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A Collaborative Study of

HPLC METHODS FOR THE DETERMINATION OF PATULIN IN APPLE JUICE

Prepared for publication by

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A collaborative study of HPLC (high performance liquid chromatography) methods for the determination of patulin in apple juice

Abstract - Two HPLC methods for the determination of patulin in apple juice were collaboratively tested in 12 laboratories from 10 countries. The collaborators themselves spiked the previously pasteurized apple juice samples with patulin standard solution before analysis. Two samples at three levels of fortification and one blank sample were analyzed for each tested method. Although both of the methods were based on reversed phase HPLC they employed different clean-up procedures: partitioning extraction and column chromatography. Mean recoveries of patulin ranged from 77 to 85 % and mean coefficients of variation were 7.3 % and 15 % for the two methods respectively.

INTRODUCTION

Oswald et al. (ref. 1) found no tumorigenicity when patulin was administered orally to mice and rats; nevertheless there is considerable interest in this mycotoxin for two reasons:

1 Patulin is toxic and produces tumors in rats at the place of injection when injected subcutaneously (ref. 2).

2 Patulin is a good quality indicator of fruits used in the processing of apple juice, since the patulin concentration is reduced by only about 20 % during processing (ref. 3).

The absence of carcinogenicity of patulin resulted in a lack of regulatory action in most countries, although Sweden, Norway and Switzerland have action levels of 50 $\mu g.L^{-1}$ of juice (ref. 4).

The most widely used quantitative tools for patulin determination have been TLC and, more recently, HPLC (ref. 5). Scott (ref. 6) organized and described results of a collaborative study of a TLC method for the determination of patulin in apple juice. The method has been adopted by the A.O.A.C. as an official first action method for the semiquantitative analysis of patulin in apple juice. Although TLC methods predominated in the early seventies they later gave way to those based on HPLC. The following four reasons were responsible for this:

1 TLC is tedious and time consuming.

2 Confirmation is needed because of poor resolution from coextractants, especially from 5-hydroxymethylfurfural (HMF).

3 The methods provide semiquantitative results.

4 The methods are not sufficiently sensitive (20 $\mu g.L^{-1}$, ref. 7).

For these reasons and because of recent advances in HPLC technology, HPLC soon became not only an attractive alternative to conventional TLC or GC methods but is at present the method of choice for the determination of patulin in food products. Therefore it has been decided by the IUPAC Commission on Food Chemistry to establish an internationally recommended method of analysis for patulin based on HPLC. Two analytical methods were selected for the collaborative study. Although both of them are based on reversed phase HPLC they employ entirely different clean-up procedures.

ORGANIZATION OF THE STUDY

Due to the decomposition of patulin in apple juice over the several weeks time period of a collaborative study (ref. 8), and the formation of an interfering substance (HMF) in apple juice after the container was opened (ref. 7), it was decided that the collaborators themselves would spike the previously pasteurized apple juice samples just before analysis.

The participants were each provided with two (one for each method) 125 ml hypo-vials of pasteurized apple juice concentrate, twelve (six for each method) 6 ml hypo-vials containing aqueous spiking solutions of patulin in acetate buffer at pH = 4, two (one for each tested method) 6 ml vials containing acetate buffer solution and one 6 ml vial containing standard solution of patulin in acetate buffer (1.25 $\mu g/ml$). The collaborators were asked to store all the vials in the refrigerator until needed.

Each laboratory was asked first to determine the concentration of the standard solution by means of reversed phase HPLC. Then, to carry out analyses of six samples of apple juice for each method followed by one analysis of sample of acetate buffer. In order to get apple juice ready to be analyzed by the method of Tanner and Zanier 10 g of the concentrate (sample S_1) was diluted to 50 ml with distilled water and seven 5 ml portions were transferred either to centrifuge tubes or to separatory funnels for further analysis. The entire contents of vials A, B, C, D, E, F (containing spiking solutions) were added to six of the above mentioned portions of the apple juice. To reduce the losses of the spiking substance each hypo-vial was rinsed twice with 2.5 ml of ethyl acetate. In all cases both rinsings were also added to the samples followed by partitionong extraction. One sample of diluted apple juice was spiked with the acetate buffer (sample G). For the method of Stray the collaborators were instructed to dilute 100 g of the apple juice concentrate (sample S_2) to 500 ml with distilled water. Seven 50 ml portions of the diluted juice were transferred to individual 250 ml separatory funnels for further analysis. Six of them were fortified with the spiked solutions in vials H, I, K, L, M and N. The acetate buffer sample was added to the last portion of the diluted juice. All the vials containing spiking solutions and buffer acetate were rinsed with two 5 ml portions of ethyl acetate taken from the volume (50 ml) dedicated for the first partitioning extraction. The participants were asked to complete the analyses for each method in one working day.

