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Quantitative analysis of tylosin by column liquid chromatography

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ABSTRACT

A column liquid chromatographic method suitable for the quality control of tylosin A is described. The determination can be carried out on different C_8 or C_{18} columns, using a mobile phase containing acetonitrile, 0.2 M tetrabutylammonium hydrogensulphate, 0.2 M phosphoric acid and water. The flow-rate is 1 ml/min and detection is performed at 280 nm. The method shows good selectivity towards the major components tylosin A, B, C and D and demycinosyltylosin. Minor degradation products, mainly observed in solutions, are also separated. The compositions of several standards are compared and results for a number of commercial samples are presented.

INTRODUCTION

Tylosin, a macrolide antibiotic, is produced by fermentation of *Streptomyces* strains [1]. It consists of a substituted sixteen-membered lactone, an amino sugar (mycaminose) and two neutral sugars, mycinose and mycarose [2,3]. Tylosin is used extensively as a feed additive and as a therapeutic substance in the treatment of mycoplasmosis in poultry and livestock [4]. In addition to the main compound, tylosin A (TA), several related structures are coproduced during the fermentation process. Desmycosin or tylosin B (TB) [2], macrocin (TC) [5], relomycin (TD) [6] and demycinosyltylosin (DMT) [7] have been isolated and their structures determined (see Fig. 1).

The first reversed-phase liquid chromatographic (LC) methods described did not allow the separation of the different tylosins [8,9]. It is necessary to separate these tylosins as it is known that they have different antibiotic activities [4]. A method for the analysis of tylosin in fermentation broths has also been described [10]. The selectivity was poor and the pH of the mobile phase too basic (> 11) for continuous use with reversed-phase columns. Later, an improved method at lower pH was published by the same author [11]. The separations were carried out on C_{18} and C_8 columns. The mobile phase consisted of tetrahydrofuran (THF), acetonitrile (CH_3CN), sodium pentanesulphonate, acetic acid and water. The selectivity was further improved by increasing the temperature to 55°C [11,12]. The major disadvantage of this method was the poor selectivity towards impurities, eluted close to the main component.

This was much improved by the method described by Fish and Carr [13]. Three stationary phases were found suitable: Nucleosil ODS, Zorbax C_8 and Hypersil Cs. The mobile phase consisted of CH_3CN in 0.85 *M* sodium perchlorate adjusted to pH 2.5 with 1 *M* hydrochloric acid. The high salt concentration combined with the corrosive chloride ion is a drawback of the method. The variance in selectivity for different batches of the same stationary phase sometimes results in a decrease in resolu-

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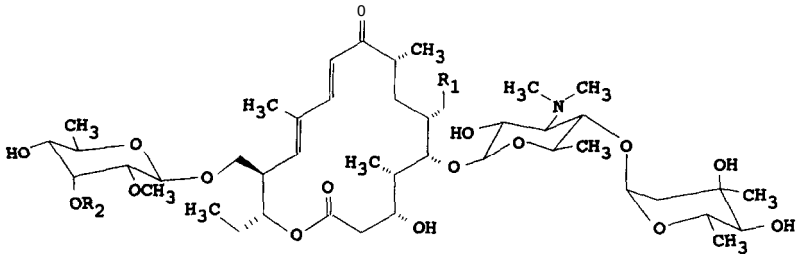
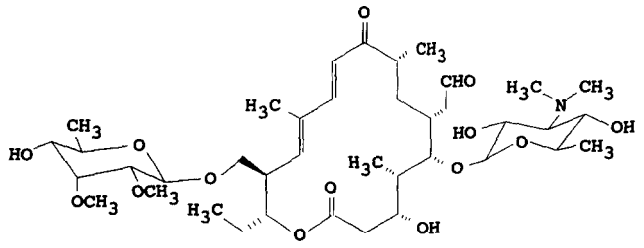
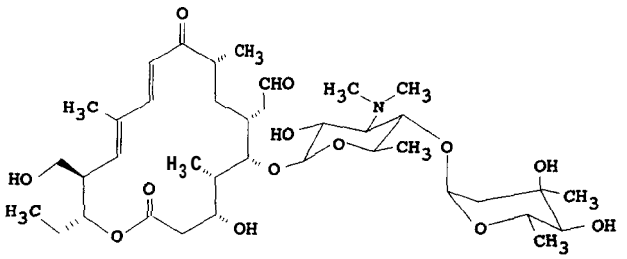


