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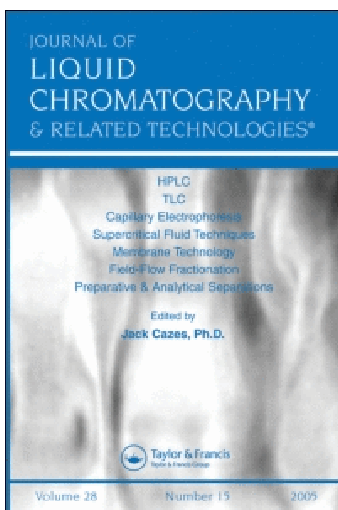

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ROUTINE HPTLC IN INDUSTRIAL ANALYSIS: PROCESS CONTROL OF FERMENTATIONS

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ABSTRACT

Quantitative HPTLC is a cost saving and reliable chromatographic routine method for the control of many fermentation processes, as for example the fermentation of penicillin V. The following substances are analyzed: lactose or sucrose, soya oil (against foaming), phenoxyacetic acid (precursor), penicillin V and p-hydroxypenicillin V, as final products. The derivatization after chromatography is performed with an automated spraying device, the measurement - perpendicularly to the direction of chromatography - and evaluation are computerized. The time requirements per sample range from 6 to 15 minutes, the pure analysis time per sample from 5 to 12 minutes. The break-down time of the complete HPTLC apparatus system is about 0,6 % of the working time, all substances to be determined can be measured with one scanner, in every interval and sequence desired. The accuracy, expressed by the coefficients of variation (N = 8 - 9, on one plate), ranges from 1,6 to 3,0 %, for very low concentrations up to 6,6 %.

INTRODUCTION

Reducing costs is a must in all fields of economy; it is also advisable to look, where analytical costs in research, development and production can be decreased. Especially for process control, additional methodic criteria, besides costs, are of importance, namely: speed, simplicity, reliability and accuracy.

Lecture, presented at the "Second International Symposium on Instrumental HPTLC" (2 - 5 May, 1982, in Interlaken)

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A chromatographic method fulfilling these requirements is quantitative HPTLC, a method, which has already been used for 6 years in many laboratories. This method allows the routine process control of many substances in every sequence with only one measuring instrument.

Industrial fermentations for the production of antibiotics are characterized by the need of information about the concentration of different substances in the fermentation liquid during fermentation in certain intervals: carbohydrates and precursors for the biosynthesis of the antibiotic, oils against foaming and the antibiotic to be produced as well, so that adding different substances, batch by batch or continuously, can be performed in the fermenter if necessary.

An on-line coupling of an HPLC device directly to a fermenter for a continuous and automated analysis would be - from the point of view of automation - an optimum, but contradictions to this concept are the uncertainty of representative sampling, the extraction and dilution steps (1), and the break-down time of HPLC devices. Although quantitative HPTLC can be only particularly automated, this method offers an extremely high reliability. This reliability is a "conditio sine qua non", because the liquid in a fermenter represents an enormous financial value; an incorrect or delayed process control would result in high financial loss. In this paper, the process control of a penicillin V fermentation is presented.

The interesting parameters of a penicillin fermentation are the C-sources lactose or sucrose, vegetable oils, as soya oil for example, the precursor phenoxyacetic acid, and the final product penicillin V and p-hydroxyphenicillin V, a by-product, in low concentration, not desired.

EXPERIMENTAL

The plates used were commercially available HPTLC-plates; samples and standards, dissolved in chloroform, were applied with a Pt-Ir-capillary (250 nm), whilst solutions of water or water/methanol were applied with a CAMAG-Nano Applicator or CAMAG-Nanomat. For chromatography, TLC chambers (from TLC kit Merck no 11622) or twin-through chambers (CAMAG), lined with filter paper, were used.

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The cleaning up of the samples from the penicillin fermenters is done in different ways, according to the biological matrix and the substances to be determined. The separations are performed conventionally in the linear manner over 3 to 5 cm, the derivatizations for several reasons preferably with a special spraying device (2, 3).

