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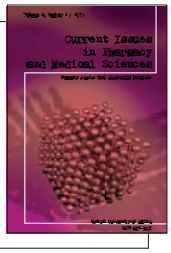

A comparison of the selectivity of nano-HPTLC systems used for determination of patulin in fruit juices from the internet stores and pharmacies

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ABSTRACT

The new nano-HPTLC method was proposed for improved separation of patulin from the admixture of the three naturally occurring natural components of fruit juices, which in last years could be often obtained from the internet functional food stores and pharmacies. Separation of patulin (P) from quercetin (Q), ascorbic acid (A) and 5-hydroxymethylfurfural (H) was performed using three different ready-for-use plates NanoAdamant (NA), Nano-SIL-20 (NSIL) and Nano-Durasil-20 (ND) covered with the non-modified silica gel layer. After development on the 8.0 cm distance, the nano-HPTLC plates were sprayed with 0.5% aqueous solution of the 3-methyl-benzothiazolinone hydrazone hydrochloride hydrate (MBTH). Most satisfactory separation of patulin from the each mentioned compounds was obtained using the Nano-SIL-20 plates and toluene-ethyl acetate-98% formic acid (25:20:5, v/v/v) as the mobile phase. In this best nano-HPLTC method the limit of detection of patulin was near 2.5 times lower (4 ng/spot) in comparison to other used here chromatographic systems. Thus, this optimized nano-HPTLC mode could be used for analysis of the solid-phase extracts of patulin from the fresh apples, clarified apple juices and other fruit products.

Keywords: HPTLC, normal-phase chromatography, patulin, apple juice

INTRODUCTION

Patulin, chemically named as (4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one), is a micotoxin biosythesized by certain genus of fungi classified to the *Penicillium, Aspergillus*, *Byssochlamys* and *Paecilomyces* species (see Table 1) which growing on a fruits as a brown rot [2]. Patulin was recognized as compound indicating a neuro-, immuno- and genotoxic action and probably can be carcinogenic [11]. Patulin has been found in apples, apple juices and tinned apples, tomatoes, apricots, cherries, blackcurrants, quinces and blue cheeses [9,3].

In the EU countries the maximum acceptable concentration of patulin in apple products should not be greater than 50 μg/kg, but in apple products for young children – below 10 μg/kg [2]. Fruits with brown rot do not present direct danger to people because their eating is usually avoided. Unfortunately, such damaged fruits are used in some instances to produce juices and tinned fruits [9]. Most common problem during patulin analysis in apple juices is phenomenon of its coextraction with quercetin (abbreviated as Q), ascorbic acid (A) and 5-hydroxymethylfurfural (H) as the common natural components of these products.

Table 1. Occurrence of specific species of fungi producing patulin.

No	Genus	Species	Ref.
1	*Penicillium*	*P. expansum, P. carneum, P. clavigerum, P. concentricum, P. coprobium, P. dipodomyicola, P. clandicola, P. roqueforti, P. sclerotigenum, P. vulpinum, P. cyclopium, P. chrysogenum, P. cyaneo-falvum, P. brevicompactum, P. crustosum, P. olsonii, P. griseofulvum*	[2,11,14]
2	*Aspergillus*	*A. clavatus, A. giganteus, A. terreus*	[2]
3	*Byssochlamys*	*B. fulva, B. nivea*	[2]
4	*Paecilomyces*	*P. variotii*	[2]

Patulin is still often determined by using the standard thin-layer chromatography (TLC) method. Scott and Kennedy [8] determined patulin, as metabolite of *Penicillium expansum,* in apples with TLC system using the wide pore and high particle size silica gel deposited (layer 0.25-0.30 mm thick) on the glass plates [8]. Recently this method has been modified by Elhariry et al. [2] by using the Whatman TLC aluminum-backed Kieselgel 60 plates and

