Technical Paper

COMPARISON OF DIFFERENT METHODS FOR METABOLITE EXTRACTION FROM *ASPERGILLUS NIGER* MYCELIUM

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Abstract

Two ways of quenching metabolism and four procedures for extraction of intracellular metabolites were elaborated with the filamentous fungus *Aspergillus niger*. Inactivation of metabolism by rapid quenching of the mycelium in liquid nitrogen or in 60% methanol solution kept at –40 °C gave similar, yet too low results after extraction with boiled buffered ethanol. Stability of analytical grade metabolites in solutions of known concentrations after acid and alkaline treatments resulted in minor deviations of 4-7%. Results obtained by extraction in buffered boiling ethanol were up to 30% too low, suggesting the reliability of the method questionable. Concentrations of metabolites present in fungal mycelia during fermentation varied with time. Amounts of pyruvate in *Aspergillus niger* mycelia varied from 0.5 to 2.0 μmol/g dry biomass, 2-oxoglutarate ranged between 0.2 and 0.8 μmol/g dry mycelium and malate from 3.0 to 16.0 μmol/g respectively, depending on time of cultivation. Both, intracellular and extracellular metabolites could be measured. Extractions of *Aspergillus niger* mycelia by perchloric acid and by sodium hydroxide were efficient and reliable, whereas treatment with buffered boiling ethanol returned too low amounts of metabolites examined.

Key words: quenching ofmetabolism, *Aspergillus niger*, extraction of metabolites, enzymes, <<a niger-2-1v3.jpg>>fermantation

Introduction

The filamentous fungus *Aspergillus niger* is able to accumulate and excrete high concentrations of organic acids and different extracellular enzymes, such as amylases, pectinases, lipases etc. In studies on metabolic regulation the knowledge about the concentrations of intermediary metabolites is required to determine metabolic pathways involved in their biosynthesis. It should be kept in mind that the concentration of metabolites in the cell is generally low and that some activating or inhibitory effects are only detectable under certain conditions, such as neutral, acidic or basic pH, appropriate concentrations of substrates etc.[1] Another very important task is the method of extraction employed. Metabolic rates are usually high, so during sampling enzymes must be inactivated at once. The goal is to stop all enzymatic reactions rapidly and simultaneously to get an instantaneous snapshot of the concentrations. Thus extreme

care must be undertaken to stop metabolism immediately. Although not all handling may result in changes of metabolites it is important to be aware of this problem. Thus fast cell sampling and inactivation of metabolism and complete extraction of desired metabolite should be introduced.

Fast sampling methods have been developed mainly for yeast[2-5] and bacteria,[6,7] whereas for the determination of *Aspergillus niger* metabolites results reported were obtained upon rapid quenching of metabolism with buffered solution of 60% methanol at –40 °C.[8]

Besides different sampling techniques different methods for the determination of metabolite concentrations were also applied, enzymatic ones,[2,6,8] HPLC,[9,10] NMR[11] and tandem MS coupled to HPAEC[5] and to LC-ESI.[7]

We have tested different methods for the extraction of metabolites with subsequent determination of metabolite concentrations by enzymatic methods. The obvious advantage of enzymatic methods is their high selectivity, with the weak point in need for relatively high sample volumes. One should be aware when determining the level of intermediates that with a chosen extraction method the metabolites are not destroyed. Conflicting results found in the literature might be partly explained by the use of different extracting reagents. Taking into consideration the mentioned problems we have applied and evaluated some of the methods with the filamentous fungus *Aspergillus niger*. We have elaborated some of metabolites of Krebs cycle recognized to be of importance in citric acid production.

Experimental

Microorganism and cultivation medium

Aspergillus niger A60 (NRRL 2270) was used throughout all experiments. Spores from 7 days old wort agar slants were suspended in 25 mL of 0.1% sterile Tween 80 solution. The beginning experiments were run on rotary shaker (New Brunswick Scientific Co., Edison, NJ) with 10^7 spores in 100 mL medium in 500 ml baffled flasks at 30 °C and 100 rpm. The fermentations were run in a glass stir tank bioreactor (Infors ISF-100) with a 5 L working volume. Approximately 5.10^8 spores were used for inoculation of 5 L medium. The defined medium, previously developed for citric acid production,[12] contained 140 g of commercial sugar, 1 g KH_2PO_4, 2.5 g NH_4NO_3, 250 mg

$MgSO_4 \cdot 7H_2O$ and 40 mg $CuSO_4 \cdot 5H_2O$ per liter of distilled water, with pH adjusted to 2.5. The temperature was kept at 30 °C, and the medium aerated with 5 liters of air/min with stirring at 300 rpm.

