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Osmium assay in fixatives and stained rat tissues by means of acid and *o,o'*-dihydroxo substituted monoazo dyes and some flavonoids

Research Article

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Abstract: New simple, rapid and accurate spectrophotometric techniques for osmium assays in fixatives and stained tissues have been elaborated using acid (Tropaeolin O, Tropaeolin OOO-I, Tropaeolin OOO-II, Orange G), *o,o'*-dihydroxo substituted azo dyes (Eriochrome Blue Black R, Acid Chrome Dark-Blue, Eriochrome Black T) and flavonoids (morin, quercetin, luteolin). Methods are based on sensitive osmium(IV) reactions with reagents and the formation of stable coloured compounds. Tolerance ratios of main matrix components of histological specimens during Os(IV) spectrophotometric determination with reagents has been established. Results of osmium determination in fixatives and fixed tissues, obtained by means of different reagents, appeared to be sufficiently similar, although effects of matrix components differ. The accuracy of spectrophotometric osmium assay in fixatives with azo dyes has been confirmed with voltammetric investigations. Results obtained from osmium uptake by rat gum tissues are valuable for clinical testing of dental drugs, indicating the influence of drug treatment on the gums.

Keywords: *Osmium determination • Azo dyes • Flavonoids • Fixatives and stained tissues*

1. Introduction

Osmium tetroxide (OsO_4) is more widely used than any other fixative to stain and fix biological specimens for electron and optical microscopy [1-7], which has provided most of what is known about the ultrastructural organization of tissues, cells and organelles [4]. Osmium(VIII) is highly electropositive and gives rise to strong electron scattering from electron donor ligands. Osmium tetroxide is known to preserve the cell structure better than other fixatives and provides a desirable degree of contrast to the image, so this staining method is suitable for electron microscopy [2,8,9]. Often osmium tetroxide is also used in combination with some other agent such as iron(II) cyanide, iodine, glutaraldehyde, organic and inorganic ligands, to give enhanced contrast [6,10]. The reactivity of cell components with OsO_4 has been generally explained by the osmium ester formation theory, according to which OsO_4 reacts with ethylene bonds of liquid or solid unsaturated fatty acids of tissues to form mono- and di-esters of osmium(VI). These compounds subsequently may undergo hydrolysis and result in the formation of a reduced osmium(IV) black oxide OsO_2, responsible for the gradual blackening of a fixed tissue and for a high accumulation of osmium in the stained tissue region [1,2,5,6,11,12]. Fixed tissues also contain osmium in nominal oxidation states of VIII, VII and VI, chelated by appropriately placed donor atoms in the macromolecule tissue matrix [10].

Various chemical and instrumental analytical techniques, in particular, gravimetry, NMR, mass spectrometry, atomic absorption, IR-, UV-VIS and X-ray spectroscopy, voltammetry, chromatography, are used both for qualitative and quantitative examination of OsO_4 reaction products with tissue components in order to study a staining mechanism as well as to interpret the results of different biological and medical researches of tissues [2,7,10-16].

Moreover, the estimation of osmium content in stained tissues and fixatives is of significance for the environmental management and engineering, since samples of this type usually contain considerable

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amounts of osmium in different forms, including OsO_4. It is well known that volatile osmium tetroxide even in minute quantities possesses a toxicity on human health, *viz.* it causes the irritation of mucous tunics of upper air passages, bronchiospasm, lacrimation, and the prolonged inhalation of OsO_4 provokes pneumonia, headache, nausea and nephritis [17] (the maximum permissible concentration of OsO_4 is 0.002 mg m^{-3}). Thus, used materials of histochemical investigations of biological tissues require special control and utilization [18,19]. It should be noted, that osmium when getting into the environment from such materials may transmute into toxic tetroxide in the presence of atmospheric oxygen as well as get into soil and groundwater. Additionally, since waste fixatives and stained tissues accumulate significant quantities of osmium, it would be reasonable to recover this most expensive platinum group element (PGE).