	R_1	R_2
Tylosin A	CHO	CH_3
Tylosin D	CH_2OH	CH_3
Tylosin C	CHO	H



Tylosin B



Demycinosyltylosin

Fig. 1. Structures oftylosins.

tion for minor components [13]. Using this method, it is necessary to adjust the composition of the mobile phase to provide sufficient resolution between the tylosin components. This method is now prescribed by the British Veterinary Pharmacopoeia (BP Vet) [14] and by the French Pharmacopoeia [15].

The LC method described here ensures a selectivity that is better than that obtained with the BP Vet method. The mobile phase is not corrosive and its composition needs less adjustment when the method is used on different stationary phases.

EXPERIMENTAL

Reagents and samples

Organic solvents were obtained from Janssen Chimica (Beerse, Belgium). Tetrahydrofuran (THF) was distilled after monitoring for the absence of peroxides. Tetrabutylammonium (TBA) hydrogensulphate was also obtained from Janssen Chimica. Other reagents were of analytical-reagent grade (Merck, Darmstadt, Germany). Water was freshly distilled from glass apparatus. Buffer solutions were prepared by mixing 0.2 *M* phosphoric acid with 0.2 *M* potassium dihydrogenphosphate until the desired pH was reached. The 0.2 *M* TBA solutions incorporated in the mobile phases were neutralized to the same pH as the mobile phase buffer with dilute sodium hydroxide.

The World Health Organization (WHO) international reference preparation of tylosin (WHO-IS, 1000 I.U./mg), the British Pharmacopoeia chemical reference substance (BP-CRS, batch 1226) and E. Lilly standards of tylosin A (lot 294-F 14-180- lx) and of tylosin (lot 186TD 1, 1073 I.U./mg) were available. House standards were prepared of TA (89.2%) TB (93.9%), TC (93.0%) and TD (98.9%) this content (% w/w) being expressed on the substance "as is". Bulk samples of tylosin were of known origin (Bulgaria, Italy and USA). A small amount of demycinosyltylosin (DMT) was also prepared but the purity of this compound was not determined precisely as it is only a minor impurity.

Columns

The columns (250 × 4.6 mm I.D.) were packed in the laboratory following a published method [16]. The reversed-phase stationary phases were Zorbax (DuPont, Wilmington, DE, USA), Nucleosil (Macherey-Nagel, Düren, Germany), Partisil (Whatman, Clifton, NJ, USA), RSil and RoSil (Bio-Rad, Eke, Belgium), Hypersil (Shandon, Runcorn, UK) and Spherisorb (Phase Separations, Queensferry, UK). The columns were always heated at 30°C.

LC apparatus and operating conditions

Isocratic elution was performed throughout the study. The apparatus consisted of a Milton Roy mini-pump (Laboratory Data Control, Riviera Beach, FL, USA), a Model CV-6-UHPa-N60 injector (Valco, Houston, TX, USA) equipped with a 20-μl loop, a Model LC 3 UV variable-wavelength detector (Pye Unicam, Cambridge, UK) set at 280 nm and a Model 3390A integrator (Hewlett-Packard, Avondale, PA, USA). For the final analyses of standards and samples a RoSil C_8 (8 μm) column was used with acetonitrile-O.2 *M* TBA-0.2 *M* phosphoric acid-water (20:8:5:67, v/v) as mobile phase. The mobile phase was degassed by sonication and the flow-rate was 1.0 ml/min. For all samples an amount equivalent to 50.0 mg of tylosin base was dissolved in water an diluted to 50.0 ml. The solutions were stored at 6°C in the dark.

RESULTS AND DISCUSSION

Development of the chromatographic method

The LC method was developed using a Zorbax C_8 column. A mobile phase containing CH_3CN, phosphoric acid and water was examined first. It was observed that the addition of quaternary ammonium ions improved both efficiency and symmetry. Tetrabutylammonium (TBA) gave better results than tetramethylammonium and therefore TBA was used in all further experiments. TBA masks the residual silanol activity and acts as a competing analyte [17,181. This causes the tylosin components not only to be better separated but also eluted faster. The influence of the pH of the mobile phase was investigated by replacing the 0.2 *M* phosphoric acid with 0.2 *M* phosphate buffers of pH up to 4.0. Increasing the pH caused an increase in retention times and symmetry factors and a decrease in resolution. The use of 0.2 *M* sulphuric acid gave results similar to those obtained with 0.2 *M* phosphoric acid. The latter was chosen for further work.