Fungal biomass was determined by gravimetric analysis after filtration of cell samples through preweighed filter papers, thoroughly washed with distilled water and dried to constant weihgt at 100 °C.

Extraction methods

Acid extraction of whole fungal culture (A). Partly modified method for the extraction of yeast metabolites was applied.[3] 5 mL of fungal cultures were directly dropped from the fermenter into liquid nitrogen. 1.5 mL 65% $HClO_4$ and 0.64 mL of 1M imidazol were added to the frozen cultures and let to thaw on ice. Extraction of metabolites was carried out by three freeze-thaw cycles with intensive shaking between. After 5 min of centrifugation at 10000 rpm and at 0 °C in Sorval RC 5C centrifuge the supernatant was neutralized with 10 M KOH and precipitate separated by centrifugation. The supernatant, containing intracellular plus extracellular metabolites, was kept on ice for immediate determination of metabolites or stored at –80 °C until use.

Acid extraction of fungal mycelia (B). 10 mL of fungal cultures were directly filtered through precooled thick metal filter net (incubated at –20 °C), quickly washed with ice cold water and put into liquid nitrogen. 2 mL of 15% $HClO_4$ was added to the frozen mycelium, kept on ice to thaw, shaked vigourously and frozen again. Freezing – thawing was repeated for another three times. Resulting suspension was centrifuged at 10000 rpm for 5 min at 0 °C, supernatant neutralized with KOH and centrifuged in cold again. Supernatant was analysed for the presence of intracellular metabolites.

Alkaline extraction (C). Procedure used was adapted and partly modified method of Francois et al.[13] 25 mL of fungal culture was filtered directly from fermenter through filter net precooled in deep freezer to –20 °C and immediately washed with ice cold water. Filter cake was instantly frozen in liquid nitrogen, mixed with 5 mL 0.1 M NaOH and heated at 80 °C for 15 min. After centrifugation at 10000 rpm for 5 min the supernatant was immediately used for metabolite determination or frozen at –80 °C.

Direct extraction with ethanol (D). Mycelium separated in cold from 5 mL of fungal culture as quickly as possible was mixed with 21 mL of boiling ethanol

containing 2 mL 1 M Hepes pH 7.5 and heated for 3 minutes at 80 °C. After cooling on ice the sample was dried using a rotavapor and residue resuspended in 1 mL of double-distilled water. After centrifugation at 4 °C for 10 min at 5000 rpm the supernatant was checked for intracellular metabolites.[3]

Freezing in 60% methanol at –40 °C and extraction with ethanol (D1, D2). Solution of 60% methanol and 10 mM Hepes pH 7.5 was precooled to –40 °C. 5 mL of fungal culture was directly poured into 26 mL of cold methanol mixture and let for another 5 minutes at –40 °C. Mixture was centrifuged for 5 min at 0 °C. Mycelial pellet was mixed with 21 mL of buffered boiled ethanol (D1) and elaborated as described for direct extraction with ethanol (D). In other cases fungal culture (5 mL in 26 mL of cold methanol suspension) was filtered through precooled metal net and washed with –40 °C methanol suspension (D2) and further elaborated as described for direct extraction with ethanol (D).[8]

Determination of metabolites

Analyses were conducted on Beckman UV/VIS spectrophotometer, all at 340 nm, following the formation or consumption of NADH. Pyruvate was determined essentially as described in Bergmeyer[14] in 50 mM triethanolamine, 5 mM $MgCl_2$, pH 7.6. 2-oxoglutarate was measured in the presence of 0.2 mM NH_4Cl, 0.1 mM ADP, 10 μM NADH with addition of 2-4 U/mL glutamate dehidrogenase.[3] Malic acid was determined in reaction mixture composed of 0.4 M hydrazine/0.5 M glycine buffer pH 9.0 with 2.55 mM NAD and 10 U/mL of malate dehydrogenase.[15]

The concentration of metabolites is expressed in micromol per g dry biomass. Conversion into intracellular concentration can be calculated by considering that 1 g *Aspergillus niger* dry biomass corresponds to 1.8 mL of cell sap.[16]

Experiments with analytical grade metabolites (SIGMA) were performed with maximum of 1 μg of compound per mL of assay mixture.