Spectrophotometry is one of the simple and accessible methods, used for the quantitative measurement of osmium uptake by biological material, which also is of value in the study of either physicochemical and biomedical properties of tissues. The number of reagents, used for spectrophotometric determination of osmium in stained and fixed tissues, is rather limited. Common spectrophotometric techniques for the assaying of free and protein-bound osmium are Majumdar and Sen Gupta test and Bahr test for free and tissue-bound osmium [20], based on the well established reactions of osmium(VIII, VI) with *o*-aminophenol-*p*-sulphonic acid [21] and of osmium(VIII) with thiourea [22,23] respectively. However, both these methods have a few drawbacks, *e.g.* their sensitivity is only on the microgram level, which, obviously, is not very sensitive [21-23]. A highly sensitive spectrophotometric technique for osmium determination in stained tissues is required when dealing with a very small amount of a sample, *e.g.* the cut section of a tissue, when a penetration of OsO_4 into a material is examined [20]. In addition, Majumdar and Sen Gupta test is suitable only for the determination of osmium in higher oxidation states, though along with Os(VIII, VI), a fixed tissue also contains Os(IV) [1,2,5,6,11,12]. The Bahr test demands complicated and time-consuming sample preparation, which includes an alkaline digestion of the tissue, subsequent oxidation with $K_2Cr_2O_7$ dissolved in H_2SO_4. OsO_4 thereby produced must then be distilled into a thiourea solution in 6 mol L^{-1} HCl [20], which may result in osmium losses, taking into account a high volatility of osmium tetroxide and possible shortcomings of the distilling apparatus. Therefore, the application of other reagents in order to develop new, simple and rapid spectrophotometric techniques for the estimation of osmium content in stained tissues and fixatives is an important task.

In this work we present the results of an osmium spectrophotometric assay in stock fixing fluid, waste fixatives and stained rat tissues by means of simple and rapid analytical procedures by applying organic reagents of different nature, *viz.* acid monoazo dyes (Tropaeolin O, Tropaeolin OOO-I, Tropaeolin OOO-II, Orange G), *o,o'*-dihydroxo substituted azo dyes (Eriochrome Blue Black R, Acid Chrome Dark-Blue, Eriochrome Black T) and flavonoids (morin, quercetin, luteolin). Literary data concerning the use of acid monoazo dyes and luteolin in spectrophotometry are not numerous, unlike *o,o'*-dihydroxo substituted azo dyes, quercetin and morin, as it is shown in Table 1. We have proposed to use these reagents for the spectrophotometric determination of osmium(IV) [24-32].

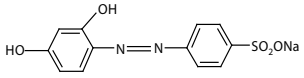
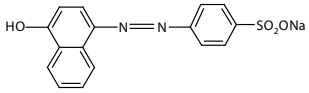
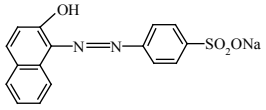
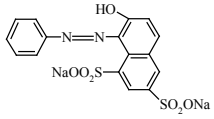
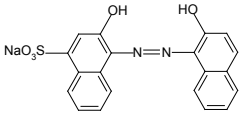
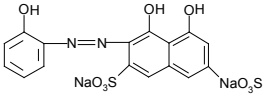
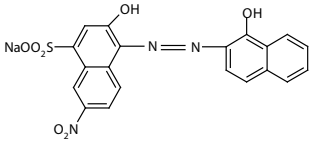
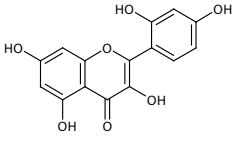
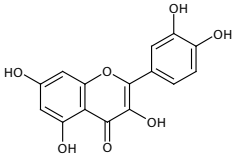
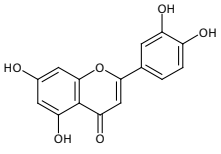
We have established, that the reactions of osmium(IV) with mentioned azo dyes and flavonoids resulted in the formation of stable coloured products. We have specified the optimum conditions for obtaining maximum analytical signals (media acidity, base electrolyte, duration of a reaction at room temperature or on heating on a water bath, reagents concentration) and elaborated new spectrophotometric methods for Os(IV) determination (see Section 2.2.). The validation of the techniques are summarized in Table 2. So spectrophotometric methods for osmium determination by azo dyes and flavonoids are sensitive (effective molar absorptivities $\varepsilon_\lambda \sim 10^3$-$10^4$ L mol^{-1} cm^{-1}), reproducible and applicable for osmium quantification in a wide concentration range.

The selectivity of developed techniques was tested at osmium(IV) determination in the presence of platinum group elements, rare earths, alkali-earth, heavy metals as well as inorganic and oxyacids anions [26-28]. The results of the selectivity investigations are shown in Table 3 on the examples of TpOOOI, EBBR and Mor as the representatives of each class of the examined reagents. The interfering influence of extraneous ions was tested to that concentration (molar) tolerance ratio Os(IV):Ion, when the deviation of the absorbance value in the presence of interferent exceeded 10% of the absorbance of Os(IV) solution without any additional foreign ions.