The influence of the amount of TBA in the mo-

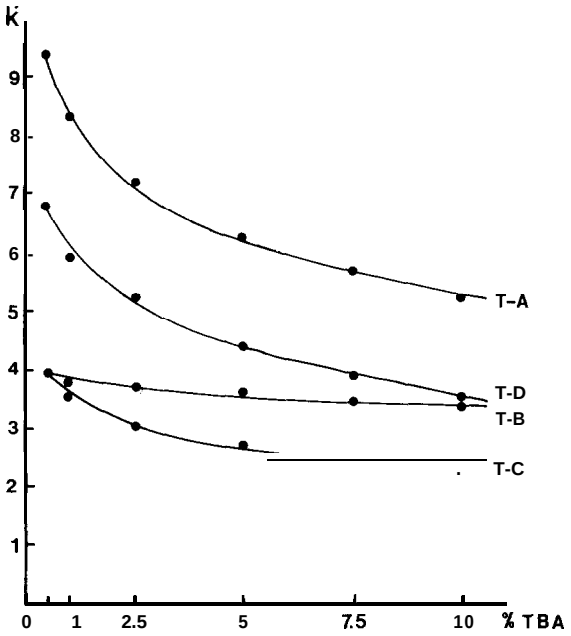


Fig. 2. Influence of the concentration (%, v/v) of 0.2 A4 TBA in the mobile phase on the separation of tylosin A, B, C. D. Column, Zorbax C_8 (8 μm); mobile phase, CH_3CN–0.2 M TBAO.2 M phosphoric acid-water [23:x:5:(72 – x)].

bile phase is shown in Fig. 2. The retention times decrease with increasing TBA concentration. The best resolution was obtained between 5 and 8% TBA. An amount of 5% TBA was chosen for further work. The influence of the organic modifier was also investigated. The capacity factors are reported in Table I. The amount of organic modifier was adjusted to obtain comparable retention times or to improve the separation. The best selectivity and symmetry were obtained with CH_3CN, which was chosen for further work.

In order to examine the general applicability of the mobile phase thus developed, a sample containing tylosin A, B, C, D and some minor impurities was analysed on columns packed with different C_8 and C_{18} stationary phases. The capacity factors for tylosin A, B, C and D are given in Table II. The elution order was the same on all the columns examined. The selectivity towards minor impurities, eluted before TC and immediately before and after TA, was similar. It was also observed that the retention was weaker on the older Zorbax column, but the selectivity was similar. A more important influence of column age was reported previously for the LC of erythromycin [19]. In a series of separate experiments it was observed that the presence of TBA improves the reproducibility of the selectivity on the different columns. In the presence of TBA there is less difference between C_8 and C_{18} materials. The separation between major (TA, TB, TC, TD) and minor components can be improved also by variation of the 0.2 M TBA content in the mobile phase between 3 and 8% (v/v). Throughout the entire study a column temperature of 30°C was maintained to improve the uniformity of working conditions. Another temperature between 20 and 35°C may be chosen with no effect on the selectivity.

Fig. 3 shows the typical chromatogram accompanying the BP-CRS batch 1226 and obtained with the BP Vet method. TAD is an impurity called aldol impurity. Fig. 4 shows a typical chromatogram of the same substance obtained with the proposed

TABLE I

INFLUENCE OF THE ORGANIC MODIFIER ON THE CAPACITY FACTORS OF TYLOSIN A. B, C AND D

Column, Zorbax C_8; mobile phase, organic modifierq.2 M TBA-0.2 M phosphoric acid-water [x:5:5:(90 – x), v/v].