METHODS

The following two HPLC methods were collaboratively studied on the basis of the previously prepared literature survey (ref. 13):

1 method developed by Stray (ref. 3),

2 method by Tanner and Zanier (ref. 9). This method had been published by the same authors earlier (ref. 10) before it was adopted by the Federal Commission for the Swiss Food Manual as official (1984).

Fig. 1 and Fig. 2 show flow diagrams of the method of Tanner and Zanier and the method of Stray respectively.

The statistical analyses of the results were carried out following guidelines recommended by the International Standards Organization (ref. 11). Additionally, analyses of variances according to Steiner (ref. 12) were also performed.

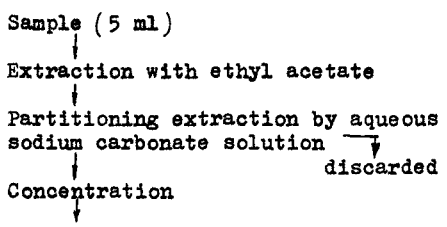

Fig. 1. Schematic representation of Tanner and Zanier procedure

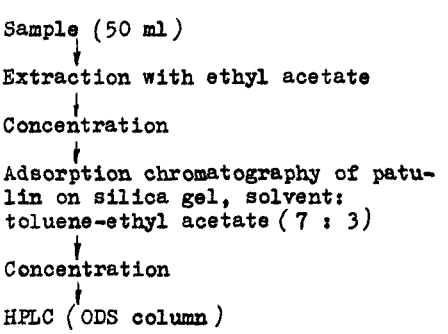

Fig. 2. Schematic representation of Stray procedure

TABLE 1. The collaborative results for the determination of patulin in the standard solution

Lab.	Standard solution (1.25 µg/ml)
1	1.15
2	1.62
3	1.50
4	1.28
5	1.14
6	1.38
7	1.22
8	1.19
9	1.25
10	1.80
11	1.60
12	1.10
Mean	1.35
Standard deviation	0.22
Rel. Std. Dev. (%)	16.8

RESULTS

The collaborators were provided with the samples in July, 1986 and the results were returned by October 15, 1986. Of the 21 laboratories invited to participate in the study, 13 agreed to take part. Finally 12 laboratories from 10 countries submitted results (see Acknowledgments).

From the collaborators results for the concentration of the standard solution sample (ST), determined by reversed phase (ODS column) HPLC, the 1.25 µg/ml solution in acetate buffer (pH = 4) appears to be stable (Table 1). Some laboratories (nos. 2, 3, 10 and 11) failed to reproduce the true concentration with sufficient accuracy ($\leq$ 10 %).

The results obtained by the participants for the concentration of patulin in the spiked apple juice (samples A through F and samples H through N) as well as in the blank apple juice (samples G and O) are tabulated in Table 2 for the method of Tanner and Zanier and in Table 3 for the method of Stray. The samples were spiked in duplicate at three known concentrations; 5, 50 and 250 $\mu g.L^{-1}$. The first spiking concentration was either at the limit of detection (method of Tanner and Zanier) or very close to it (method of Stray, limit of detection = 1 $\mu g.L^{-1}$). Most of the findings obtained for these samples indicate that the limits of detection reported by the authors of the methods are beyond the reach of most laboratories. Because of the presence of interfering substances in extracts as analyzed by HPLC the real limits of detection are between 10 and 20 $ug.L^{-1}$. Occassionally false positives estimated to be at the level of 10 $\mu g.L^{-1}$ were recorded for the blank samples G and O.

It is easily seen from a cursory examination of the data in Table 2 and Table 3 that some laboratories are outliers. The results provided by the laboratories nos. 5, 8, 10 and 11 for the method of Tanner and Zanier and the laboratories nos. 3, 4 and 11 for the method of Stray deviate so much from comparable entries from other laboratories that they may be considered as irreconcilable with the other data without applying Dixon's outlier test. It was apparent that these laboratories did not have the methods under control. Additional enquiry sent to the participants revealed that all the laboratories except one identified as outliers had never used the methods before. In most cases poor HPLC resolution of patulin from concurrent interfering substances was responsible for the erroneous results. An apple juice concentrate relatively rich in interfering substances was deliberately selected for this study. The rejected outliers in Table 2 and in Table 3 are put in parenthesis.

Average recoveries obtained by the collaborators for the concentration of patulin in the spiked apple juice were 85 % (samples B and E) and 83 % (samples C and F) for the method of Tanner and Zanier and 80 % (samples I and M) and 77 % (samples K and N) for the method of Stray for the spiking levels 50 $\mu g.L^{-1}$ and 250 $\mu g.L^{-1}$ respectively. The differences between the mean recoveries found for both the spiking levels and the methods were not statistically distinguishable (t - test).