The accuracy of the methods has been successfully tested on model solutions of various content and real samples (RSDs ≤5%) [28,31].

Table 4 contains the data concerning the most common spectrophotometric methods for the determination of osmium in different samples, including histochemical specimens (*o*-aminophenol-*p*-sulphonic acid and thiourea) in order to compare them with the proposed

Table 1. Organic reagents used for osmium assay in the research and their spectrophotometric application

Reagent	Structural formula	Analytes
Tropaeolin O (TpO)		Pd(II) [33], albumin, casein [34]
Tropaeolin OOO-I (TpOOOI)		organic medicines [35,36]
Tropaeolin OOO-II (TpOOOII)		Pd(II) [33], organic drugs [37,38], albumin [39]
Orange G (OG)		organic drugs [40], proteins [41,42]
Eriochrome Blue Black R (EBBR)		Mg, Al [43]
Acid Chrome Dark Blue (ACDB)		Mg, Ca, Zn, Al, Be [44,45]
Eriochrome Black T (EBT)		Ca, Mg, Zn, Mn, Cd, Pb, Ga, In, Al, Zr, P [46,47]
Morin (Mor)		Al, Be, Ce, Ga, In, Sb, Sc, Sn, Th, Zr, Au, Nb, Tl, rare earths [48,49]
Quercetin (Quer)		Cr, Al, Fe, Th, Mo, Ge, Sn, Tl [48,50]
Luteolin (Lut)		Au [51]

methods. One can conclude, the suggested methods for osmium determination with azo dyes and flavonoids compete favourably with well known techniques both in their sensitivity and selectivity. In addition, they are more complicated to do, *e.g.* the method with nitroso-R-salt demands the heating of solutions for 1.5 hours on a boiling water bath [52].

Thus, the spectrophotometric methods for osmium determination with Tropaeolin O, Tropaeolin OOO-I, Tropaeolin OOO-II, Orange G, Eriochrome Blue Black R, Acid Chrome Dark-Blue, Eriochrome Black T, morin, quercetin and luteolin can be effectively applied to fixatives and stained tissues assays.

Table 2. Effective molar absorptivities of coloured products and the validation parameters for the spectrophotometric determination of osmium(IV) with azo dyes and flavonoids

Reagent	$\varepsilon_\lambda \times 10^{-3}$ (L mol^{-1} cm^{-1})	Ratio Os:Reagent	Linearity range C_{Os} (μg mL^{-1})	Limit of Detection (μg mL^{-1})	Correlation Coefficient R
TpO	2.1	3:2	0.5-22.0	0.2	0.9994
TpOOOI	23.0	1:4	0.005-0.150	0.002	0.9981
TpOOOII	3.6	1:1	0.2-11.5	0.07	0.9998
OG	11.0	2:1	0.03-2.90	0.01	0.9991
EBBR	1.0	1:3	0.6-12.5	0.6	0.9993
ACDB	4.8	2:1	1.2-6.2	1.0	0.9999
EBT	7.4	1:1	3-140	0.8	0.9999
Mor	15.0	1:1	0.07-0.72	0.07	0.9991
Quer	13.0	1:4	0.18-1.45	0.15	0.9992
Lut	7.4	1:1	0.45-11.50	0.35	0.9984

Table 3. Tolerance ratios of extraneous ions during the spectrophotometric determination of Os(IV) with TpOOOI, EBBR and Mor

Ion	$C_{Os}:C_{Ion}$			Ion	$C_{Os}:C_{Ion}$		
	TpOOOI	EBBR	Mor		TpOOOI	EBBR	Mor
Ru(IV)	1:5	1:0.5	1:1	Ni(II)	1:1	1:2	1:1
Rh(III)	1:2	1:7*	1:6	Co(III)	1:5	1:0.1	1:0.5
Pd(II)	1:5	1:0.5	1:1.5	Zn(II)	1:5	1:1	1:8
Pt(IV)	1:1	1:0.5	1:8	Al(III)	1:3	1:0.1	1:20
Ir(IV)	1:3	1:0.5	1:3	Fe(III)	1:3	1:<0.1	1:5
Ag(I)	1:20	1:1	1:5	F^-	1:500*	1:15	1:50
Au(III)	1:2	1:0.5	1:10*	$P_2O_7^{4-}$	1:100	1:50	1:5
Ce(III)	1:2	1:1.5	1:1	EDTA	1:60	1:500	1:2
Nd(III)	1:40	1:1	1:7	Cit^{3-}	1:100	1:10	1:10
Cu(II)	1:3	1:2	1:0.5	$Tart^{2-}$	1:30	1:100	1:100