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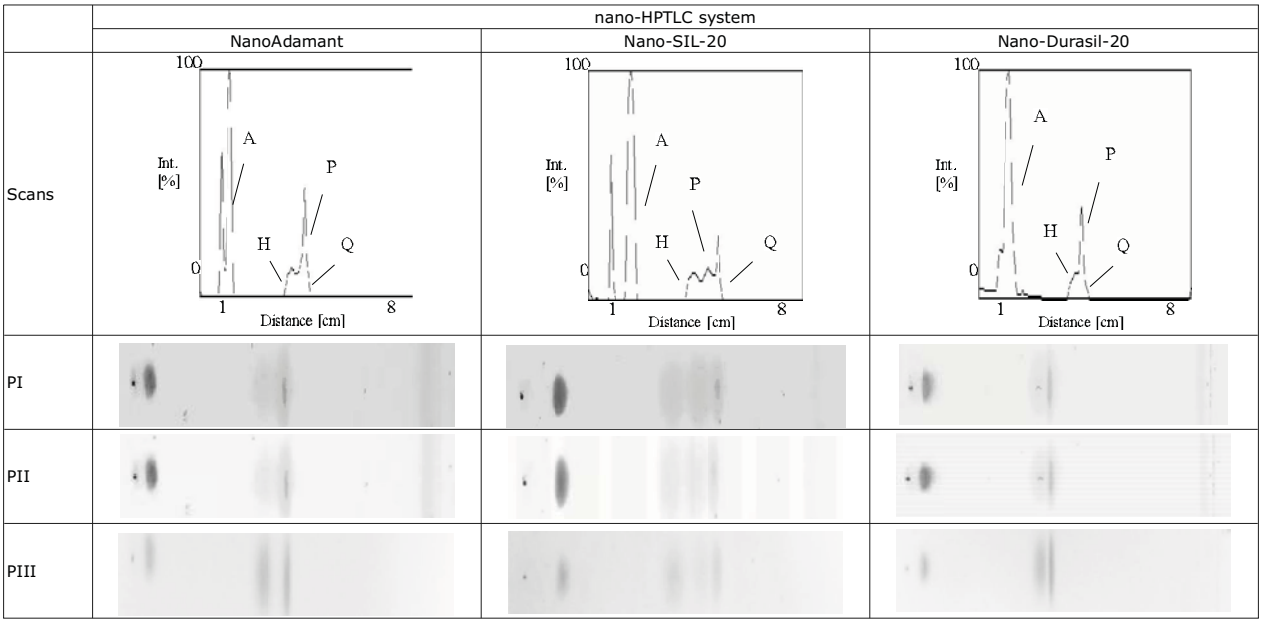
toluene-ethyl acetate-formic acid (25:20:5, v/v/v) as the mobile phase. Martins et al. [6] determined traces of patulin in seven varietes of apples with use of the silica gel based SIL G-25 HR TLC plates from Macherey Nagel [13]. The highest concentration of this mycotoxin (80.5 mg/kg) was identified with this approach in the Richared variety of apples [2]. Welke et al. [14] used analogous TLC method to isolate patulin produced by range of *Penicillium expansum* and *Penicillium griseofulvum* strains from apples during storage. The purpose of our study was to optimize separation conditions of listed above compounds with use of the nano-HPTLC method. The nano-HPTLC systems using identical polar-acidic mobile phase but the three various commercial stationary phases in form of glass plates covered with layers of the low particle size, irregular, unmodified, non-wettable silica gel as the stationary phase have been compared in this study.

rich and prepared at a concentration of 0.5% by dissolving in the freshly deionized water. All these solutions were stored in the temp. of 4°C. Deionized water (0.06 µS/cm) used for analysis was obtained from the HLP Smart 2000 purification system (Hydrolab, Gdańsk, Poland).

TLC analysis. Nano-HPTLC were performed on the 10 × 10 cm silica gel 60 coated glass plates: 1) NanoAdamant (NA) – cat. no. 821140, 2) Nano-SIL-20 (NSIL) – cat. no. 811012 and 3) Nano-Durasil-20 (ND) – cat. no. 812010 (Macherey Nagel, Duren, Germany). These plates possessed 0.2 mm irregular, unmodified, nano-silica gel layers of particles size 2-10 µm, mean pore size 60 Å and specific pore volume 0.75 mL/g. According to the producer data these types of the nano-HPTLC plates differed only by the type of polymeric material which binding silica gel layer with glass support plate [13]. Chromatograms were developed to the distance 8.0 cm in the horizontal teflon-glass chambers DS-II (Chromdes, Lublin, Poland)

Table 2. Typical scans and photos of the nano-HPLTC plates with separation effects of ascorbic acid (A), 5-hydroxymethylfurfural (H), patulin (P) and quercetin (Q). PI – photo after 30 min, PII – photo after 2 hours , PIII – photo after 10 months of storage.



