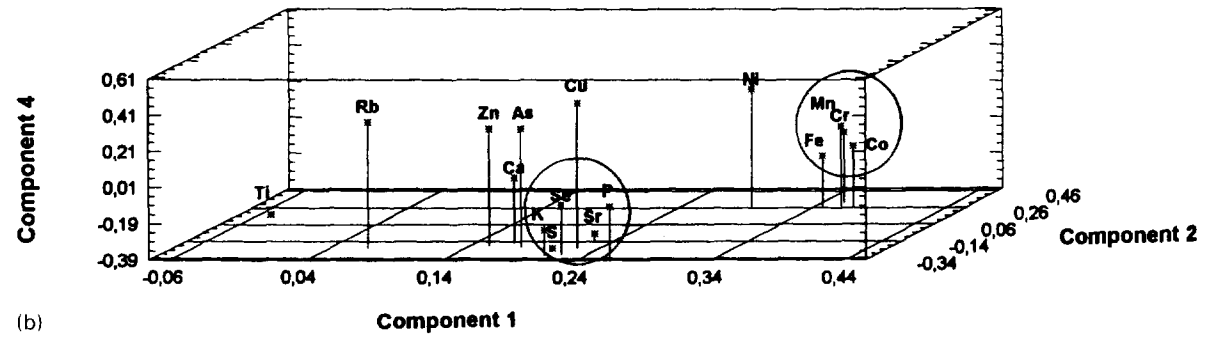
exists between the variables and the data. Ideally, only two variables are needed to reach an accuracy of 100% for classification.

To obtain acceptable accuracy and to preserve the clear identification of clusters, the relationships between five synthetic variables are calculated and illustrated as PC plots of component weights in Fig. 3.

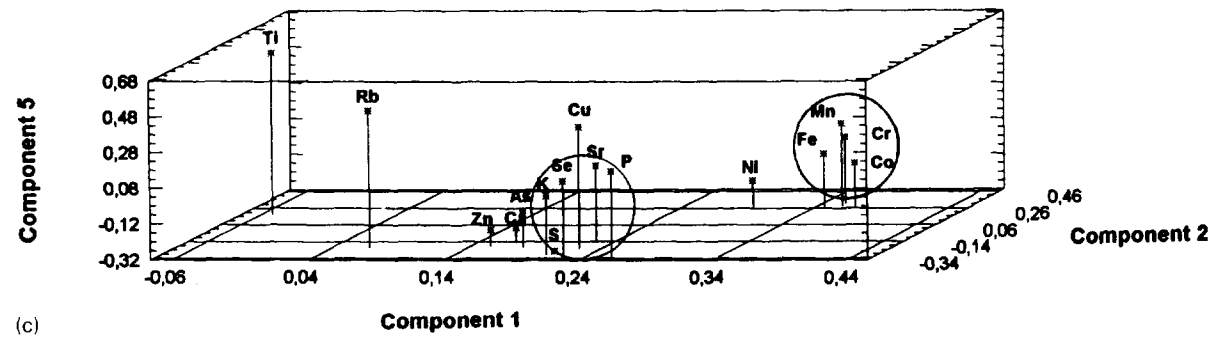
The elements are arranged in a three dimensional array. The grouping of elements derived from the three PC plots shows so-called "point cloud" variables. A basic requirement of such an arrangement is that the point cloud of the variables, or the group of elements, is similar over all the plots. In the demonstrated case the elements Mn, Cr, Co, and Fe can be


Component 3
0,38
0,18
-0,02
-0,22
-0,42
Rb
K
S
Cr
Fe
Co
Mn
Ni
Zn
P
Tl
Cu
Se
Ca
As
Sr
-0,06
0,04
0,14
0,24
0,34
0,44
Component 1
0,46
0,26
0,06
-0,14
-0,34
Component 2

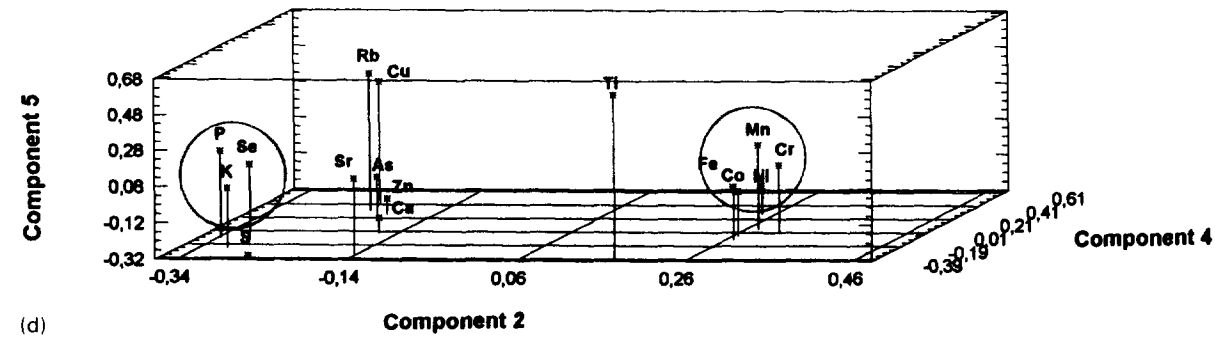
(a)