** higher concentrations of marked ions were not investigated*

2. Experimental procedure

2.1 Reagents and apparatus

All aqueous solutions utilized in the research have been prepared using distilled water. All chemicals used in the research were of analytical grade.

The stock solution of Os(IV) (H_2OsCl_6) was prepared by dissolving the exact mass of OsO_4 from the hermetically sealed glass ampoule in concentrated hydrochloric acid [28]. The obtained osmium solution was stored for one month to complete the reduction of OsO_4 to $[OsCl_6]^{2-}$. The identification of Os(IV) solutions has been carried out spectrophotometrically comparing the electronic absorption spectra of the obtained solutions with literature data [53]. The standardization of the obtained stock osmium solutions has been carried out titrimetrically by means of potassium iodide [54]. The standard working Os(IV) solutions were prepared by dissolving an aliquot of osmium(IV) stock solution in 1 mol L^{-1} HCl.

Organic reagents solutions were prepared from commercially available chemicals. The solutions of Tropaeolin O (Synbias, Ukraine), Tropaeolin OOO-I, Tropaeolin OOO-II, Orange G (Shostkinsky Chemical Plant, Ukraine) Eriochrome Blue Black R (Merck, Germany), Acid Chrome Dark-Blue (POCh, Poland) and Eriochrome Black T (VTC MC UKhII 97-58, Mosgor, Russia) were prepared by dissolving the exact mass of the reagent in the distilled water. The solutions of morin (LOBA CHEMIE, Czech Republic), quercetin (CHEMAPOL, Czech Republic) and luteolin (Ukrainian Scientific Centre of Medicines Quality, Ukraine) were prepared by dissolving the exact mass of the reagent in 96% v/v ethanol.

UV-VIS measurements were performed using UV-VIS scanning spectrophotometer CARY.WIN – UV-VIS-50, Varian, USA (spectral range 190-800 nm with resolution of 0.1 nm; absorbance range 0-4; standard deviation of absorbance measurement 1% at A=1; photometric noise 0.0018 at A=2 and λ=500 nm), spectrophotometer UV-VIS SPECORD M 40, Carl Zeiss Jena, Germany (spectral range 190-1100 nm with resolution of 1 nm; absorbance range -0.3-2; standard deviation of absorbance measurement 0.5% at A=1) and photometer KFK-2 MP, Zagorsky mechano-optical plant, Russia (spectral range 315-980 nm; width of absorption filters transmission band from 25±5 to 45±5 nm depending on a filter; absorbance range 0-2; standard deviation

Table 4. Some common spectrophotometric methods for the determination of osmium

Reagent	Analyte	Linearity range C_{Os} (μg mL^{-1})	λ_{max} (nm)	Ratio Os:Reagent	Interferents
o-Aminophenol-*p*-sulphonic acid [21,52]	Os(VIII, VI)	2-8	440	1:2	PGE, Cr, Zr, Mg
Thiourea [22,23,52]	Os(VIII, IV)	8-40	480	1:6	Oxidants, Cl^-, PGE
Tyron [52]	Os(IV)	8-24	480	1:1	Al, Ca, Ba, Sn
Pyridinazoresorcinol [52]	Os(IV)	0.35-5.7	533	1:2	–
Selenourea [52]	Os(VIII, VI, IV)	2-60	600	–	–
Sulphanilic acid [52]	Os(VIII, VI)	2-8	490	1:2	PGE
Nitroso-R-salt [52]	Os(VIII)	0.76-15.2	520	–	–

– no data

of separate absorbance measurement up to 0.3%).The path length of cuvettes *l* was 1 cm for spectrophotometers and in the range of 1-5 cm for optimal measurements with the photometer. All absorbance measurements were performed at ~20°C.

