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Use of Acid, Alkali and Enzymes for the Extraction of Nucleic Acids from Pollen

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Abstract. Various methods for extracting nucleic acids from pollen were tested to find a suitable procedure for obtaining a pure preparation of nucleic acids uncontaminated by polysaccharides and polyphosphates without the use of ion exchangers. Extraction was carried out with perchloric acid, potassium hydroxide, ribonuclease and deoxyribonuclease, sodium tetraborate, and combinations of these. In all fractions, residues of precipitates and residues of extracted pollen matter, the quantity of RNA, DNA, proteins and concomitants, *i.e.* polysaccharides and polyphosphates, was determined. The purity of preparations was checked by means of UV-spectra. The criterion of nucleic acid purity was agreement between the nucleic acid amounts calculated on the basis of measurement of absorption in UV-region, orcinol reaction and content of phosphorus. It was found that in our material only a few methods would be applicable and with great limitation, because many polysaccharides and polyphosphates appeared in nucleic acid fractions.

Quantitative determination of nucleic acids in plant material is commonly made difficult by high content of polysaccharides and polyphosphates with UV-absorbing compounds, which pass mostly into extracts of nucleic acids and interfere with chemical and spectrophotometrical determination of nucleic acids and with work with radioisotopes (WANG and MANCINI 1966). Few methods exist for extraction of nucleic acids from plant material and therefore in this paper the classical methods of extractions of nucleic acids from animals were also used. All basic methods and their modifications have been described by HUTCHISON and MUNRO (1961).

Material and Methods

Experimental Plants

Comparison of chosen methods of extraction was carried out on pollen of hazel (*Corylus avellana* L.), which was stored in the refrigerator at about −10°.

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Preparation of Samples

Homogenisation in an electric propeller homogenizer MSE (Measuring and Scientific Equipment Ltd., London) in the presence of ballotini beads grade 10 proved very suitable for the mechanical breaking of pollen. After three minutes of homogenisation of 200 mg pollen with 2 g of ballotini beads in 2 ml of initial washing solution in 5 ml homogenisation flasks at the highest revolutions of the homogenizer, all pollen grains were broken, which was not possible with manual homogenisation in a grinding mortar with sand. Broken pollen was successively washed and extracted exactly according to cited methods. At the end of every extraction procedure all residues of appropriate precipitates and extracted pollen matter were extracted additionally 2 × 30 minutes with 1 N PCA according to STEFFENSEN (1966) at 75°.

Analytical Methods

Nucleic acids were measured in extracts spectrophotometrically according to CHARGAFF and ZAMENHOF (1948), via phosphorus according to BERENBLUM and CHAIN (1938) using the modification of MARTIN and DOTY (1949), with orcinol (CERIOTTI 1955). DNA was determined with diphenylamine (GILES and MYERS 1965). Purity of preparations in regard to presence of UV-absorbing compounds was checked by measuring spectra in the region between 200 and 320 nm against 1 N PCA. In order to obtain further information on the content of contaminating substances, the sum of Lowry-positive substances was determined (LOWRY *et al.* 1951). Reaction of extracts with phenol reagent was probably caused chiefly by the presence of proteins. For simplicity the content of all reacting substances is expressed in micrograms of proteins, measured according to a calibration curve obtained with a standard solution of lyophylised human albumine.

Chemicals Used

Chemicals were of the highest purity and were purchased from the firm Lachema Brno, PCA from VEB Laborchemie Apolda, H_2O_2 and orcinol from E. Merck AG, Darmstadt. RNase from bovine pancreas 1 × crystallised and lyophylised was from Reanal Finomvegyszergyár Budapest, DNase 2 × crystallised, from NBC Cleveland. DPA from Lachema Brno was 10 × crystallised from ethanol.

Apparatus

The content of nucleic acids was determined in UV-region on a Hitachi Perkin-Elmer UV-VIS spectrophotometer, UV-spectra on a Beckman DB spectrophotometer with recorder. Colorimetric measurement was also carried out on the Beckman DB spectrophotometer. A vörtex-mixer was used to wash precipitates and to mix samples. After each washing or extraction the liquid was separated from pollen matter or precipitate by centrifugation for 10 minutes at 3 000 × g in a Janetzki T 20 centrifuge. Volumes of all measured fractions were brought up to 5·0 ml.