Results and Discussion

Determination of analytical grade metabolites

To elucidate the influence of different extraction methods on the stability of metabolites we started our work with analytical grade metabolites. We have verified the

effect of acid, alkali and neutral (buffered ethanol) treatment on their stability. Results of enzymatic determination of differently treated metabolites are gathered in Tables 1-3. Treatment with perchloric acid (Table 1), as well as with alkali (Table 2) resulted in good recovery (±6%). Approximately 30% loss of pyruvate, malate and 2-oxoglutarate was observed after treatment with buffered boiling ethanol (Table 3). It seemed that metabolites are most probably instable in hot ethanol or even react with it.

Table 1. Effect of acid ($HClO_4$) treatment upon the stability of analytical grade metabolites (Method B).

	amount (mg/L) before acid treatment	amount (mg/L) after acid treatment	Recovery (%)
Pyruvate	521 ± 4	506 ± 27	97
Malate	513 ± 16	502 ± 10	97
2-oxoglutarate	510 ± 19	482 ± 28	94

Analytical grade metabolites (Sigma) were treated with 15% $HClO_4$ as described in Experimental. Measurements are the average ± standard deviation of three experiments, each with five parallel measurements.

Table 2. Effect of alkali (NaOH) treatment upon the stability of analytical grade metabolites (Method C).

	amount (mg/L) before alkali treatment	amount (mg/L) after alkali treatment	recovery (%)
Pyruvate	515 ± 11	543 ± 30	105
Malate	523 ± 5	535 ± 21	102
2-oxoglutarate	517 ± 6	546 ± 4	106

Analytical grade commercial metabolites (Sigma) were treated with 0.1 M NaOH as described in Experimental. Measurements are the average ± standard deviation of three experiments, each with five parallel measurements.

Table 3. Effect of ethanol treatment upon the stability of analytical grade metabolites (Method D).

	amount (mg/L) before ethanol treatment	amount (mg/L) after ethanol treatment	recovery (%)
Pyruvate	516 ± 6	359 ± 26	70
Malate	498 ± 19	348 ± 22	71
2-oxoglutarate	506 ± 13	374 ± 11	74

Analytical -grade metabolites- (Sigma) were treated with hot buffered ethanol as described in Experimental. Measurements are the average ± standard deviation of three experiments, each with five parallel measurements.

Metabolites in fungal cultures

The first step was to quench the metabolism instantly by pouring the sample as quickly as possible into liquid nitrogen. Next, metabolites were extracted at extreme pH, enabling inactivation of most enzymes, with either strong acid or strong alkali, or in neutral with buffered boiling ethanol. For metabolites under investigation acid and alkali extraction were better with higher recovery than the extraction in boiling ethanol (Table 4). Aliquotes were taken from the same fungal culture on third day of cultivation and were treated with acid, alkali and buffered ethanol as described in Experimental. Similar values for pyruvate, malate and 2-oxoglutarate were obtained after acid and alkali treatment, whereas results obtained with ethanol were 20-30% lower. The loss with ethanol treatment could be accounted to instability of metabolites in boiling buffered ethanol rather than because of incomplete extraction or loss of sample during extraction procedure, since the recovery compared to results obtained for analytical grade metabolites (Table 3) was in same observed range. With the examined metabolites, malate, pyruvate and 2-oxoglutarate, acid and alkali extraction proved to be more suitable than extraction with boiling buffered ethanol.

Table 4. Concentrations of malate, pyruvate and 2-oxoglutarate during cultivation of *Aspergillus niger* on rotary shaker. Metabolites were extraced from whole medium or from fungal mycelia after 3 days of cultivation by different methods as described in Experimental.