The measurement of the spots with a Zeiss-chromatogram-spectrophotometer is performed perpendicular to the direction of chromatography, to and from, the technique of automated evaluation is the same as shown earlier (2), with two exceptions: a balance (for weighing the fermentation broth samples) is coupled to the computer, and a fast integrator (HP 3390 A) is used, so that the speed of scanning can be raised to 100 mm/min. As function for the calibration line that of $area^2$/concentration, sometimes log a/log c have proved; the computation of the calibration line was not done by the least-square method, but by the percentual method, as described earlier (3).

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Determination of lactose/sucrose

Materials: HPTLC plates silicagel 60 F_{254}, 20 x 10 cm, Merck no 5642
CAMAG-Nano Applicator, 230 nl

Standards: lactose and/or sucrose
1 1 mg/ml CH_3OH/H_2O = 1/1
2 2 mg/ml - " -
3 3 mg/ml - " -

Solvent system: 1-butanol/acetic acid/water = 40/50/7,5

Spray reagent: 2 ml aniline + 2 g diphenylamine + 10 ml H_3PO_4 + 88 ml CH_3OH

Samples: The fermentation liquid is diluted with CH_3OH/water = 1/1 and filtered or centrifugated.

Procedure: Samples A - I and standards 1 - 3 are applied as follows:
A B 1 C D 2 E F 3 G H I A B 1 C D 2 E F 3 G H I

The separation (distance 5 cm) takes about 30 minutes. The plate is dried for 30 minutes at 120°C, sprayed automatically with the reagent and placed in a drying

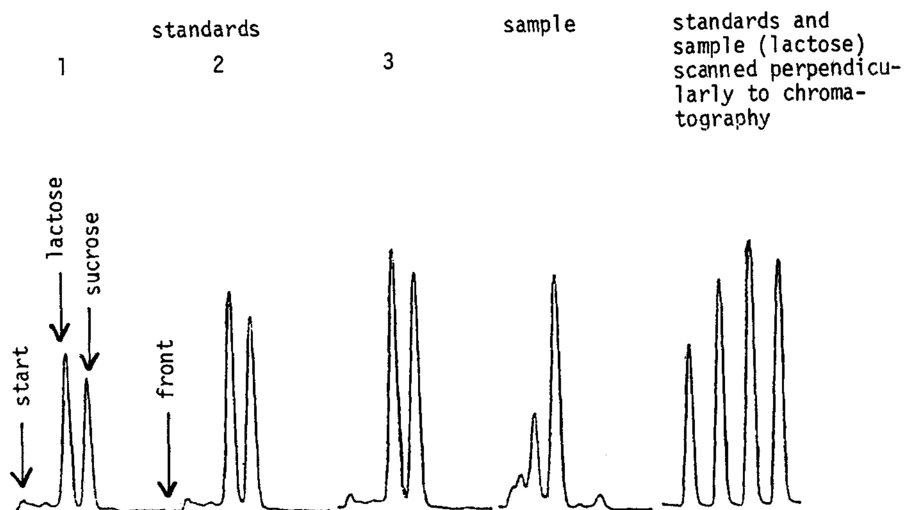

FIGURE 1
Chromatogram of Lactose/Sucrose Separation

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oven for 15 minutes (110°C). Lactose (R_f = 0,16) shows grey-blue spots, sucrose (R_f = 0,26) brown-green spots.

Conditions: M-Pr, A/I/7, λ = 523 nm, remission, slit length 3,5 mm, slit width 0,5 mm, calibration line: a^2/c

Determination of soya oil

Materials: HPTLC plates silica gel 60 F_{254}, 20 x 10 cm, Merck no 5642
Pt-Ir-capillary 250 nl

Standards: soya oil
1 0,6 mg/ml $CHCl_3$
2 1,2 mg/ml - " -
3 1,8 mg/ml - " -

Solvent system: petrol ether/diethyl ether/acetic acid = 135/15/1,5

Samples: The broth is extracted and diluted with $CHCl_3$.

