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APPLICATION OF QUANTITATIVE HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY IN THE ANTIBIOTIC INDUSTRY

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SUMMARY

For the in-process control of antibiotic fermentations and for routine assays of samples for scaling up and for pure products, quantitative high-performance thin-layer chromatography (HPTLC) can be used with advantage. Rapid chromatography on high-performance layers, combined with an automatic spraying device for exact derivatization on the plate and precise computation of the calibration line within an automatic measurement and evaluation, represents a new, inexpensive analysis system. There are only 2 min of labour time (one fifth of that required in thin-layer chromatography) required for one sample and the total analysis time varies from 3 to 9 min (one third to one quarter of that required in thin-layer chromatography) based on one plate with 12 samples. The 95% confidence limits ($N = 10$) range between 0.5 and 3.0%.

INTRODUCTION

For controlling antibiotic fermentations in small and large fermenters, for analyzing samples from flask fermentations, for evaluation of mutants, for optimizing fermentation liquids and for analyzing pure fermentation products chromatographic methods are needed. Of the methods available, high-performance liquid-chromatography (HPLC) and thin-layer chromatography (TLC) are preferred. For analytical control of fermentations, not only the antibiotic to be produced but also other components of the fermentation liquids, such as precursors, sugars and constituents of the biomass (for example, ergosterol), are of interest. According to the fermentation process and the composition of the fermentation liquid, different metabolites have to be determined in a matrix that frequently varies because the starting materials for the fermentation liquids may have to be changed within a short time period owing to alterations in the prices of the raw materials. The analyst has to cope with such changes with maximum flexibility as far as the available methods of analysis are concerned. To connect complicated analysers directly to fermenters is not advisable because of their high cost and because of the possible changes mentioned above, with some special exceptions. The centralized analysis of samples of different origins is preferable. The decision between using TLC or HPLC for the specific substances to be determined depends on their advantages and disadvantages in particular instances.

For many problems in the antibiotic industry, quantitative TLC is an efficient method. The dissolution of chromatography and detection offers the possibility of analyzing various substances from different fermentations in any sequence with one scanner, which is an important advantage in production processes.

The application of quantitative HPTLC was described recently for the first time[1,2]. In contrast to TLC, HPTLC offers the following advantages in routine assay:

(1) three times more samples can be applied on one plate;

(2) the separation is effected 4–5 times faster;

(3) the chromatography gives such symmetrical results that the spots can be measured at right-angles to the direction of separation;

(4) each plate can be used on both sides if the migration distance is not longer than 3 cm.

APPLICATION

For the manual application of solutions, platinum–iridium capillaries[3] (*ca.* 7 mm long, I.D. 0.25 mm, volume *ca.* 250 nl), fixed to an iron rod, have proved successful. The capillary is rinsed three times with the next sample each time before use. The application is carried out in the following sequence ("data-pair technique")[4]: P1, P2, P3, S1, P4, P5, P6, S2, P7, P8, P9, S3, P10, P11, P12, P1, P2, P3, S1, P4, P5, P6, S2, P7, P8, P9, S3, P10, P11, P12, where P1–P12 are samples and S1–S3 are standards.

DERIVATIZATION

All substances that do not have sufficient specific colour or UV absorption and which cannot be excited so as to produce fluorescence must be made visible by spraying a reagent on to the thin-layer plate. This is done with an automatic spraying device[5] (according to Kreuzig, produced by Anton Paar KG, Graz, Austria), so that former inaccurate results[6] from manual spraying are avoided. The determination of gramicidin (spraying with 4-dimethylaminobenzaldehyde) shows that the results obtained in UV and after derivatization (Tables II and III) are of the same precision. Visible spots facilitate adjustment of the plate before measurement.

MEASUREMENT AND AUTOMATED EVALUATION

A Zeiss Chromatogrammspektralphotometer and Merck HPTLC plates were used. Measurements were made at right-angles to the direction of separation with a speed of 50 mm/min to and fro. This means a measuring time of 8 min, or 10 min including adjusting and printing for 12 samples. The peaks are integrated (Perkin-Elmer SIP 1) and the integrated values transmitted by an interface, which directs the start and return of the plate table, to a computer (DCS 116, 16 bits, 32 kW), which computes the results by means of the calibration line and prints them at the terminal. The program is written in Timesharing Basic.