Voltammetric measurements were performed using an oscillopolarograph PO-5122 model 03, Russia, with a digital setup equipped with a computer and a three-electrode cell (current range 0-10 μA with resolution of 0.1 μA; potential range -2-2 mV with resolution of 0.01 mV). A dropping mercury electrode, a platinum electrode and a saturated calomel electrode served as indicator, auxiliary and reference electrodes, respectively. The analytical investigations were carried out at the potential sweep rate of 1.0 V s^{-1}. All voltammetric measurements were performed at ~20°C. Dissolved oxygen was removed by sparging with purified argon for 15 min.

The pH measurements were carried out with pH-meter model pH-150 M equipped with a glass electrode, Gomelsky Plant of Measuring Devices, Belarus (pH range 1-14 with resolution of 0.01). The pH of each solution was established using diluted HCl and NaOH solutions.

2.2. The procedure of osmium assay in the histochemical specimens

The sample (stock fixing fluid, used fixative or a stained tissue) was placed into a test tube, filled with hydrochloric acid (~40 mL, 6 mol L^{-1}) and kept for five days in the dark at room temperature for the complete extraction and conversion of free and bound osmium(VIII, VI, IV) into the soluble forms of Os(IV) chlorides [55]. Then, after quantitative transfer of the obtained solution into a 50.0 mL volumetric flask, distilled water was added to complete the volume. For osmium determination 0.5-2.0 mL of analytes' aliquots has been taken and undergone the proper analytical procedure according to the following scheme. The necessary quantities of solutions of buffers or sodium salts and the proper organic reagents (according to Table 5) were placed into a 25.0 mL volumetric flask. Then the analytes' aliquots were added. The distilled water was added to a total volume of ~15-20 mL. Then the pH was adjusted by means of NaOH and HCl diluted solutions. After that, distilled water was added to complete the volume, and the solutions were heated on a boiling water bath (~98°C), if necessary, or kept at room temperature (~20°C) during the definite time (Table 5). Then solutions were then cooled to room temperature and the absorbance measurements were carried out against blank solution at the appropriate wavelength in 1-5 cm cuvettes. The osmium content was evaluated using the methods of a normal calibration curve or standard additions (all tests were performed at the alpha level of 5%; the number of individual measurements n was 5; confidence limits were calculated as mean ± St/√n, where S is a standard deviation and t=2.78).

3. Results and discussion

We have applied the elaborated spectrophotometric techniques for osmium determination with acid monoazo dyes, *o,o'*-dihydroxo substituted azo dyes and flavonoids for osmium assays in stock fixing fluid containing nominally 2% w/w OsO_4, in these fixatives, used for rat gum and teeth tissues staining and fixing, and in the gum tissues of intact rats, sick and treated with a drug.

Fixatives were prepared in one of the commonly utilized buffer systems, namely 0.2 mol L^{-1} cacodylate buffer (pH=7.4), containing sodium dimethylarsenate $NaAsO_2(CH_3)_2$ for the permanent storage of the fixative [1]. As it is widely known, biological materials, including rat tissues, contain appreciable amounts of major mineral elements, *e.g.* calcium and magnesium, which probably can readily get into a fixative during the staining process as well as into the extract during acidic treatment of a tissue. That is why we have studied the influence of

Table 5. The conditions of the spectrophotometric determination of osmium(IV) in fixatives and stained tissues by means of azo dyes and flavonoids

Reagent	$C_{Reagent} \times 10^5$ (mol L^{-1})	Medium	pH	Time of reaction (min)	λ (nm)	l (cm)
TpO	12.0	0.30 mol L^{-1} acetate buffer	5.2	10*	540	5
TpOOOI	5.4	0.03 mol L^{-1} sodium tetraborate	8.0	30*	364	2
TpOOOII	6.0	0.50 mol L^{-1} acetate buffer	4.8	20*	340	3
OG	6.0	0.20 mol L^{-1} acetate buffer	5.8	30*	540	2
EBBR	4.5	0.05 mol L^{-1} sodium chloride	10.5	10**	400	3
ACDB	3.0	0.05 mol L^{-1} sodium chloride	11.2	2*	340	3
EBT	3.0	0.05 mol L^{-1} sodium chloride	2.5	7*	480	3
Mor	15.0	1.20×10^{-4} mol L^{-1} sodium tetraborate	9.5	30*	485	1
Quer	6.6	0.10 mol L^{-1} sodium chloride	10.0	5*	440	1
Lut	1.5	10% v/v C_2H_5OH; 0.10 mol L^{-1} sodium chloride	6.0	2**	400	1