	Method A μmol/ml broth	Method B μmol/g dry myc.	Method C μmol/g dry myc.	Method D μmol/g dry myc.
Malate	0.121 ± 0.021	6.97 ± 0.18	7.52 ± 0.36	6.23 ± 0.25
Pyruvate	0.112 ± 0.009	1.28 ± 0.13	1.37 ± 0.32	0.97 ± 0.13
2-oxoglutarate	0.090 ± 0.032	0.63 ± 0.12	0.72 ± 0.02	0.51 ± 0.09

A-extraction from whole medium, B-acid extraction of mycelium, C-alkali extraction of mycelium, D-neutral extraction of fungal mycelium.

Direct quenching of whole culture medium in liquid nitrogen allows fast and repeated sampling when metabolites to be tested are not present in the medium. One should be aware that during extraction metabolite levels were not changed by enzymatic or chemical conversion, that the extraction was complete, and that they were not destroyed during extraction. We were trying to detect metabolites present intracellularly, so the cell separation from the medium was necessary, since metabolites were also

present extracellularly. So we also measured metabolite concentrations in the filtrate (F) (Table 5). If our measurements were correct the sum of results obtained for mycelia (method B) and for filtrate should be the result obtained for method A (extraction of complete fermentation broth); or substraction of values for filtrate from complete broth should be in correlation with the amounts determined in the mycelium. Table 5 presents results showing good correlation between measured and calculated results. At the same time good repeatability between individual fermentations could also be observed. Method A (extraction of broth) with concomitant determination of metabolite in the filtrate could be convinient for comparison of amounts of intracellular and extracellular metabolites, since simple substraction of the amount obtained for filtrate gives the amount of metabolite in the mycelium.

Table 5. Concentration of metabolites present intracellularly and extracellularly after cultivation of *Aspergillus niger* for 5 days in 5 l fermenter.

	Method A mg/L broth (myc. with filtrate)	Method B mg in dry myc. from 1 L broth	Filtrate F mg/L filtrate	Calculated (A–F) mg in dry myc. from 1 L broth
Malate I	17.807 ± 0.918	2.988 ± 0.063	15.00 ± 0.48	2.807
Malate II	20.930 ± 1.301	3.413 ± 0.203	17.62 ± 1.76	3.310
2-oxoglutarate I	10.860 ± 0.086	0.406 ± 0.087	10.50 ± 0.69	0.360
2-oxoglutarate II	7.088 ± 0.891	0.320 ± 0.009	6.72 ± 0.59	0.368

Results were obtained with two individual cultivations of *Aspergillus niger* in laboratory fermenter (I and II). Measurements are the average ± standard deviation of three parallel extractions. (A-acid extraction from whole media, B-acid extraction from mycelia).

Cultivation in fermenter

Intracellular concentrations of pyruvate, 2-oxoglutarate and malate were followed for 6 days of *Aspergillus niger* cultivation in fermenter. Extractions in cold conditions (at –20 to 0 °C) after quenching of metabolism in liquid nitrogen, and immediate cooling of samples in 60% methanol cooled to –40 °C enabled by direct sampling device fitted on fermenter were performed. Cooled samples were further elaborated after centrifugation (method D1) or after filtration and washing mycelia with cold methanol (method D2). Results are gathered in Figure 1 for pyruvate, in Figure 2 for 2-oxoglutarate and Figure 3 for malate. Results obtained by boiling ethanol (methods D, D2) were noticable lower compared with acid and alkaline extraction what was already

observed in beginning experiments with analytical grade metabolites. Regarding these results we have concluded that extraction with buffered boiling ethanol was not suitable for metabolites examined. Huet et al.[17] reported that the extraction of metabolites by the boiling buffered ethanol could not be used, because it destroyed oxaloacetate due to its instability in neutral pH. Kubicek and Roehr[18] also observed the instability of 2-oxoacids during their extraction from mycelia. Method D1 gave erroneous results, since we did not wash mycelia, so we also measured extracellularly present metabolites. Acid (B) and alkali (C) extraction were again more accurate, especially regarding malate (Figure 3), whereas results obtained with pyruvate (Figure 1) and 2-oxoglutarate (Figure 2) were somehow little more scattered.

