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Comparison of thin-layer chromatography and overpressured layer chromatographic techniques for the separation of ascorbigen and 1′-methylascorbigen

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Abstract

Simple and efficient methods are described for the separation of biologically active ascorbigen and 1′-methylascorbigen by normal-phase TLC, conventional as well as new personal overpressured layer chromatographic (OPLC) systems using the same eluent. Results obtained with these techniques were compared on the basis of the separation parameters with special emphasis on the presentation of the advantages of the automated personal OPLC system. For the characterization of the personal OPLC system dependence of average plate height and resolution on the external pressure, front velocity and development distance was examined on TLC and HPTLC sorbent layers using ascorbigen and 1′-methylascorbigen as model compounds.

Keywords: Ascorbigen; Methylascorbigen

1. Introduction

Ascorbigen (ASC) (Fig. 1) is a natural derivative of L-ascorbic acid (AA) and was identified as a biotransformation product of the alkaloid glucobrassicin [1]. ASC can be isolated from some fresh, non-fresh or sour cruciferous vegetable tissues (cabbage, kohlrabi, savoy cabbage, etc.) [2,3].

ASC and some of their derivatives have been synthesized for systematic analytical and pharmacological investigations [1,3–6]. Their biological evaluation showed that the most active substance is 1′-methylascorbigen (MeASC) (Fig. 1) which inhibits tumor growth in animals [1,9,10], protects animals from some bacterial and viral infections [1,8,10], and also has an immunomodulating activity [1,9]. MeASC has a pronounced apoptotic effect [11]

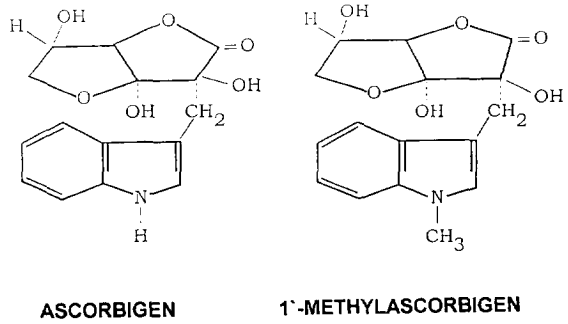

Fig. 1. Chemical structure of ASC and MeASC.

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in which formaldehyde from the methyl group of MeASC plays a crucial role. The presence of MeASC in plants has not yet been proven. An interesting characteristic of ascorbigens is their ability to release AA in physiological conditions, so they are real AA pools [10].

Samples containing ASC and its derivatives of natural and synthetic origin were already analysed using liquid chromatographic techniques as TLC [4,7,12,13] and HPLC [14–16]. The special biological activity of MeASC and the eventual parallel occurrence of ASC and MeASC-like substance in plant kingdom in itself provides the motivation for the development of efficient methods for the separation of ASC and MeASC.

According to recent investigations overpressured thin-layer chromatography (OPLC) [17–20], which integrates the advantages of TLC and HPLC, is suitable for the efficient separation of different substance groups. The aim of this paper is to demonstrate the comparison of TLC and OPLC techniques for the separation of ASC and MeASC as model compounds with special emphasis on the efficiency of personal OPLC.

2. Experimental

2.1. Materials

Authentic ASC and MeASC were synthesized essentially according to the previously described methods [1,3–6]. All chemicals were of analytical grade and purchased from Reanal (Budapest, Hungary). Impres II (Labor Instruments, Budapest, Hungary) polymer suspension was used to obtain sealed chromatoplates for OPLC.

2.2. Instrumentation

Linomat III (Camag, Muttenz, Switzerland) sample applicator was used for applying samples to the chromatoplates.

TLC separations were performed in conventional Camag chambers.

OPLC separations were carried out by means of the Chrompres 10 (Labor Instruments) and the personal OPLC (OPLC-NIT, Budapest, Hungary) instruments. The newly developed automated personal OPLC system includes the separation chamber and the liquid delivery system [20]. The separation chamber is capable of providing high external pressure (max. 5.0 MPa) and has four main parts: holding unit, hydraulic unit, layer cassette and an attached drain valve. The cassette envelops the sealed chromatoplate. On the inside part of the PTFE cover of the cassette which directly contacts the sorbent layer there are two directing channels (troughs). One trough at the beginning of the sorbent layer generates the formation of the straight front line and the other one at the end of the sorbent layer serves to collect the eluent in the case of unexpected overflow. This second trough can be used for the continuous development or on-line separation mode at preparative OPLC (on-line OPLC). The computer controlled liquid delivery system has two pump heads, one for the eluent delivery, the other one for the hydraulic liquid. All parameters for simple or repeated developments can be given and stored in the software.