Organic modifier	x (%, v/v)	k'			
		TC	TB	TD	TA
Methanol	40	3.7	4.5	6.8	6.7
2-Propanol	17	4.8	6.1	9.1	9.4
2-Ethoxyethanol	27.5	4.2	5.0	6.6	7.5
Tetrahydrofuran	17.5	3.1	3.8	4.9	6.2
Tetrahydrofuran + acetonitrile	8.75 + 11.5	3.1	3.8	5.1	6.5
Acetonitrile	23	3.0	3.8	4.7	6.6

TABLE II

CAPACITY FACTORS OF TYLOSIN A, B, C AND D ON DIFFERENT COLUMNS

Mobile phase, CH_3CN–0.2 *M* TBA-0.2 A4 phosphoric acid-water [x:5:5:(90 − x), v/v].

Stationary phase	Particle size (μm)	*k'*				x
		TC	TB	TD	TA	
Zorbax BP-C, (I)	8	3.0	3.8	4.1	6.6	23
Zorbax BP-C, (II)	8	2.8	3.1	4.4	5.3	21
Partisil C_8	10	2.2	2.4	3.3	4.0	20
RoSil C_8	8	3.1	3.1	4.8	6.7	22
Partisil ODS	10	4.4	4.5	7.1	7.8	20
RSil C_{18} LL	10	2.9	3.4	4.6	5.9	23
RSil C_{18} HL	10	3.2	4.2	5.4	1.7	23
Spherisorb ODS 1	5	3.0	3.5	4.8	6.3	23
Spherisorb ODS 1	10	3.3	3.8	5.3	7.0	23
Spherisorb ODS 2	5	2.3	3.0	3.8	5.0	23
Spherisorb ODS 2	10	3.1	4.8	6.3	8.8	21
Nucleosil ODS	10	2.9	3.8	5.0	7.1	23
Hypersil ODS	5	2.5	3.1	4.2	6.1	23

[a] I = New column; II = old column.

method. The general elution pattern obtained with both methods is very similar, but the selectivity towards minor impurities is better with the proposed method. The major advantage of the new method, however, is the better reproducibility of the selectivity on different stationary phases. To verify the selectivity a system suitability test was needed. It was observed that a resolution of at least 4.0 between TA and TB and a symmetry factor of less than 1.5 for TA guaranteed a sufficient separation on all the columns examined. TB was chosen for the resolution test because it can be prepared easily from TA by acid degradation. TD and TAD are not commercially available, nor can they be obtained by easy to perform chemical reactions. Using purified TA it was checked that no TB was formed in the column during analysis.

Calibration graphs and repeatability

Quantitative work was carried out on a RoSil C_8 column. Calibration graphs were obtained with the house standards for TA, TB, TC and TD. The following relationships were found, where y = peak area, x = amount of base injected in micrograms,

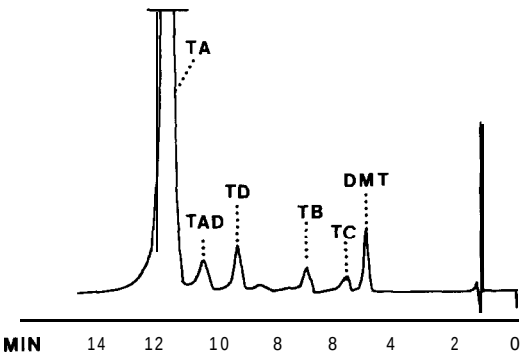


Fig. 3. Typical chromatogram accompanying the BP-CRS batch 1226 and obtained with the BP Vet method.

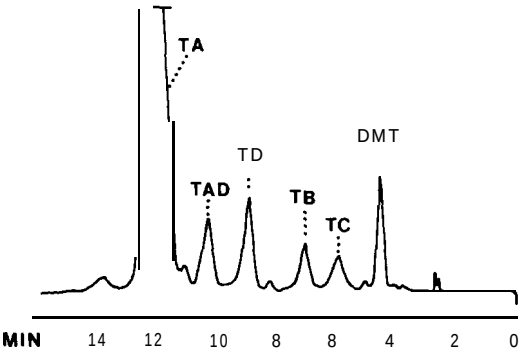


Fig. 4. Chromatogram of the BP-CRS batch 1226 obtained with the proposed method. Column, Hypersil C_{18}; mobile phase, CH_3CN–0.2 *M* TBA–0.2 *M* phosphoric acid-water (23:3:5:69).