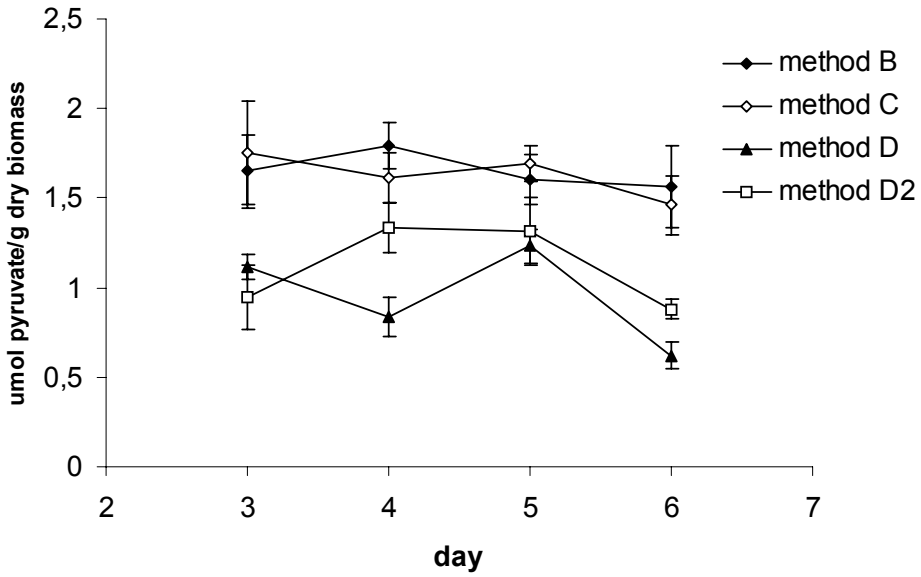

Figure 1. Concentrations of pyruvate in *Aspergillus niger* mycelia during cultivation in fermenter, obtained by different extraction methods. Measurements are the average ± standard deviation of three fermentations.

Results obtained in fermenter showed that intracellular levels of pyruvate, 2-oxoglutarate and malate and most probably other TCA acids could be quantitatively followed.

When we compared our results with the results of other authors working with fungi and yeast (Table 6), good correlation between fungal strains, fungal species and yeast *S. cerevisiae* was observed, with the exception of pyruvate from *M. Ruber*.

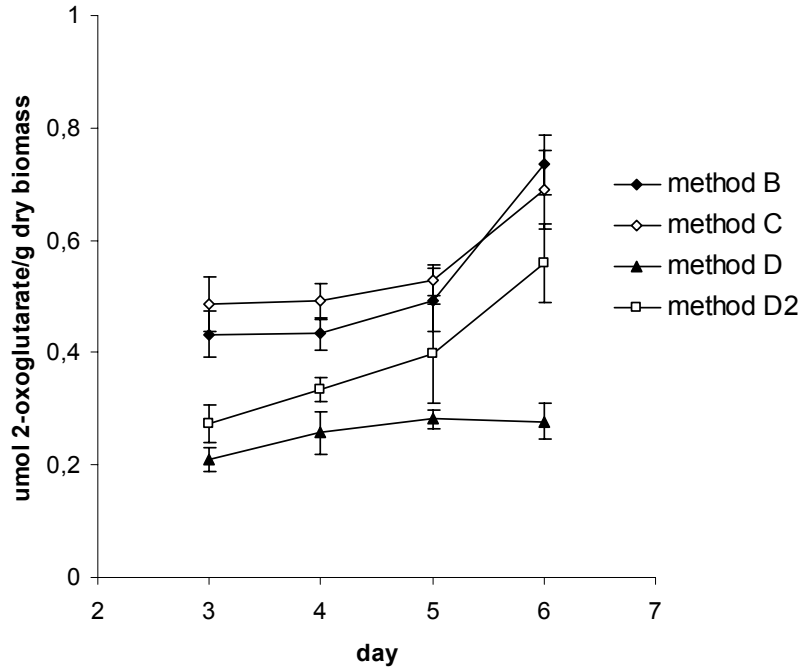

Figure 2. Amounts of 2-oxoglutarate extracted with differnet methods from *Aspergillus niger* mycelia during cultivation in fermenter for 6 days. Cultivation of *Aspergillus niger* in fermenter was performed three times. The data represent the average ± standard deviation.