MATERIALS AND METHODS

Chemicals. Patulin (≥ 98%), quercetin dihydrate (≥ 98%) were purchased from Sigma Aldrich (Poznań, Poland) and 5-hydroxymethylfurfural (≥ 98%), L(+)-ascorbic acid (extra pure) were obtained from POCh (Gliwice, Poland). Standard solutions of patulin, quercetin, 5-hydroxymethylfurfural and ascorbic acid were prepared in the HPLC grade methanol (POCh) to obtain a final concentration of 300 µg/mL (patulin – 50 µg/mL). The components of mobile phase: toluene, ethyl acetate and formic acid (98%, extra pure) were from POCh. The 3-methyl-benzothiazolinone hydrazone hydrochloride hydrate (MBTH) used as visualizing reagents was purchased from Sigma Ald-

in the temperature of 20°C with toluene-ethyl acetate-formic acid (25:20:5, v/v/v) as the mobile phase. On the sample application position (1.0 cm from the bottom edge of the HPTLC plate) they were put on 1.0 µL prepared earlier standard solutions (patulin – 50 ng/spot, quercetin, 5-hydroxymethylfurfural and ascorbic acid – 300 ng/spot) with use of Hamilton type 701 (Bonaduz, Switzerland) microsyringe. After development of chromatograms to the distance of 8.0 cm, the HPLTC plates were sprayed with freshly prepared aqueous 0.5% solution of MBTH. After spraying, the HPTLC plates were dried in air by 5 min and next heated for 30 minutes at 100°C. Next, the HPTLC plates were scanned with use of the HP LaserJet 3055 (Hewlett-Packard, Poland) 600 dpi flatbed scanner

in the visible light. The obtained digital images of chromatograms were analyzed densitometrically with use of the ScionImage v. 4.0.2. for Windows software [12].

RESULTS AND DISCUSSION

In Table 2, the typical view of the obtained chromatograms and their scans in the each studied nano-HPTLC system has been shown. The time of chromatogram development was the longest (37 min) on the Nano-SIL-20 (coded as NSIL) plates, but the little shorter (22 min) on the Nano-Durasil-20 (coded as ND) plates as compared to the NanoAdamant (coded as NA) plates where it was equal to 25 min. The mean values of characteristic data (zone colour, dimension and symmetry Φ) for these chromatograms (n = 3 each) have been summarized in Table 3. Densitometric analysis of the HPTLC chromatograms gives objective possibility to calculate retention parameters of each analyte with better precision [7]. For each analyzed compound, the highest zone symmetry Φ was observed on the Nano-SIL-20 plates. Single zones of ascorbic acid (A) and 5-hydroxymethylfurfural (H) was symmetrical and oval (Φ = 1), but the spots of patulin and quercetin were oval in horizontal direction (Φ< 1) [4].

Next, the set of parameters characterizing retention (retention factor R_f, retardation factor R_m, capacity factor k') and separation efficiency (number of theoretical plates N, height equivalent of theoretical plate H_a, difference in retention factor ΔR_f, resolution R_s, selectivity factor α) were calculated for the each analyte in studied nano-HPTLC system [1,4,5,7] and summarized in Table 4 and 5. The R_f values were calculated using equation $R_f = a/b$, where a is migration distance of the center of analyte zone from the sample application position on the HPTLC plate (mm) and b is migration distance of the mobile phase front up to 8.0 cm from the sample application position. The observed R_f values of patulin were as follows: 0.500, 0.566 and 0.480 on the NA, NSIL and ND plates, respectively (see Table 4). In studied HPTLC systems the highly ionized ascorbic acid (A) was adsorbed stronger (see Table 3,4,5) followed by 5-hydroxymethylfurfural (H), patulin (P) and quercetin (Q). This fact was confirmed by the highest R_m values for ascorbic acid, *i.e.* 1.28, 0.91, 1.28 on the NA, NSIL and ND plates, respectively. The zones for the critical pair of analytes as patulin (P) and quercetin (Q) were overlapped (no separation) in case of the plates NanoAdamant ($R_{f\ Q\text{-}P} = 0.51/0.50$ and $\Delta R_{f\ P\text{-}Q} = 0.01$) and Nano-Durasil-20 ($R_{f\ Q\text{-}P} = 0.48/0.48$ and $\Delta R_{f\ P\text{-}Q} = 0.00$). This effects give a false positive results during qualitative and quantitative determination of patulin by currently applied TLC or HPTLC methods [2,6,8,14] after its preliminary isolation with use of the solid-phase extraction (SPE) step from the various apple juice samples [11].