** on heating on a boiling water bath (~98°C); ** at room temperature (~20°C)*

Table 6. Tolerance ratios of ions of fixatives and tissues matrices when determining osmium(IV) spectrophotometrically with azo dyes and flavonoids (C_{Os}:C_{Ion})

Ion	Reagent									
	TpO	TpOOOI	TpOOOII	OG	EBBR	ACDB	EBT	Mor	Quer	Lut
$(CH_3)_2AsO_2^-$	1:50*				1:40	1:10	1:20	1:50	1:50	1:5
Ca^{2+}	1:1000*				1:1	1:1	1:2	1:100	1:150	1:100
Mg^{2+}	1:1000*				1:2	1:1	1:4	1:100	1:20	1:10

** higher concentrations of matrices ions were not investigated*

main matrix components of fixatives and stained tissues on the possibility of Os(IV) determination with azo dyes and flavonoids in order to elaborate new accurate spectrophotometric methods for osmium assays in histological specimens. The results of the selectivity investigations of osmium(IV) spectrophotometric determination towards matrix components are presented in Table 6.

According to tabulated data, in the case of acid monoazo dyes (TpO, TpOOOI, TpOOOII and OG) the presence of large amounts of cacodylate, calcium and magnesium ions does not interfere Os(IV) spectrophotometric determination. Concerning *o,o'*-dihydroxo substituted azo dyes (EBBR, ACDB and EBT), the very excess of sodium cacodylate over osmium in stock fixative (C_{Os}:$C_{Cacodylate}$~1:5) does not affect the osmium assay, but Ca^{2+} and Mg^{2+} significantly interfere, since EBBR, ACDB and EBT are known to be metallochromic indicators in chelatometry [44]. Flavonoids (Mor, Quer and Lut) are more selective relative to the main matrix components of fixatives and stained tissues than *o,o'*-dihydroxo substituted azo dyes. The interfering influence of sodium cacodylate causes the signal to decrease probably because of the complexation of Os(IV), while the presence of Ca and Mg results in an absorbance increase. Thus the results of selectivity investigations of Os(IV) interaction with reagents must be taken into the consideration when estimating the amount of osmium in histochemical specimens.

Table 7 contains the results of osmium determination in stock and waste fixatives. Osmium content in stock fixing fluid, established by means of utilized organic reagents, was somewhat lower that the nominal one. It can be explained knowing that at fixative preparation the proper amount of OsO_4 is not weighed with a precise analytical balance, so the nominal concentration of osmium in the fixative is usually approximate, but for comparing osmium uptake by different tissues the exact concentration was necessary. The application of flavonoids for osmium assaying in the stock fixative resulted in even lower values of Os, probably due to the presence of sodium cacodylate (Table 6).

In accordance with Table 7, osmium uptake by gum tissue is substantially greater, than by teeth tissue, as was concluded from the residual osmium concentrations in fixatives after gum and teeth sample staining. Osmium concentration in used fixatives, determined spectrophotometrically with *o,o'*-dihydroxo substituted azo dyes and flavonoids, was higher, than the one with acid monoazo dyes, most likely due to the influence of calcium and magnesium ions (Table 6), which possibly get into the fixative solution from biological materials. It should be noted, that osmium assays in used fixatives [56] is *pari passu* with stained tissues valuable in clinical research, especially taking into consideration that liquid

Table 7. Results of the spectrophotometric assay of osmium in stock fixing fluid and used fixatives by means of azo dyes and flavonoids

Reagent	Stock fixing fluid			Fixative, used for rat gum tissue staining		Fixative, used for rat teeth tissue staining	
	$C_{Os}^{calc.}$ (mg mL^{-1})	C_{Os} (mg mL^{-1})	RSD, %	C_{Os} (mg mL^{-1})	RSD, %	C_{Os} (mg mL^{-1})	RSD, %
TpO	15.0	14.4±0.7	4.0	3.3±0.1	2.0	5.8±0.2	2.7
TpOOOI		14.0±0.9	5.2	2.9±0.1	2.3	5.3±0.2	3.0
TpOOOII		14.6±0.6	3.4	3.0±0.1	3.5	5.6±0.3	4.3
OG		14.2±0.6	3.4	3.0±0.2	4.5	5.4±0.2	3.6
EBBR		14.2±0.5	3.1	4.3±0.1	2.2	6.8±0.2	2.7
ACDB		14.6±0.3	1.7	4.9±0.2	2.9	7.3±0.3	3.0
EBT		14.3±0.4	2.3	4.5±0.1	2.5	7.0±0.2	2.8
Mor		13.4±0.8	5.3	3.6±0.2	5.0	6.4±0.4	4.7
Quer		13.9±0.9	5.4	2.9±0.2	5.1	5.3±0.3	5.0
Lut		12.9±0.6	4.0	2.9±0.2	4.8	6.0±0.3	4.4