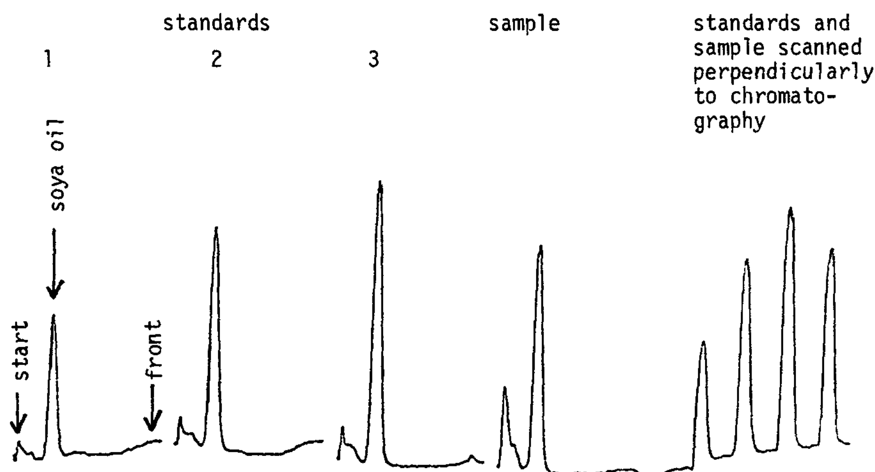

FIGURE 2
Chromatogram of Soya Oil Separation

Spray reagent: 3 g NH_4 molybdate + 25 ml water + 30 ml 0,1 N HCl + 15 ml $HClO_4$ (60 %) + 70 ml CH_3OH

After spraying the plate automatically, it is heated for 2 minutes at 115°C in a drying oven. R_f = 0,16, the spots are blue.

Conditions: M-Pr, A/I/7, λ = 630 nm, simultaneously in transmission and reflectance mode, $f_R : f_T$ = 100 : 20, slit length 6 mm, slit width 1,0 mm, calibration line: a^2/c

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Determination of phenoxyacetic acid

Materials: HPTLC plates silica gel 60 F_{254}, 20 x 10 cm, Merck no 5642

CAMAG-Nano Applicator, 230 nl

Standard: phenoxyacetic acid

1 2 mg/ml water

2 4 mg/ml water

3 6 mg/ml water

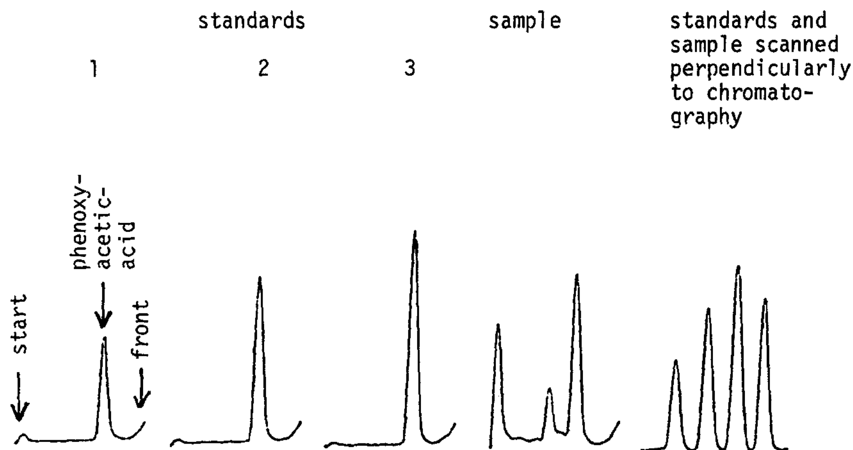

FIGURE 3
Chromatogram of Phenoxyacetic Acid Separation

To raise the solubility of the standard, the water must contain 1 mg $NaHCO_3$/ml.

Solvent system: butylacetate/methylenechloride/acetic acid = 80/10/10

Sample: The fermentation broth is filtered and the filtrate diluted with water.