Component 4
0,61
0,41
0,21
0,01
-0,19
-0,39
Rb
Cu
Ni
Zn
As
Mn
Cr
Fe
Co
Ca
Tl
Se
P
K
Sr
S
-0,06
0,04
0,14
0,24
0,34
0,44
Component 1
0,46
0,26
0,06
-0,14
-0,34
Component 2

(b)


Component 5
0,68
0,48
0,28
0,08
-0,12
-0,32
Tl
Rb
Cu
Mn
Fe
Cr
Co
Sr
Se
P
Ni
K
As
Zn
Ca
S
-0,06
0,04
0,14
0,24
0,34
0,44
Component 1
0,46
0,26
0,06
-0,14
-0,34
Component 2

(c)


Component 5
0,68
0,48
0,28
0,08
-0,12
-0,32
Rb
Cu
Tl
P
Se
K
Sr
As
Zn
Ca
S
Mn
Cr
Fe
Co
Ni
-0,34
-0,14
0,06
0,26
0,46
Component 2
0,61
0,41
0,21
0,01
-0,19
-0,39
Component 4

(d)

grouped into a cluster and so can the elements P, K, S, and Se. It appears that these elements show a pattern according to the classification of neoplastic and normal colon tissue. Ti and Rb occupy a special position. A possible explanation is that Ti and Rb are not essential elements, but they are incorporated into the organism as a result of environmental exposure. The incorporation could be the result of nutrition (absorption into the colon mucosa) or inhalation (transferred by blood). The contribution of Rb and Ti in carcinogenesis remains to be proved by further investigations.

The results obtained by PCA mainly agree with the results obtained by the spreadsheet program and by cluster analysis. A comparative study of the significant elements in the colon carcinoma determined by TXRF after wet digestion and determined by direct TXRF measurements of microtome tissue sections [22] shows correspondence in the majority of cases. In both cases Cr, Co and Fe seem to be important for the identification of neoplastic or normal tissue. Se, S and Rb could not be observed in microtome sections as being relevant because of the low mass of these elements in tissue sections. In digest solutions of colon tissues, Mn is defined as significant, whereas Ni is defined as important through the results obtained by analysis of microtome sections. These results correspond to the results published by Drake and Sky-Peck [4]. Further confirmation of the results presented here can be found in a paper by Issaq. He discusses the role of metals in tumor development and inhibition in respect to numerous basic references [23]. It is noticeable that most of the elements identified as significant are metal carcinogens (Cr, Co, Ni, and Mn). The common property of these elements is their propensity to form chelation complexes with biological molecules, including nucleic acids and proteins. The influence of these elements on carcinogenesis is very complex and cannot clarified at this point of the research.

4. Conclusion

The determination of element concentrations in colon carcinoma and normal tissues from the same patient by TXRF analysis can be performed satisfactorily in combination with high pressure wet digestion. Patterns of element distribution of neoplastic versus normal colon tissues can be recognized, but should be validated by studying a larger number of samples. From the viewpoint of simple sample preparation, the direct analysis of microtome sections is preferred over the time-consuming sample preparation by wet digestion. Another advantage of direct measurements on tissue sections is the possibility of determining the element distribution of adenocarcinoma of the colon in dependence on the histological colonic layers (mucosa, submucosa, muscularis propria, subserosa, serosa) corresponding to the stage of the cancer. It remains to be proved by further experiments whether histological grading (differentiation of cancer cells) plays a role in the element distribution of colon carcinoma. Indications of such a relationship, obtained for prostatic carcinoma, has been published by Feustel and Wennrich [24–26]. In another study of the trace elements in tissue prostate cancer and metastases, it was established which factors determine the organ pattern of metastases and the primary tumor [27].

For evaluation of the complex relationship between such factors as the stage of the cancer and metastases, the individual patient data, nutritional habits and trace element concentrations in cancerous tissues, multiway principal component analysis seems to be a suitable aid to recognizing potential connections.

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Fig. 3. Plot of component weights calculated by five-way principal component analysis for classification of human colon tissue: (a) one, two and three principal components; (b) one, two and four principal components; (c) one, two and five principal components; (d) two, four and five principal components.

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