The results showed the low variation anticipated for a method that is based on HPLC compared with TLC (ref. 6). Coefficients of variation were 7.5 % (samples B and E) and

TABLE 2. Results reported by participants (method by Tanner and Zanier)

Lab.	5 $\mu g.L^{-1}$		50 $\mu g.L^{-1}$		250 $\mu g.L^{-1}$		0
	A	D	B	E	C	F	G
1	2	4	40	37	203	201	Trace
2	Results not reported						
3	5.8	3.9	42.4	36.6	177.3	202.3	2.0
4	26.7	n.d.	40.0	46.7	186.2	179.3	n.d.
5	n.d.	n.d.	(n.d.)	(n.d.)	(25)	(360)	n.d.
6	20	20	49.2	47.2	209.2	246.2	20
7	8.9	n.d.	45.6	50.8	218.4	217.1	n.d.
8	10	20	(10)	(interf.)	(interf.)	(208)	10
9	10	5	40	35	220	250	5
10	91	580	(235)	(139)	(471)	(266)	115
11	Destroyed	-	(1500)	(1400)	(-)	(1900)	1300

( ) rejected outliers

7.2 (samples C and F) for the method of Tanner and Zanier and 12 % (samples I and M) and 18 % (samples K and N) for the method of Stray for the spiking levels of 50 $\mu g.L^{-1}$ and 250 $\mu g.L^{-1}$ respectively. The mean coefficients of variation were 7.3 % for the method of Tanner and Zanier and 15 % for the method of Stray. Calculation of within and between-laboratory components of total variances (ref.12) for the two sample sets (spiking levels) for each method revealed the large random error contribution to the total variance (Table 4 and Table 5). F-ratios S_d^2/S_r^2 gave no evidence for the presence of significant systematic errors among the laboratories. The values of r (repeatability) and R (reproducibility) computed according to the ISO guidelines are shown in Table 6. They mean that the difference between two single determinations found either in one laboratory or in two different laboratories on identical test material will exceed the repeatability (r) or reproducibility (R) respectively not more than in 5 % of the cases (95 % probability).

TABLE 3. Results reported by participants (method by Stray)

Lab.	5 $\mu g.L^{-1}$		50 $\mu g.L^{-1}$		250 $\mu g.L^{-1}$		0
	H	L	I	M	K	N	O
1	n.d.	n.d.	31	Trace	162	133	12
2	6.0	n.m	40.8	31.2	211.4	145.4	0.4
3	10	10	(33.9)	(-)	(124.3)	(76.7)	10
4	n.d.	n.d.	(16.7)	(8.3)	(86.4)	(65.0)	n.d.
5	n.d.	n.d.	23	30	163	150	n.d.
6	10	14.8	36.9	47.6	199.2	270.6	10
7			Results not reported				
8	10	10	47.2	45.7	220.6	138.0	10
9	5	15	50	45	250	250	5
10	49	54	n.a.	48	203	160	16
11	Destroyad	1770	(1410)	(1710)	(1670)	(890)	1480
12	0	0	38.1	40.9	220.6	216.3	0

( ) rejected outliers

TABLE 4. Within-and between-laboratory variances of patulin analyses method of Tanner and Zanier

Samples (spiking level)	Average patulin found ($\mu g.L^{-1}$)	Average recovery (%)	Total		Within-lab.		Between-lab.	
			S_d^2	C.V. (%)	S_r^2	C.V. (%)	S_b^2	C.V. (%)
B, E (50 $\mu g.L^{-1}$)	42.2	80.0	24.2	11.6	10.2	7.56	14.0	8.86
C, F (250 $\mu g.L^{-1}$)	107.0	82.8	512.4	10.9	223.9	7.23	288.5	8.20

TABLE 5. Within-and between-laboratory variances of patulin analyses (method of Stray)

Samples (spiking level)	Average patulin found ($\mu g.L^{-1}$)	Average recovery (%)	Total		Within-lab.		Between-lab.	
			S_d^2	C.V.(%)	S_r^2	C.V.(%)	S_b^2	C.V.(%)
I, M (50 $\mu g.L^{-1}$)	39.7	79.4	71.6	21.3	24.2	12.4	47.4	17.3
K, N (250 $\mu g.L^{-1}$)	193.3	77.3	1967.4	22.9	1197	17.9	770.4	14.3

TABLE 6. Computed values of s.d., r and R

Samples (spiking level)	s.d. (S_r)	r	s.d. (S_b)	R
	Method by Tanner and Zanier			
B, E (50 $\mu g.L^{-1}$)	3.19	8.9	3.74	10.5
C, F (250 $\mu g.L^{-1}$)	14.9	41.9	16.9	47.5
	Method by Stray			
I, M (50 $\mu g.L^{-1}$)	4.91	13.7	6.88	19.3
K, N (250 $\mu g.L^{-1}$)	34.6	96.8	27.7	77.7

s.d. standard deviation; S_r within-laboratory s.d.; S_b between-laboratory s.d.

RECOMMENDATIONS

Both the tested methods for the determination of patulin in apple juice by means of HPLC should be officially recommended by IUPAC.

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