Table 8. Results of osmium voltammetric assays in stock fixing fluid and used fixatives by means of azo dyes

Reagent	Stock fixing fluid			Fixative, used for rat gum tissue staining		Fixative, used for rat teeth tissue staining	
	$C_{Os}^{calc.}$ (mg mL^{-1})	C_{Os} (mg mL^{-1})	RSD, %	C_{Os} (mg mL^{-1})	RSD, %	C_{Os} (mg mL^{-1})	RSD, %
TpO	15.0	14.6±0.5	3.0	3.5±0.2	4.0	6.0±0.3	3.7
TpOOOI		14.2±0.6	3.2	3.1±0.1	3.8	5.5±0.2	3.0
TpOOOII		14.4±0.6	3.4	3.2±0.1	3.5	5.7±0.3	4.2
OG		14.0±0.8	4.4	3.0±0.2	4.5	5.6±0.3	4.6
EBBR		14.0±0.8	4.8				
ACDB		14.3±0.7	4.3				
EBT		14.2±0.9	5.3				

fixatives do not require specific procedures of osmium extraction for analysis, unlike complex tissues, from which osmium may be not completely extracted.

In order to test the accuracy of osmium spectrophotometric determination in fixatives with azo dyes, we have carried out the assay of osmium in these samples using the electrochemical technique, *viz.* an oscillopolarography with a linear potential sweep on a dropping mercury electrode. The voltammetric studies of Os(IV) interaction with acid and *o,o'*-dihydroxo substituted azo dyes have shown that the presence of osmium(IV) results in sufficient decrease of a cathodic peak of azo group on the voltammograms of each examined dye. Also it has been established that in the conditions of spectrophotometric determination Os(IV) with azo dyes the decrease of a cathodic peak of the dye is proportional to the osmium concentration in a solution. Thus we have utilized the acid and *o,o'*-dihydroxo substituted monoazo dyes for the indirect voltammetric osmium quantification in fixatives, measuring the decrease of azo group current in the presence of osmium relative to the current of a blank azo dye solution. Osmium oscillopolarographic determination has been carried out according to the same method, which is presented in Table 5. Osmium content was evaluated using the methods of standard additions. The results of the osmium voltammetric assays in stock fixing fluid and used fixatives are presented in Table 8.

Voltammetric determination of osmium by means of azo dyes has provided similar results of osmium concentration in fixatives to ones obtained by means of spectrophotometric assays. Hence, the elaborated spectrophotometric methods for osmium estimation in fixatives using azo dyes are compatible.

Table 9 contains the results of testing variances and differences between means of osmium spectrophotometric and voltammetric determination in fixatives using acid monoazo dyes for all the relevant pairs of assays by means of most commonly used statistical tests, viz. F-test and Student's t-test respectively.

The statistical analysis has shown that there is no significant difference between the accuracy and precision of the proposed methods, since the calculated t- and F-values did not exceed the tabulated ones.

As was already mentioned, a quantitative estimation of osmium uptake by different tissues is often used, particularly, in biomedical investigations. So using azo dyes and flavonoids we have examined osmium content in rat tissues specimens from the clinical testing of dental drugs. The amount of osmium in stained tissues, given in the terms of micrograms of osmium present in each sample, is shown in Table 10 (before staining each

Table 9. Statistical analysis of the results of osmium spectrophotometric and voltammetric assays in fixatives using acid monoazo dyes

Reagent	Stock fixing fluid		Fixative, used for rat gum tissue staining		Fixative, used for rat teeth tissue staining	
	F-test*	t-test**	F-test*	t-test**	F-test*	t-test**
TpO	1.7	0.6	4.5	1.8	2.0	1.6
TpOOOI	2.6	0.5	3.1	2.0	1.1	1.9
TpOOOII	1.0	0.6	1.1	1.9	1.0	0.7
OG	1.6	0.6	1.0	0.4	1.8	1.4