Procedure: Samples A - I and standards 1 - 3 are applied as follows:
A B 1 C D 2 E F 3 G H I A B 1 C D 2 E F 3 G H I
The separation (distance 5 cm) takes about 7 minutes.
The plate is dried for 30 minutes at 120^{0}C. R_f = 0,51.
Conditions: M-Pr, F/II/1, λ= 268 nm, remission, slit length 3,5 mm, slit width 1,5 mm, calibration line: a^2/c

Determination of penicillin V and p-OH-penicillin V

Materials: HPTLC plates silica gel 60 F_{254}, 20 x 10 cm, Merck no 5642, CAMAG-Nano Applicator, 230 nl

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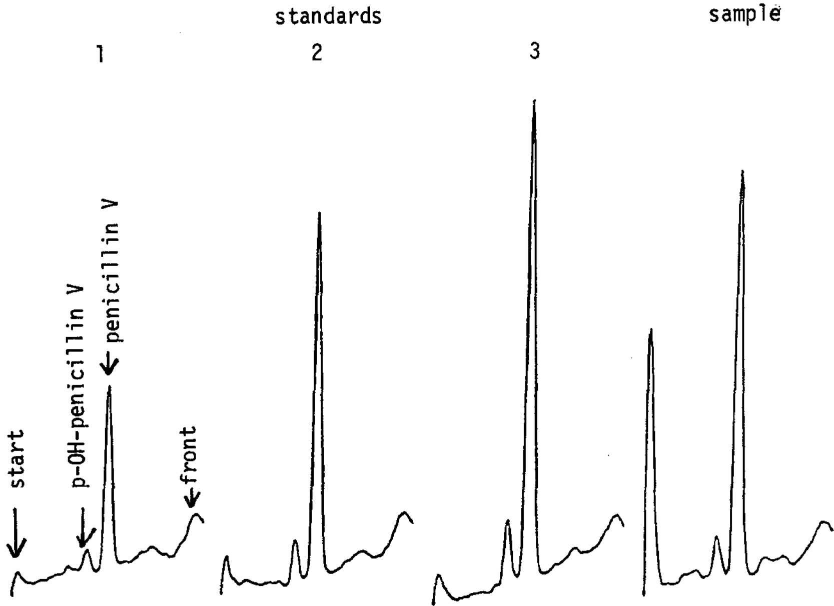

FIGURE 4

Chromatogram of Penicillin V and p-OH-Penicillin V Separation

Standard:	penicillin V, K-salt; p-OH-penicillin V, Na-salt
	1 2 mg penicillin V + 0,2 mg p-OH-penicillin V/ml water
	2 4 mg - " - + 0,4 mg - " -
	3 6 mg - " - + 0,6 mg - " -
Solvent system:	toluene/ethylacetate/acetic acid = 40/40/20
Sample:	1 volume of fermentation liquid + 1 volume of CH_3CN, the precipitate is filtered and the filtrate diluted with water.
Procedure:	The samples A - H and the standards 1 - 3 are applied in the following way: A B 1 C D 2 E F 3 G H A B 1 C D 2 E F 3 G H The separation distance of 5 cm is reached within 9 minutes, the plate is dried for 20 minutes at 120°C.

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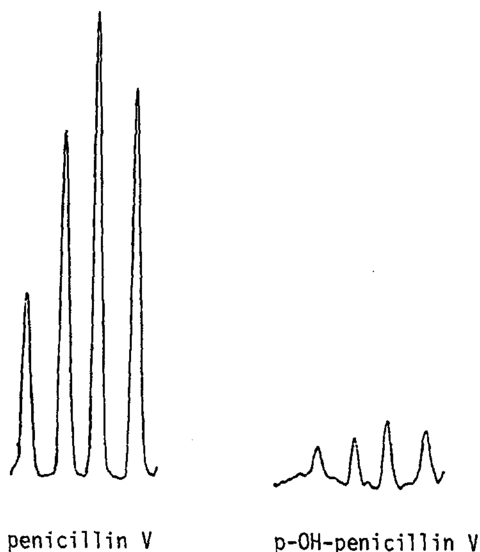

FIGURE 5

Chromatogram of Penicillin V and p-OH-Penicillin V Separation Standards and Sample, scanned perpendicularly to Chromatography

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The R_f-value of penicillin V is 0,44, for p-OH-penicillin V 0,34.