The results in Table 5 showed that each of studied nano-HPTLC system would be useful for good separation of patulin (P) from 5-hydroxymethylfurfural (H) and ascorbic acid (A). As indicating the values of calculated selectivity factor α (Table 5) in case of the Nano-SIL-20 plates a sharp separation of patulin (P) from quercetin (Q) ($\alpha = 1.13$) and patulin (P) from 5-hydroxymethylfurfural (H) ($\alpha = 1.17$) have been observed. The desired separation of patulin (P) from quercetin (Q) was only achieved on the Nano-SIL-20 plate where $\Delta R_{f\ P\text{-}Q}$ parameter was equal to 0.07. Thus, using the Nano-SIL-20 plates the most precise and accurate separation of patulin (P) from quercetin (Q) could be obtained. On the Nano-SIL-20 plates R_f value for ascorbic acid (A) was the highest (0.11) as compared to other studied here HPTLC plates (0.05). In case of NA

Table 3. Characteristic data (n = 3) of analytes zones on each studied nano-HPTLC system for ascorbic acid (A), 5-hydroxymethylfurfural (H), patulin (P) and quercetin (Q)

HPTLC	Parameter	Compound			
		A	H	P	Q
NA NSIL ND	Color of the spot in visible light	orange	green	yellow	yellow
NA	dimension	2.0 × 2.0	4.0 × 4.0	4.0 × 2.0	3.0 × 1.0
NSIL	[width × height]	3.0 × 3.0	5.0 × 5.0	4.0 × 3.0	4.0 × 2.0
ND	(mm)	3.0 × 3.0	4.0 × 4.0	4.0 × 1.0	1.5 × 1.0
NA		1.00	1.00	0.50	0.33
NSIL	$\Phi = d_1 / d_2$	1.00	1.00	0.75	0.50
ND		1.00	1.00	0.25	0.67

d_1 – height and d_2 – width of each compound zone (mm) on the HPTLC plate

Table 4. Retention parameters (n = 3) of ascorbic acid (A), 5-hydroxymethylfurfural (H), patulin (P) and quercetin (Q) on different HPTLC plates: NanoAdamant (NA), Nano-SIL-20 (NSIL) and Nano-Durasil-20 (ND)

HPTLC	Parameter	Compound				Parameter	Compound			
		A	H	P	Q		A	H	P	Q
NA		0.05	0.43	0.50	0.51		1280	3120	3360	6116
NSIL	$R_f = a / b$	0.11	0.48	0.56	0.63	$N=[16 \times l \times z] / w^2$	1565	1997	3760	4160
ND		0.05	0.44	0.48	0.48		569	2880	3200	23324
NA		1.28	0.12	0.00	-0.02		0.06	0.03	0.02	0.01
NSIL	$R_m = logk'$	0.91	0.03	-0.10	-0.23	$H_a=[(0,25 \times w)^2] / z$	0.05	0.04	0.02	0.01
ND		1.28	0.10	0.03	0.03		0.10	0.03	0.02	0.003

and ND systems, the low R_f value was a consequence of the increased adsorption of ionized ascorbic acid on the sample application position. As seen from Table 5, the best selectivity of each pair of analyzed compounds was observed on the Nano-SIL-20 plate. Graphical relation between changes in the calculated R_f values of studied analytes and type of applied here nano-HPTLC system in the same mobile phase were shown in Figure 1.

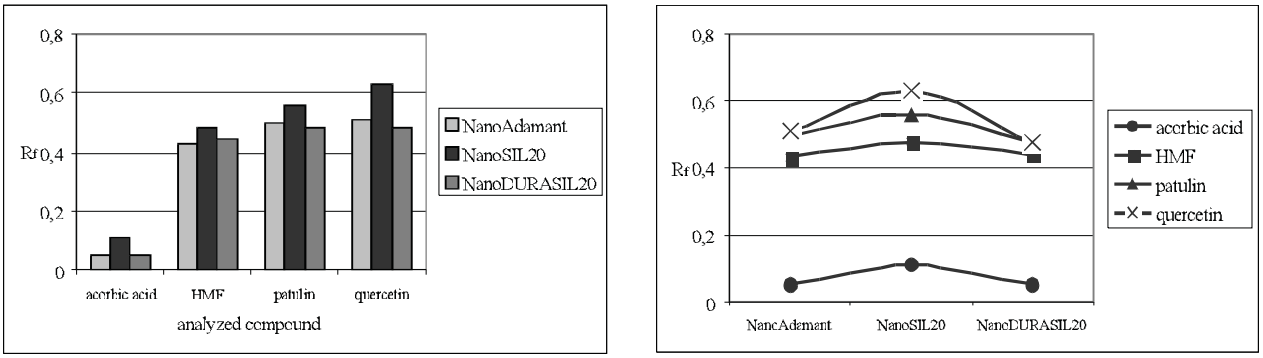


Figure 1. Changes in R_f values of studied compounds in three studied nano-HPTLC systems