** tabulated F-value for 4 and 4 degrees of freedom at P=0.95 is 6.388;*
*** tabulated t-value for 4+4 degrees of freedom at P=0.95 is 2.306*

Table 10. Results of the spectrophotometric assay of osmium in rat tissues, stained with osmium tetroxide, by means of azo dyes and flavonoids

Reagent	Gum of an intact rat		Gum of a sick rat		Gum of a rat, treated with the tested drug	
	m_{Os} (mg)	RSD, %	m_{Os} (mg)	RSD, %	m_{Os} (mg)	RSD, %
TpO	7.3±0.4	3.8	10.8±0.5	4.0	8.2±0.3	3.1
TpOOOI	7.5±0.2	2.1	11.0±0.4	3.3	8.4±0.5	4.5
TpOOOII	7.8±0.3	3.3	11.3±0.5	3.7	8.8±0.4	3.4
OG	7.7±0.5	4.6	11.1±0.6	4.5	8.8±0.5	4.8
EBBR	8.5±0.3	3.0	12.0±0.6	3.9	10.7±0.3	2.2
ACDB	8.6±0.3	2.7	11.2±0.4	2.5	9.6±0.4	3.5
EBT	8.9±0.2	1.7	11.7±0.5	3.3	10.4±0.3	2.6
Mor	9.8±0.5	4.3	12.6±0.6	3.9	11.1±0.6	4.7
Quer	8.3±0.4	4.4	11.4±0.7	4.6	9.7±0.6	5.0
Lut	8.7±0.6	5.1	11.9±0.4	2.8	10.5±0.5	4.1

of tissue samples weighed ~15 mg, so the results of osmium content in either tissue may be compared with the sufficient accuracy).

As can be concluded from the tabular data, spectrophotometric assaying of osmium by means of all utilized organic reagents have shown, that gums of a rat treated with a tested drug absorbed more osmium than that of intact, but less than of sick rat. Osmium uptake by a tissue and the depth of OsO_4 penetration are in considerable dependence on a tissue microporosity and friability, so the testers of this drug may suggest its efficiency in the treatment of a dental disease. A similar effect was previously [14] observed, *viz.* a tissue with a porous structure or containing much unsaturated lipids greatly absorbs osmium during fixation.

Comparing the results of osmium determination in stained tissues, obtained with the spectrophotometric organic reagents, one can conclude that *o,o'*-dihydroxo substituted azo dyes and flavonoids provide overestimated results concerning osmium uptake relative to acid monoazo dyes. Again, the most reasonable explanation of such results is also the interfering influence of Ca^{2+} and Mg^{2+}, extracted with hydrochloric acid from biological tissues.

Hence, in spite of the difference in the conditions of osmium spectrophotometric assays by means of acid and *o,o'*-dihydroxo substituted azo dyes and flavonoids, in the sensitivity and selectivity of the techniques, we have obtained sufficiently commensurable results concerning osmium content in the studied fixatives and stained rat tissues. Therefore the examined reagents are suggested for the accurate osmium quantification in the samples of histochemical tests.

In addition, our investigations have confirmed that biological tissues after staining as well as waste fixatives contain considerable amounts of osmium. That is why they require special utilization in order to prevent osmium from getting into environment. Also, used histochemical samples should be recycled for osmium recovery.

4. Conclusions

We have elaborated new, simple and rapid spectrophotometric techniques for osmium assays in stock fixing fluid, waste fixatives and stained rat tissues containing OsO_4 by means of acid and *o,o'*-dihydroxo substituted azo dyes and flavonoids. The selectivity of the reagents relative to main matrix components of histological specimens, *viz.* dimethylarsenate, calcium and magnesium ions, has been studied. Acid monoazo dyes (Tropaeolin O, Tropaeolin OOO-I, Tropaeolin OOO-II and Orange G) appeared to be the most selective among the examined organic reagents, and TpOOOI

seemed to be the best one for sensitive, rapid and selective determination of osmium in fixatives and stained tissues. The accuracy of the spectrophotometric determination of osmium in fixatives with azo dyes has been also confirmed by voltammetric techniques. Osmium content in rat tissue specimens from the clinical testing of dental drugs using azo dyes and flavonoids has been examined. The results of osmium determination in histochemical samples by means of different reagents are sufficiently similar. The developed spectrophotometric methods are applicable for osmium assaying in fixatives and stained tissues, and they do not require any expensive equipment, complicated sample preparation or use of harmful organic solvents.

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