Abbreviations used: PCA — perchloric acid, TCA — trichloroacetic acid, NA — nucleic acids, r-RNA — ribosomal RNA, RNase — ribonuclease, DNase — deoxyribonuclease, NA-P — nucleic acid phosphorus, RNA-P — ribonucleic acid phosphorus, DNA-P — deoxyribonucleic acid phosphorus, DPA — diphenylamine, O — orcinol, residue PAE — residue after PCA extraction, residue EPM — residue of extracted pollen matter, LPS — Lowry positive substances.

Results

Method of OGUR and ROSEN (1950)

After homogenisation in 70% ethanol (v/v) and washing of broken pollen grains with 70% (v/v) ethanol, 70% (v/v) ethanol acidified with 0·2 N PCA, mixture of ethanol-ether 3 : 1 (v/v) at boiling, and cold 0·2 N PCA, RNA was extracted 18 hours with 1 N PCA at 4°. Pollen matter was washed 2 × with cold 1 N PCA, and these washings were added to the main extract, called RNA fraction. Then DNA was extracted 2 × 20 minutes with 0·5 N PCA at 70°. It can be seen in Table 1 that the RNA fraction also contains some DNA, phosphates and polysaccharides. In the DNA fraction more RNA than DNA was found, less contaminating phosphates than in the RNA fraction, but more orcinol-positive compounds and substances reacting with phenol reagent. From measured values at 260 nm and 235 nm (Fig. 1) it is evident that both RNA and DNA fractions are contaminated also with UV-absorbing substances. In the sample from the residue of pollen matter after original extractions Lowry-positive substances and polysaccharides prevail on both RNA and DNA.

TABLE 1

THE NA-CONTENT of the fractions according to the method of OGUR and ROSEN (1950)

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
RNA fraction	102·8	159·0	0·5	610	680
DNA fraction	39·4	44·0	1·7	712	1197
Residue EPM	11·2	6·3	0·7	117	2097

Two Procedures According to MARCUS and FEELEY (1962)

Pollen was homogenized in cold 1% PCA (w/v) and then washed with 70% ethanol (v/v), with a boiling mixture of ethanol-ether-chloroform 2 : 2 : 1 (v/v/v) and with boiling ether. From washed samples nucleic acids were extracted according to two procedures. Procedure A consists in extracting only RNA 3 × 24 hours with 1 N PCA at 0°. Procedure B is a modified method of SCHMIDT and THANNHAUSER (1945) and consists in hydrolysing washed samples with 0·3 N KOH for 17 hours at 37°. After acidifying to pH between 1 to 2 with 5 N HCl, 1 N PCA is added in the cold to make final concentration 0·2 N. Precipitate formed during standing in the cold was separated by centrifugation from the water layer containing hydrolysate of RNA. DNA, which was contained in the precipitate, was obtained in extraction 2 × 20 minutes with 0·2 N PCA at 70°. According to procedure A RNA was extracted with minimal amount of DNA contaminated mainly with polysaccharides (Table 2). According to procedure B RNA fraction contained no DNA, but polysaccharides and a considerable amount of proteins are present in higher quantity than in the RNA fraction of procedure A (Table 3). The DNA fraction was contaminated with RNA, Lowry-positive substances and polysaccharides. In the residue of extracted pollen matter a part of DNA was found with concomitants. Extract obtained with 1 N PCA from the residue of pollen matter according to procedure A gave characteristic spectrum of nucleic acids (Fig. 2), which was evidently caused

TABLE 2

THE NA-CONTENT of the fractions according to the method of MARCUS and FEELEY (1962), procedure A

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
RNA fraction	107·0	150·0	0·4	721	438
Residue EPM	52·0	39·0	2·4	726	4236

by presence not only of DNA, but also of a considerable amount of RNA. From relatively high optical density in UV-region it is possible to judge that DNA fraction obtained in procedure B was contaminated with impurities (Fig. 3).