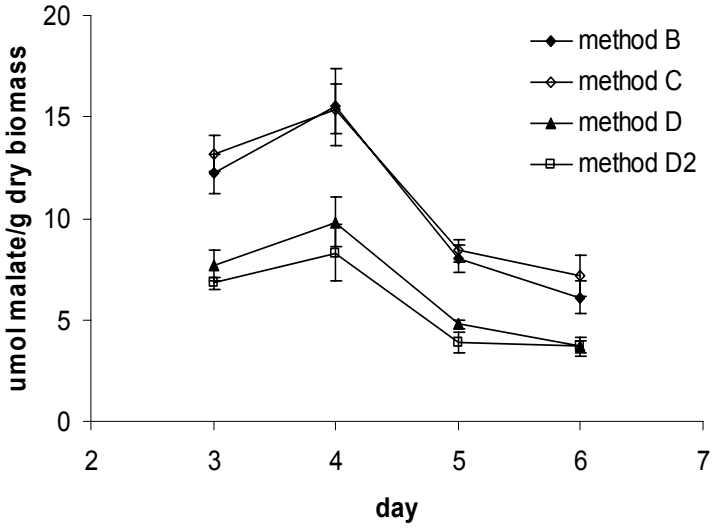

Figure 3. Malate concentrations in *Aspergillus niger* mycelia grown in fermenter for 6 days extracted by different methods. Results are the average ± standard deviation of three separate fermentations.

Together with knowledge of the kinetic properties of enzymes, metabolite levels can be used to quantify flux distribution in metabolic pathways and to determine factors which control metabolic flux in vivo. Since we have been interested in the regulation of metabolic pathways leading to the production of organic acids in *Aspergillus niger*, we followed pyruvate, 2-oxoglutarate and malate as metabolites recognized to have a crucial part in regulation of citric acid accumulation. Glycolytic pyruvate is converted by

Table 6. Concentrations of intracellular metabolites determined in different microorganisms.

microorganism	pyruvate (μmol/g dry biom.)	2-oxoglutarate (μmol/g dry biom.)	*malate* (μmol/ g dry biom.)	Reference
A. niger	0.13-0.82	0.5-1.2	7.0-16.0	*18*
B. emersonii	0.544	1.677	3.583	*19*
S. cerevisiae	0.2-1.6			*20*
A. niger			2.6-11.9	*21*
M. ruber	4.7-4.9			*22*
S. cerevisiae	1.83	1.07		*23*
A. niger	0.50-2.00	0.20-0.80	3.0-16.0	present work

pyruvate carboxylase to oxaloacetate and further to malate by cytosolic malate dehydrogenase. It has been postulated that cytosolic malate may serve as the co-substrate of the mitochondrial tricarboxylic acid carrier and that an enhanced malate concentration may stimulate export of citrate from the mitochondrion. Several explanations for citric acid accumulation are based on postulation of a metabolic inhibition of the NADP-specific isocitrate dehydrogenase by citrate or glycerol which would create a bottleneck in the TCA cycle reflecting in change of 2-oxoglutarate.[24] The knowledge about intracellular concentrations of metabolites together with corresponding enzyme activities could contribute to better understanding of regulation of individual steps within metabolic pathways leading to citric acid accumulation.

Conclusions

Most intermediary metabolites occur in cells at concentration in the micromolar to millimolar range amongst a mixture of thousands of other substances, some of which could be chemically similar. Measurement of their concentrations therefore requires sensitive method and enzymic analysis using spectrophotometric detection proved to be

such a method. From present work it can be seen that acid and alkali extraction of metabolites were better than boiling buffered ethanol. Although no extensive metabolic analysis has been presented, the methods described here presents the opportunity to follow changes in metabolic activities of *Aspergillus niger* during cultivation or under various cultivation conditions. Work has illustrated the validity of these methods and has also shown that intracellular levels of TCA metabolites could be quantitatively measured.

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Povzetek

Primerjali smo dva načina hitre zamrznitve metabolizma in štiri metode za ekstrakcijo intracelularnih metabolitov iz micelija glive *Aspergillus niger*. Metodi trenutne prekinitve metabolizma sta enako uspešni. Metodi alkalne in kisle ekstrakcije metabolitov iz glivnega micelija sta zanesljivi in kažeta manjša odstopanja (4-7%), medtem ko so rezultati dobljeni pri ekstrakciji z vrelim zapufranim etanolom celo do 30% prenizki. Koncentracije metabolitov v miceliju so se spreminjale s časom gojenja glive. Množine piruvata, 2-oksoglutarata in malata so se gibale v okviru koncentracij opisanih v literaturi.