The computation of the calibration line is not done by the gaussian least-squares method but by the percentage method[7], which minimizes the squares of the relative errors of X-values. Hence samples of low concentrations, which can be read

on the lower range of the calibration line, are analyzed more exactly than by the gaussian method, when the analytical conditions are not optimal (inconstant temperature, end of capillary not face-ground; see Table I). The quality of the calibration line is not indicated by the correlation coefficient, but by the quality cofficient (g):

$$g = \sqrt{\frac{(\%\ \text{deviation})^2}{n-1}}$$

$$\text{Deviation}\ (\%) = \frac{X_{\text{re-calibrated}} - X_{\text{given}}}{X_{\text{given}}} \cdot 100$$

When these coefficients exceed 10%, the analysis is repeated.

The more exactly the chromatography is executed, the lower are the quality coefficients and the variation in the results obtained from both kinds of evaluation (see Tables II and III). The quality coefficients from the gaussian evaluation are always higher than those from the percentage evaluation (Tables I–III), which means that the latter method is more exact. This can be seen by means of the coefficients of var iation with statistical verification.

TABLE I

GRAMICIDIN: QUANTITATIVE HPTLC (METHOD NOT OPTIMIZED) AT 570 nm

A = least-squares method; B = percentage method; r = correlation coefficient; g = quality coefficient (%); S.D. = standard deviation; CV = coefficient of variation; CL = 95% confidence limits.

A		*B*	*Samples (gramicidin content in μg/ml)*							
r	*g*	*g*	*1*		*2*		*3*		*4*	
			A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
0.9912	12.8	8.9	352	448	486	452	952	934	1760	1658
0.9960	11.3	7.9	440	402	542	608	816	976	1624	1644
0.9888	11.3	5.6	380	444	510	560	928	990	1668	1618
0.9796	12.7	7.0	352	462	506	562	936	1060	1512	1688
0.9916	10.5	5.2	380	430	576	560	924	1008	1612	1568
0.9778	13.3	7.7	448	420	606	592	908	976	1700	1572
0.9833	15.7	8.0	452	434	508	610	963	1006	1740	1510
0.9942	8.0	7.0	400	400	562	590	960	922	1692	1608
0.9773	11.4	9.1	450	456	560	576	948	980	1644	1618
0.9882	10.6	6.7	466	440	518	608	944	976	1656	1624
		Average:	412	434	537	581	928	982	1660	1610
		S.D.:	44.0	21.1	35.6	24.2	44.4	38.6	70.6	50.6
		CV (%):	10.7	4.9	6.6	4.2	4.8	3.9	4.2	3.1
		CL (%):	7.6	3.5	4.7	3.0	3.4	2.8	3.0	2.2

The percentage method offers an increase in accuracy of analytical data in routine work, which does not always run only under optimal conditions.

The analysis times in TLC and HPTLC for the analysis of 12 samples of gramicidin and ergosterol are compared in Table IV.

TABLE II

GRAMICIDIN: QUANTITATIVE HPTLC (OPTIMIZED METHOD) AT 281 nm

Symbols as in Table I.

A		*B*	*Samples (gramicidin content in μg/ml)*													
r	*g*	*g*	*1*		*2*		*3*		*4*		*5*		*6*		*7*	
			A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
0.9937	8.8	6.4	383	342	448	411	620	595	890	882	1465	1493	1797	1845	1815	1865
0.9966	6.4	4.6	375	344	442	415	614	595	873	867	1456	1478	1876	1918	1779	1816
0.9901	9.4	7.2	394	354	457	421	613	586	881	871	1378	1397	1782	1825	1722	1762
0.9900	9.9	7.5	402	359	461	422	614	585	877	866	1421	1445	1767	1814	1808	1858
0.9943	8.2	5.9	391	354	448	414	611	587	823	812	1300	1316	1730	1772	1706	1746
0.9957	7.3	5.3	381	348	452	422	613	592	873	866	1417	1438	1812	1853	1822	1864
0.9883	10.2	8.0	393	351	455	417	625	597	854	846	1379	1398	1812	1857	1809	1854
0.9891	11.8	8.8	417	365	470	423	626	591	854	837	1374	1399	1829	1890	1759	1814
0.9909	10.5	7.8	406	360	466	425	618	587	842	827	1332	1351	1841	1897	1840	1896
0.9934	9.1	6.6	387	345	448	410	618	591	873	863	1449	1476	1988	2050	1961	2022
		Average:	393	352	455	418	617	591	865	854	1397	1419	1823	1872	1802	1850
		S.D.:	12.6	7.6	8.9	5.3	5.2	4.2	20.2	22.3	54.4	57.6	70.5	75.7	71.0	76.9
		CV (%):	3.2	2.2	2.0	1.3	0.8	0.7	2.3	2.6	3.4	4.1	3.9	4.1	3.9	4.2
		CL (%):	2.3	1.5	1.4	0.9	0.6	0.5	1.7	1.9	2.8	2.9	2.8	2.9	2.8	3.0