Extraction with RNase and DNase (STANGE *et al.* 1962)

From the sample homogenized in 70% ethanol (v/v) and washed with 70% (v/v) ethanol, destilled water, mixture ethanol-ether 3 : 1 (v/v) at boiling, and with boiling ether, RNA was first obtained by the action of RNase 3×3 hours at 37° (concentration of the enzyme 1 mg/1 ml H_2O).

TABLE 3

THE NA-CONTENT of the fractions according to MARCUS and FEELEY (1962), procedure B

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
RNA fraction	175·0	175·0	0	817	3750
DNA fraction	21·0	2·5	1·5	550	1625
Residue EPM	14·0	2·0	0·5	269	3300

After each incubation samples were washed with H_2O and after the third one with 0·005 M $MgSO_4$. The DNA fraction was obtained by the action of DNase (concentration 0·4 mg of the enzyme in 3 ml 0·005 M $MgSO_4$ in acetate buffer pH = 5) 3×3 hours at 37°. From Table 4 it is evident that RNA fraction contains traces of DNA and polysaccharides while DNA fraction contains mostly RNA. A large part of DNA with an abundance of orcinol-positive substances and compounds reacting with Lowry phenol reagent was present only in residue of extracted pollen matter. A relatively high amount of nucleic acids remains unextracted and passes into 1 N PCA at final extraction (Fig. 4).

TABLE 4

THE NA-CONTENT of the fractions according to the method of STANGE *et al.* (1962)

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
RNA fraction	101·0	126·0	0·1	227	458
DNA fraction	13·0	12·0	0·3	69	950
Residue EPM	33·0	67·0	2·0	1036	5058

SCHNEIDER Extraction (1945) in Modification of SMILLIE and KROTKOW (1960)

Pollen was homogenized in 98% (v/v) methanol and then washed with 98% (v/v) methanol, 0·05 M formic acid in 98% methanol (v/v), cold 5% TCA (w/v), with 70% ethanol (v/v) at room temperature and at boiling, with a boiling mixture of ethanol-ether 2:1 (v/v) and finally with boiling ether. In contrast to SCHNEIDER (1945), who extracted all nucleic acids with 5% TCA (w/v), SMILLIE and KROTKOW (1960) used 5% PCA (w/v). In our experiments nucleic acids were extracted according to the shortened procedure

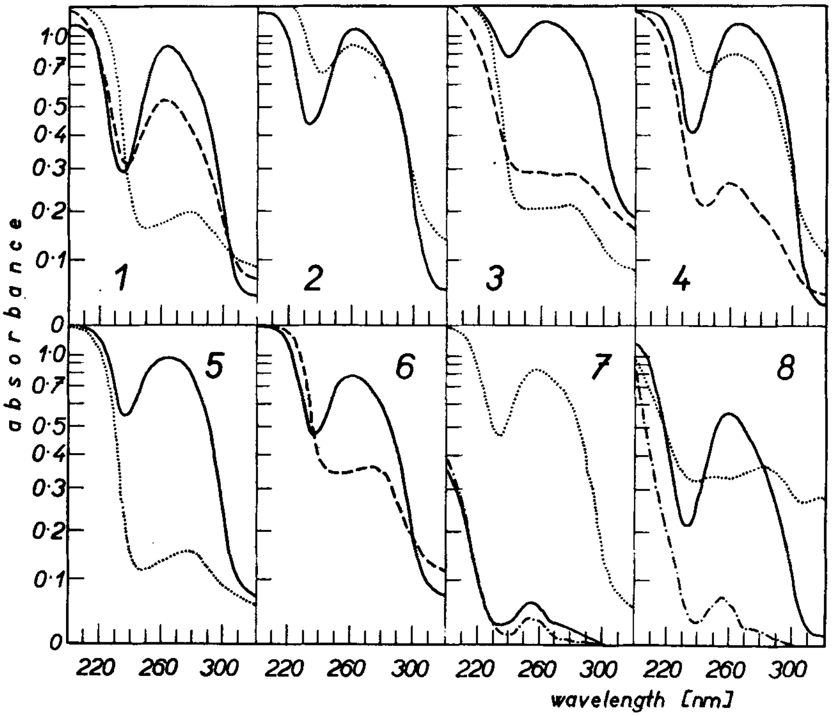

Fig. 1. UV-spectrum of the extracts according to OGUR and ROSEN (1950) in the region from 200 to 320 nm. ——— 20 fold diluted RNA fraction, ------- 10 fold diluted DNA fraction, ········ 10 fold diluted fraction from residue EPM.