n = number of analyses, r = correlation coefficient, $S_{y,x}$ = standard error of estimate and R = range of injected mass examined: TA, $y = 67 + 8913x$, $n = 10$, $r = 0.9989$, $S_{y,x} = 61$, R = 12–24 μg; TB, $y = 120 + 9009x$, $n = 10$, $r = 0.9996$, $S_{y,x} = 150$, R = 0.1–0.35 μg; TC, $y = 16 + 8698x$, $n = 10$, $r = 0.9999$, $S_{y,x} = 17$, R = 0.1-3 μg; TD, $y = 88 + 8961x$, $n = 10$, $r = 0.9998$, $S_{y,x} = 72$, r = 0.1–2 μg. The limit of quantification was 0.05%. When the house standard was analysed ten times over a period of 4 days, the relative standard deviation (R.S.D.) was 0.6%.

Comparison of tylosin standards

The composition of the tylosin A house standard was determined as follows. The total base content expressed as TA was determined by titration with perchloric acid in non-aqueous conditions, using acetic acid as the solvent. The mean result was 91.1% ***(n = 4,*** R.S.D. = 0.4%). The watercontent (9%) was determined by Karl Fischer titration ***(n = 5,*** R.S.D. = 2%). The total mass was therefore accepted to be explained by tylosin bases and water. The chromatographic purity was determined using the proposed method. As the values of the specific absorbance for the different tylosins must be very close, as can be concluded from the similarity of the slopes of the calibration graphs and as only small amounts of impurities were present, their content was expressed as TA. The content of the TA house standard was therefore accepted to be 100 − (9.0 + 1.4 + 0.4) = 89.2%.

Using this TA house standard, the standards were compared by the proposed method and the results are reported in Table III. Titrations were not carried out on the standards, owing to the limited amount available. The deviation between the total mass explained and 100% may be due to the presence of solvents in the WHO and Lilly standards. The WHO standard was dried over phosphorus pentoxide before being sealed in ampoules and it is possible that the very hygroscopic sample took up some water during the packing procedure and/or after the opening of the ampoule. The BP CRS content is very low, probably because it is a tartrate salt. It should be emphasized that this CRS is not used for quantitative work. The content of the Lilly standard exceeds the theoretical amount of 1000 μg/mg because the micrograms concerned have to be interpreted as micrograms of activity, determined by microbiological assay against a standard, and not as micrograms of mass. This is confirmed by the deviation between declared content and that found by LC. This situation can be a source of confusion [20].

TABLE III

COMPOSITION OF TY LOSIN STANDARDS

Values in percent (w/w) in terms of the base; relative standard deviations (R.S.D., %) are given in parentheses.

Component	House standard TA (n = IO)	WHO (1000 I.U./mg) (n = 16)[a]	BP-CRS (n = 3)[a]	E. Lilly house standard (1073 μg/mg) (n = 7)	E. Lilly tylosin A (n = 5)
DMT	1.4 (5.8)	0.3 (15)	2.6 (8.3)	0.3 (14)	0.1 (6.2)
TC	< 0.05	0.2 (9.3)	1.4 (1.6)	5.8 (5.8)	0.1 (8.9)
TB	< 0.05	0.2 (15)	1.6 (9.4)	0.2 (12)	0.3 (23)
TD	0.4 (31)	2.5 (3.0)	3.9 (3.8)	2.9 (1.4)	0.6 (13)
TAD	10.05	< 0.05	2.8 (3.6)	< 0.05	< 0.05
TA	89.2 (0.6)	93.0 (0.5)	73.4 (0.9)	86.0 (0.8)	96.6 (0.9)
Others	< 0.05	1.0 (9.9)	0.1	< 0.05	0.3 (3.6)
Subtotal	91.0	97.2	85.8	95.2	98.0
Water[b]	9.0 (2.0) (n = 5)[a]	0.0[c]	ND[d]	ND	ND

[a] Number of analyses.
[b] Karl Fischer.
[c] As mentioned on the label.
[d] ND = Not determined owing to the limited amount of sample.

TABLE IV

COMPOSITION OF BULK SAMPLES OF TYLOSIN

Results are expressed in % (w/w). Tylosins and related substances arc calculated as tylosin A. Water was determined by Karl Fischer titration.