TABLE III

GRAMICIDIN: QUANTITATIVE HPTLC (OPTIMIZED METHOD) AT 570 nm

Symbols as in Table I.

A		*B*	*Samples (gramicidin content in µg/ml)*													
r	*g*	*g*	*1*		*2*		*3*		*4*		*5*		*6*		*7*	
			A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
0.9971	5.1	3.8	331	307	434	414	649	636	917	914	1468	1485	1776	1804	1797	1825
0.9926	6.4	5.5	346	325	432	413	633	620	869	863	1441	1452	1874	1898	1725	1744
0.9976	3.5	2.9	330	317	421	410	611	604	890	888	1404	1412	1756	1770	1798	1813
0.9861	8.9	8.3	333	311	440	420	632	616	899	890	1402	1404	1719	1729	1711	1720
0.9947	4.5	4.3	319	310	415	407	619	613	866	862	1375	1378	1756	1763	1730	1737
0.9946	6.4	6.4	312	312	425	424	630	627	911	906	1431	1423	1766	1755	1690	1680
0.9971	4.8	4.2	326	311	420	406	615	605	867	864	1374	1381	1785	1801	1762	1778
0.9761	8.8	8.4	338	318	442	425	640	626	912	903	1423	1423	1802	1809	1701	1700
0.9989	3.1	2.6	323	310	419	409	622	615	880	878	1377	1384	1814	1829	1802	1817
0.9924	6.2	6.0	306	297	406	398	635	628	891	887	1446	1445	1855	1857	1846	1848
		Average:	326	312	425	413	629	619	890	886	1414	1419	1790	1802	1756	1764
		S.D.:	12.0	7.4	11.5	8.5	11.8	10.4	19.4	18.7	33.0	34.6	47.3	50.7	52.4	3.2
		CV (%):	3.7	2.4	2.7	2.1	1.9	1.7	2.2	2.1	2.3	2.4	2.6	2.8	3.0	3.2
		CL (%):	2.6	1.7	1.9	1.5	1.3	1.2	1.6	1.5	1.7	1.7	1.9	2.0	2.1	2.3

TABLE IV

COMPARISON OF ANALYSIS TIMES (min) FOR 12 SAMPLES

Sample	*Operation*	*TLC (3 plates)*	*HPTLC (1 plate)*
Gramicidin	Application	45	12
	Separation	55 (10 cm)	12 (3 cm)
	Drying	60	30
	Spraying with 4-DMABA	9	3
	Drying	35	35
	Measurement and evaluation	60	10
	Total time	264	102
	Total time per sample	22	8.5
	Manipulation time	114	25
	Manipulation time per sample	10	2
Ergosterol	Application	45	12
	Separation	12 (10 cm)	3 (3 cm)
	Drying	5	5
	Measurement and evaluation	60	10
	Total time	122	30
	Total time per sample	10	2.5
	Manipulation time	105	22
	Manipulation time per sample	9	2

CONTROL OF ERRORS

In order to find errors due to inaccurate adjustment of the plates or incomplete filling or rinsing of the metal capillary (there is no possibility of visual control), the program has been conceived in such a way that, if the peak areas of a data pair differ more than 10%, this is printed on the terminal. The capillaries rarely become obstructed (an average of one obstruction per 200 dosages).

ACCURACY

For the evaluation of the accuracy some samples were analyzed on 10 different plates and the coefficients of variation (CV) and the 95% confidence limits (CL) were computed as follows:

Gramicidin (570 nm): CV = 1.7–3.4%, CL = 1.2–2.3% (see Table III)
Gramicidin (281 nm): CV = 0.7–4.2%, CL = 0.5–3.0% (see Table II)
Ergosterol* (282 nm): CV = 1.2–1.8%, CL = 0.8–1.3%.

* The CL of 5.3% obtained earlier[1] was due to an unsuited platinum–iridium–glass capillary and to the use of non-automated evaluation.

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