Fig. 2. UV-spectrum of the extracts according to the method of MARCUS and FEELEY (1962), procedure A, in the region from 200 to 320 nm.——— 20 fold diluted RNA fraction ······· 10 fold diluted fraction from residue EPM.

Fig. 3. UV-spectrum of the extracts according to MARCUS and FEELEY (1962), procedure B in the region from 200 to 320 nm. ——— 20 fold diluted RNA fraction, ------- 10 fold dilutde DNA fraction, ········ 10 fold diluted fraction from residue EPM.

Fig. 4. UV-spectrum of the extracts according to STANGE *et al.* (1962) in the region from 200 to 320 nm. ——— 10 fold diluted RNA fraction, ------- 10 fold diluted DNA fraction, ········ 10 fold diluted fraction from residue EPM.

Fig. 5. UV-spectrum of the extracts according to SMILLIE and KROTKOW (1960) in the region from 200 to 320 nm. ——— 20 fold diluted NA fraction, ········ 10 fold diluted fraction from residue EPM.

Fig. 6. UV-spectrum of the extracts according to STEFFENSEN (1966) in the region from 200 to 320 nm. ——— 20 fold diluted RNA fraction, ------- 10 fold diluted DNA fraction.

Fig. 7. UV-spectrum of the extracts according to KERN (1960), procedure A, in the region from 200 to 320 nm. ——— 10 fold diluted NA fraction, -·-·-· 10 fold diluted fraction from residue PAE, ······· 20 fold diluted fraction from residue EPM.

Fig. 8. UV-spectrum of the extracts according to the method of KERN (1960), procedure B, in the region from 200 to 320 nm. ——— 10 fold diluted NA fraction, -·-·-· 10 fold diluted fraction from residue PAE, ······· 40 fold diluted fraction from residue EPM.

for *Euglena*: 2 × 15 minutes with 5% PCA (w/v) at 90°. With this procedure the NA fraction obtained contains most of RNA and DNA with an abundance of contaminating polysaccharides while in residue of pollen matter polysaccharides are present in lower quantities (Table 5). Spectrum of this NA fraction shows that it is relatively highly contaminated with UV-absorbing substances (Fig. 5). Final 1 N PCA extract is very much contaminated, which is evident from shifting of the maximum of its optical density in UV to 270 nm.

TABLE 5

THE NA-CONTENT of the fractions according to the method of SMILLIE and KROTKOW (1960)

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
NA fraction	142·5	167·0	2.3	1095	3696
Residue EPM	19·8	15·3	0·4	58	3273

Method of STEFFENSEN (1966)

This is another modification of the SCHMIDT and THANNHAUSER (1945) method. Pollen was homogenized in cold 0·2 N PCA and washed with 0·2 N PCA, 96% (v/v) ethanol saturated with magnesium acetate, mixture of ethanol-ether 2 : 1 (v/v) at 60° and finally with boiling ether. Then RNA was hydrolyzed in 1 ml 0·5 N KOH at 37° for 20 hours. After cooling of extracts, 0·06 ml 11 N PCA was added to precipitate $KClO_4$ and DNA. Precipitate separated by centrifugation was 2 × washed with cold 0·2 N PCA and washings were added to fraction of hydrolysed RNA. DNA was then extracted from the precipitate 2 × 15 minutes with 1 N PCA at 75°. The RNA fraction contains DNA and polysaccharides (Table 6), proteins are also present as shown not only by its spectrum in UV-region but mainly by the strong reaction of the fraction with phenol reagent (Fig. 6). In comparison with other methods described in this paper, the highest amount of DNA was extracted by this method. The DNA fraction however was also contaminated with RNA, polysaccharides and very probably with proteins.