Conditions: M-Pr, F/II/8, λ = 270 nm, remission, slit length 3,5 mm, slit width 1,0 mm, calibration line: log a/log c (for penicillin V)
a^2/c (for p-OH-penicillin V)

RESULTS AND DISCUSSION

The reason for the preference of quantitative HPTLC for routine analyses in antibiotic industry are, of course, the usual, wellknown benefits, as for instance speed (4, 5, 6, 7). But these benefits can be utilized to its fullest extent if chromatography and detection are optimized in such a way that measurement can be done perpendicularly to the direction of chromatography (3, 8), the saving of time is remarkable and besides that Gaussian peaks will result. With this method of mea-

surement a complete analytical system is available which is so fast that it is not displaced by HPLC. This kind of scanning HPTLC plates is practicable if the spot to be measured is separated sufficiently from neighboured spots who will often orginate from the matrix of the samples. The analyst has to select

- an appropriate method to clean up the sample (which should be a simple one-step method)
- and/or a special solvent
- and/or a selective derivatization reagent.

The optimal combination of these possibilities will always offer a proper solution of problems. It it is necessary to scan in the direction of chromatography, an automated system should be used (9).

The following table illustrates the speed of analyses:

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TABLE 1

Time Requirement (min) for preparing and analyzing 8 - 9 Samples.

substance	preparation (A)	analysis (B)	(A)+(B)	(A)+(B) / sample	(B) / sample
lactose or sucrose	25	96	121	15	12
soya oil	10	38	48	6	5
phenoxy-acetic acid	10	55	65	7	6
penicillin V + p-OH-penicillin V	30	53	83	10	7

The slowest method is that for the determination of lactose/sucrose, because the solvent is rather viscous. On the other hand, separation with HPLC will take about 25 minutes per sample.

Simplicity and reliability depend on each other. Quantitative HPTLC is, in comparison to other chromatographic methods, not a sophisticated one and can be adapted quickly to various scanning conditions, the results can be given without delay at a distinct time.

In the last two years we could reduce the break-down time (for instruments for sample application, scanner, integrator, computer, printer) to about 0,6 % of the working time. For scanning, the presence of smoke, dust and vapor of aromatic solvents must be strictly avoided.

For evaluation of the accuracy of the quantitative determination of different substances one sample was analyzed 8 to 9 times on one plate and the coefficients of variation computed as follows:

TABLE 2
Accuracy of quantitative Determination

substance	coefficient of variation (%)	N
lactose/sucrose	1,6	9
soya oil	3,0	8
phenoxyacetic acid	2,1	9
penicillin V	1,9	8
p-OH-penicillin V	6,6	8

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The value of 6,6 % is due to the low concentration of p-OH-penicillin V.

Typical quality coefficients of calibration lines are shown in table 3.

TABLE 3
Quality Coefficients of Calibration Lines

substance	quality coefficient (%)	calibration-line
lactose/sucrose	3,8	a^2/c
soya oil	4,4	a^2/c
phenoxyacetic acid	1,9	a^2/c
penicillin V	1,8	log a/log c
p-OH-penicillin V	7,5	a^2/c

These coefficients are stored on the disc of the computer and printed out on request, arranged according to substance. This information is of importance in controlling the quality of analytical work.

If one spot, standard 2, is scanned ten times, the following coefficients of variation were found:

TABLE 4
Accuracy for Scanning one Spot

substance	coefficient of variation (%)
lactose/sucrose	0,4
soya oil	0,6
phenoxyacetic acid	1,0
penicillin V	0,9
p-OH-penicillin V	2,7

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These measurements may signal the analyst, if for example the spot shape should be further optimized.

Quantitative HPTLC is the cheapest chromatographic method. The more different substances have to be analyzed with the same priority - and this is typical for process control of fermentation - the more the superiority of this method is obvious. Furthermore the scanner can be used beneath routine analyses for working out and optimizing HPTLC-methods.

If these process control analyses for the determination of penicillin V would be performed with HPLC, there would be three complete liquid chromatographs necessary.

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