As is seen in Table 5, for the most pairs of studied compounds the resolution R_s values were higher then 1.5 which means that complete separations were obtained [7]. Exception to this rule were noted the two critical pairs of compounds: 1) patulin (P)/quercetin (Q): $R_{s\,P\text{-}Q} = 0.80$ (in the NA system), $R_{s\,P\text{-}Q} = 1.43$ (in the NSIL system) and $R_{s\,P\text{-}Q} = 0.50$ (in the ND system) and 2) 5-hydroxymethylfurfural (H)/quercetin (Q): $R_{s\,H\text{-}P} = 0.88$ in the ND system. Values of R_s show that in the case of critical pair of compounds patulin (P)/quercetin (Q) the change of applied nano-HPTLC plate enabled the highly improved separation of those compounds. Selectivity factor α was the highest for the pair of quercetin (Q) and asorbic acid (A): $\alpha = 10.00$ (NA system); $\alpha = 5.09$ (NSIL system) and $\alpha = 9.60$ (ND system) because, in given polar-acidic mobile phase, the specific adsorption of the mostly ionized ascorbic acid molecules on the surface hydroxyl groups present in the surface layer of applied here silica gel type HPTLC plates, was the highest as compared to quercetin.

The results of our study suggest that in case of the Nano-SIL-20 plate decreased mobility and enhanced interactions of the mobile phase components with analyzed here compounds in the chromatographic system could lead to the highest observed development time and increased R_f values (Table 4), as well as to increased dimension of the each analyte spot in comparison to the other studied here nano-HPTLC plates (see Table 3 and 4). All of the parameters of the silica gel layers in applied here three HPTLC systems and the composition of mobile phase, were identical. Thus, our results clearly indicate that the special binder(s) system used in the each of studied here nano-HPTLC plates to connect the supporting glass plate surface with the silica gel layer strongly influenced the observed separation of patulin from the other analyzed compounds. Unfortunately, the exact chemical name, structure and properties of these binders was not enabled by the producer to the public [13]. For the Nano-SIL-20 plates the producer used "the highly polymeric product" [13] as a binder, which is resistant to aggressive reagents, but in case of the Nano-Durasil-20 plates manufacturer used a "special binder system" [13] which gives the "water-resistant and wettable silica gel layers" [13]. Due to modification of this binder system, the separation of analyzed here compounds varied significantly. In the NSIL system the R_f value for ascorbic acid was the highest (0.11) and for the other plates R_f value for this compound did not exceed 0.05. In case of the NSIL system we observed a clear separation ($\alpha = 1.13$) of patulin (P) from quercetin (Q) and patulin (P) from 5-hydroxymethylfurfural (H) ($\alpha = 1.17$). Consequently, in this best nano-

Table 5. Separation parameters (n = 3) obtained for ascorbic acid (A), 5-hydroxymethylfurfural (H), patulin (P) and quercetin (Q) in studied nano-HPTLC systems NA, NSIL and ND

HPTLC	Parameter	Pair of compounds					
		H - A	H - P	P - Q	Q - H	Q - A	P - A
NA	$\Delta R = R_{f1} - R_{f2}$	0.38	0.07	0.01	0.08	0.46	0.45
NSIL		0.37	0.08	0.07	0.15	0.52	0.45
ND		0.39	0.04	0.00	0.04	0.43	0.43
NA	$R_{s(b)}=(2\times d) / (w_{b1}+ w_{b2})$	5.11	1.67	0.80	5.33	12.33	7.78
NSIL		4.83	1.60	1.43	2.67	9.11	6.20
ND		5.33	0.88	0.50	1.67	9.00	7.00
NA	$\alpha = R_{f2} / R_{f1}$	8.60	1.16	1.02	1.19	10.20	10.00
NSIL		4.36	1.17	1.13	1.31	5.73	5.09
ND		8.80	1.09	1.00	1.09	9.60	9.60

d – distance and $w_{b1,2}$ – width of two neighbouring peaks on the scan of obtained HPTLC chromatogram.

HPLTC system NSIL, the minimal detectable amount of patulin as 4.0 ± 1.0 ng/spot after its deposition from the diluted standard methanolic solution have been observed (data not shown). This amount was near 2.5 times lower in comparison to reported earlier TLC modes [2,6,8,14] and described here two other nano-HPTLC systems. Thus, the use of Nano-SIL-20 plates should be assumed as recommendable for determination of trace amounts of patulin (e.g. 50 μg/kg) in real samples of apple juices, following its selective SPE isolation and preconcentration steps, in view of increased selectivity, sensitivity, precision and reproducibility of chromatographic process offered by this Nano-SIL-20 plates.

CONCLUSIONS

The best selectivity of patulin separation from the three commonly accompanying compounds was obtained using the Nano-SIL-20 type plates. Results of our study suggest that the choice of this nano-HPTLC plate could lead to achieve a more reproducible, accurate and reliable determination of trace amounts of patulin in apple juices.

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