TABLE 6

THE NA-CONTENT of the fractions according to the method of STEFFENSEN (1966)

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
RNA fraction	172·0	149·0	0·8	626	1063
DNA fraction	18·0	6·0	1·8	682	569
Residue EPM	7·0	4·5	0·4	—	—

Method of KERN (1960)

This is an extraction of nucleic acids from unwashed pollen material with 0·01 M $Na_2B_4O_7$ and following precipitation, with the same volume of 20% TCA (w/v). Precipitate is washed similarily as in the paper of OGUR and ROSEN (1950) and nucleic acids were extracted from it with 0·5 N PCA for 30 and 10 minutes at 70°. Homogenisation lasted 3 minutes as in other methods (procedure A), but the yield was extremely low and therefore in procedure B homogenisation was repeated 3 × 3 minutes. In the NA fraction obtained by procedure A only traces of DNA and a small amount of RNA were found. Part of the RNA was extracted from the residue of the precipitate with 1 N PCA at 75° (Table 7). The main part of both RNA and DNA remained in residue of extracted pollen matter and it was then extracted with 1 N PCA at 75°. In this last fraction a great part of polysaccharides was found. Also in procedure B, *i.e.* by 3 homogenisations in 0·01 M $Na_2B_4O_7$, nucleic acids were not extracted quantitatively (Table 8). A considerable part of both RNA and DNA remained in the pollen matter after the extraction and it was possible to extract it quantitatively with 1 N PCA. Both 1 N PCA extracts after A and B procedures are evidently highly contaminated with proteins (Figs. 7 and 8). In the precipitate of tetraborate extract with 20% TCA (w/v) DNA remained after extraction with 0·5 N PCA, but it is well extracted with 1 N PCA at 75°.

TABLE 7

THE NA-CONTENT of the fractions according to the method of KERN (1960), procedure A

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
NA fraction	6·0	2·0	0·1	12.5	25
Residue PAE	3·0	1·0	0	4·0	25
Residue EPM	123·0	147·0	2·9	1042·0	831

TABLE 8

THE NA-CONTENT of the fractions according to the method of KERN (1960), procedure B

μg/100 mg	UV NA-P	P NA-P	DPA DNA-P	O NA-P	LPS
NA fraction	47·0	52·0	0·4	84	1112
Residue PAE	5·0	4·5	0·9	60	1094
Residue EPM	117·0	156·0	1·7	1073	4850

Discussion

It follows from the above results that determination of both RNA and DNA is hampered above all by problems of their selective and quantitative extraction, the presence of polysaccharides and polyphosphates interfering for the most part. According to the method of OGUR and ROSEN (1950) RNA and DNA are not separated. Curiously, most RNA was found in the DNA fraction and a considerable part of DNA stayed in the residue of extracted pollen matter. After homogenisation all the pollen grains were broken as was seen by microscopic observation, but the time of the extraction with heating was not sufficient to break nuclei properly and to extract all DNA from them.

Cold 1 N PCA in procedure A (MARCUS and FEELEY 1962) results in great amount of contaminating compounds in RNA fraction. Small yield of DNA in procedure B can probably be explained by loss during KOH hydrolysis.

With enzymatical hydrolysis (STANGE *et al.* 1962) fractions were obtained that contained also a great amount of impurities, while large part of both RNA and DNA remained in the residue of pollen matter. Nucleic acids bound in nucleus and in cytoplasmic organelles are obviously not accessible for enzyme molecules.

Relatively high yields of both RNA and DNA were obtained by procedure according to SMILLIE and KROTKOW (1960), but a considerable part of impurities passed with nucleic acids into extracts. By prolongation of time of extraction at the same temperature it would probably be possible to obtain both RNA and DNA quantitatively and for some studies not requiring use of radioisotopes this method would be applicable.

Good yield of nucleic acids was obtained also with method of STEFFENSEN (1966), but the expected separation of RNA from DNA did not occur. The total amount of DNA extracted by this method was the highest in comparison with other methods, but about one third of the DNA was found in RNA fraction. As the NA content in the residue of pollen matter was very low, this procedure for obtaining DNA was used for additional extractions of nucleic acids after using other methods, by means of which practically all UV-positive and phosphorus-containing compounds were extracted from residues of pollen matter or eventual precipitates.