Sample origin and number	n^a	TA		DMT	TC	TB	TD	Others	Water			Total
		Mean	R.S.D. (%)						Mean	*n*	R.S.D. (%)	
A1	4	83.5	0.2	0.5	0.4	1.6	2.8	0.1	3.3	2	0.9	92.2
A2	4	81.8	0.4	1.0	0.7	1.0	4.2	0.4	4.6	2	3.3	93.7
A3	4	80.6	0.8	0.5	0.4	1.4	6.1	0.2	5.8	2	0.7	95.0
A4	4	88.0	0.4	0.5	0.7	0.9	3.1	0.4	5.4	4	4.3	99.6
A5	4	89.5	0.6	0.2	0.2	0.4	1.8	0.6	3.4	4	1.7	96.1
B1	5	90.7	0.7	0.2	1.5	0.3	4.6	0.1	2.0	5	0.6	99.4
B2	5	85.9	0.5	0.4	2.6	0.5	1.5	0.1	2.2	5	2.2	99.2
B3	8	81.2	1.4	1.0	3.4	0.3	3.2	0.4	2.3	3	3.8	91.8
B4	4	86.7	0.6	2.1	1.4	0.7	2.6	0.7	2.3	3	1.3	96.5
B5	5	86.5	0.6	1.8	1.2	0.9	2.9	<0.1	2.4	3	3.1	95.7

[a] Number of analyses.

Analysis of commercial samples

Commercial samples were analysed as described above for the standards. Table IV gives the results for tylosin bulk samples. For simplicity, R.S.D. values are not mentioned for impurities. The repeatability for the TA assay is good. The major part of the impurities is explained by DMT, TC, TB and TD, which is the major impurity. Most of the samples also contain small amounts of other related substances. The water content varied from 2.2% to 5.8%. The total mass explained ranges from 91.8% to 99.6%. It is probable that many of these samples contain salts which are not detected by LC.

Table V gives the results for tylosin tartrate sam-

TABLE V

COMPOSITION OF BULK SAMPLES OF TYLOSIN TARTRATE

Results are expressed in % (w/w). Tylosins and related substances are calculated as tylosin A base. Pure TA tartrate contains 92.4% of TA. Water was determined by Karl Fischer titration.

Sample origin and number	n^a	TA		DMT	TC	TB	TD	Others	Total tylosins as tartrate	Water			Total
		Mean	R.S.D. (%)							Mean	*n*	R.S.D. (%)	
A4	3	67.9	0.6	2.2	0.5	8.0	2.3	<0.1	87.6	5.9	4	2.6	93.5
A5	4	78.1	0.8	0.2	0.2	2.0	2.5	0.1	89.9	3.5	4	4.0	93.4
A6	4	78.7	0.1	0.1	0.3	1.7	2.7	0.1	90.5	3.5	3	1.3	94.0
B4	6	69.1	0.6	0.4	1.2	3.4	3.7	<0.1	84.2	4.8	3	2.7	89.0
B5	5	68.9	0.4	0.9	1.2	3.5	3.4	<0.1	84.3	4.8	3	4.9	89.1
B6	4	70.3	0.4	0.2	0.7	4.5	3.3	<0.1	85.5	5.0	6	4.9	90.5
C1	3	68.9	0.1	0.2	2.1	1.2	9.5	0.2	88.9	1.6	4	4.4	90.5
c2	3	73.4	0.3	0.2	0.6	1.1	8.4	0.2	90.8	1.7	5	2.8	92.5
c3	3	69.8	0.9	0.2	3.8	0.9	7.7	0.5	89.7	1.5	6	3.8	91.2

[a] Number of analyses

ples. The same impurities are present, but the total amount is higher than for the base samples. The TB content is higher than in the base samples. TB can be formed from TA by acid hydrolysis. The sum of tylosins and related substances is calculated as the tartrate salt. The water content varies from 1.5% to 5.9%. The total mass explained ranges from 89.9% to 94.0%, which is much lower than for the base samples. The low content of these samples is due to impurities which are not detected by LC. This is reflected by the sulphated ash values, which are higher for the tartrate salts than for the base samples.

CONCLUSIONS

The results obtained have shown that the LC method described is suitable for the analysis of tylosin and its tartrate salt. Advantages over existing methods are the less corrosive chromatographic conditions, a better selectivity and, above all, a better reproducibility on different stationary phases.

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