The method of KERN (1960) is a reverse procedure of the others, *i.e.* crude extract of nucleic acids was obtained initially and it was further washed. The main failure of this method when used for pollen material is that even after several times repeated extraction the yields of nucleic acids were very low.

By comparing spectra it was shown that the nucleic acid determination in UV-region has itself a definite error mainly in methods using KOH or NaOH as hydrolysing agents, because a great amount of proteins or/and their hydrolytical products, *i.e.* aromatic amino acids, has a certain influence on curve maximum characteristic for nucleic acids. The content of proteins measured in isolated fractions confirmed this.

In the determination of phosphorus the amount of NA-P was higher by the amount of phosphorus in phosphoproteins, the presence of which could not be excluded in present methods. For example inorganic phosphorus can be removed from extracts (DELORY 1938), but it could cause further loss of nucleic acids.

The determination of DNA with diphenylamine is probably highly selective but in the original method (DISCHE 1930) mucins and in general all compounds convertable to ω-hydroxy-levulic aldehyde react as DNA does. OVEREND (1951) stated that proteins and products of their hydrolysis, predominantly valine, increase the extinction of the discoloration arising from the reaction of diphenylamine with deoxyribose of DNA, but that it may originate also from impurities in diphenylamine samples. COHEN (1944) showed that fructose and its derivatives and also ascorbic acid, furfural, glyceraldehyde and agar interfere in the diphenylamine method. HOLDEN (1953) reported that diphenylamine also reacts weakly with pectines and that galacturonic acid reacting with diphenylamine has a maximum in optical region different from DNA, RNA increasing the intensity of the coloration by 5%. Also some derivatives of glucose and galactose react with diphenylamine and give a weak green coloration. We used the better and simplified method of GILES and MYERS (1965), which has lower sensitivity to saccharides different from deoxyribose, but nevertheless it is not possible to exclude that in some extracts, mainly from residues of pollen matter, greater part of polysaccharides could give some definite positive reaction with diphenylamine.

The orcinol reaction is certainly not specific for pentoses, because most substances of sugar character react similarly. In all extracts, obtained with whatever method used, the values calculated on the basis of orcinol reaction overreach highly values obtained in UV-region and via phosphorus. This means that all extracts were contaminated with saccharides and that no method from these studied permits use of the orcinol method for the determination of nucleic acids in the given pollen material.

MACIEJEWSKA-POTAPCZYK *et al.* (1969) washed hazel pollen according to SMILLIE and KROTKOW (1960) and determined nucleic acid amount according to SCHMIDT and THANNHAUSER (1945). Although they used lyophylised pollen, they obtained RNA content in UV-region 5·564 mg/g and via phosphorus 6·084 mg/g, which is roughly 3 × less than reported here, and DNA content in UV 0·168 mg/g and with diphenylamine 0·127 mg/g, which is roughly 2 × less.

It is my hope that with other material some of these methods could be applicable, unless many impurities interfere as in the case of pollen. For example, extraction of nucleic acids was carried out from young wheat seedlings according to SMILLIE and KROTKOW (1960) and it was possible to obtain pure extracts (Süss — unpublished data).

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Experimentálně byly vyzkoušeny různé metody extrakce nukleových kyselin z pylového materiálu za účelem najít dobrý postup pro získání čistého preparátu nukleových kyselin nekontaminovaného polysacharidy a polyfosforečnany bez užití iontoměničů: Extrakce s HClO$_4$, KOH, ribonukleásou a desoxyribonukleásou, Na$_2$B$_4$O$_7$ a jejich kombinacemi. U všech frakcí, zbytků sraženin a extrahované pylové hmoty byl stanoven obsah RNK, DNK, bílkovin a kontaminujících látek, tj. polysacharidů a polyfosforečnanů. Čistota preparátu byla kontrolována sledováním průběhu UV-spekter. Za kritérium čistoty byla považována shoda mezi množstvím nukleových kyselin měřeném v UV-oblasti, orcinolovou metodou a přes fosfor. Bylo nalezeno, že pro náš pokusný materiál lze užít pouze několik metod extrakce a to ještě velmi omezeně neboť ve frakcích nukleových kyselin bylo nalezeno velké množství polysacharidů a polyfosforečnanů.