Chromatograms were evaluated instrumentally using a Shimadzu CS-930 (Kyoto, Japan) dual-wavelength TLC scanner.

For regression analysis the FIT $$$ software program was used.

2.3. Methods

Chloroform–methanol–acetic acid (90:10:1, v/v) solvent mixture was used as selected mobile phase.

Developments were performed on 20×20 cm TLC and HPTLC silica gel 60 F_{254} (Merck, Darmstadt, Germany) chromatoplates. For OPLC developments chromatoplates were sealed: the sorbent layer was scrapped within a narrow strip (2–3 mm) along the edges of the plate and these strips were coated with Impres II. For Chrompres 10 three edges of the chromatoplates were coated, while for personal OPLC all four edges were sealed. In the case of Chrompres 10 the migration of eluent can be seen, while this is not possible for personal OPLC. Therefore, the second trough and the sealing of four edges of chromatoplate are useful in the elimination of the unexpected overflow of the eluent or in continuous development. For comparison of TLC with OPLC HPTLC chromatoplates were used, and the charac-

terization of the personal OPLC system was carried out on TLC and HPTLC chromatoplates.

Methanolic solutions of standard samples (5 μl) containing 1.0 mg/ml of ASC and MeASC were applied to the plates as spots having 1.5 mm average diameter. The distance between the start and directing channel (z_0) was 20 mm. For study of the correlation between the average plate height ($\bar{H}$) and the development distance ($z_f - z_0$, L) ($\bar{H}$–L curve) samples were applied to the chromatoplate in diagonal application mode.

For comparison of TLC and OPLC techniques TLC developments were performed in ascending mode with chamber saturation, the development distance was 175 mm. By developments performed with Chrompres 10 the membrane pressure (external pressure) was 1.2 MPa, the eluent front velocity was 0.9 cm/min, and the development distance was 175 mm. Using personal OPLC the selected chromatographic conditions were as follows: external pressure, 5.0 MPa; rapid eluent flush, 350 μl; eluent flow-rate, 250 μl/min (the corresponding front velocity 1.17 cm/min); development volume, 3700 μl; the software calculated development time, 902 s; and the development distance, 176 mm. The different eluent front velocities selected for OPLC and personal OPLC are optimum values based on the $\bar{H}$–u relationship. The whole development time is composed of two parts: The "real" development time, 888 s (3700 μl: 250 μl/min) and additionally 14 s which are necessary for delivery of 350 μl eluent at the beginning of the process (rapid eluent flush) to form a straight front line. This rapid eluent flush means that at the beginning of the separation process the flow-rate of the eluent is six time more than that selected for development. The eluent with high speed fills the trough and forms the straight front line during 14 s. After this period of time the eluent velocity becomes smaller corresponding to the established value. It has to be pointed out that the rapid eluent flush does not reach the start points of the samples and so does not disturb the separation process planned. At the end of development after termination of the eluent delivery the external pressure is released and the layer cassette can be taken out from the chamber.

At the efficiency study of the personal OPLC system the development conditions for study of the correlation between $\bar{H}$ and external pressure (P_{ext}) ($\bar{H}$–P_{ext} curve), and between the resolution (R_s) and P_{ext} (R_s–P_{ext} curve) were as follows: rapid eluent flush, 350 μl; eluent flow-rate, 250 μl/min; development volume, 3700 μl; and the external pressure was increased from 1.0 to 5.0 MPa. For study of the relationship between $\bar{H}$ and the front velocity (u) ($\bar{H}$–u curve) the following parameters were used: rapid eluent flush, 350 μl; development volume, 3700 μl; external pressure, 5.0 MPa; and the eluent flow-rate was changed between 100 and 1000 μl/min. In the case of the study of the dependence between H and $z_f - z_0$ (L) (H–L curve) the rapid eluent flush was 350 μl, the eluent flow-rate was 250 μl/min, the development volume was 6150 μl (continuous development), the external pressure was 5.0 MPa, and the development time was 1490 s.

After development chromatoplates taken out were dried in a stream of cold air for 5 min. Spots were visualized by UV illumination, or by using one of two reagents: Procházka's reagent [21,23], or ammonium molybdate [2,22]. Densitometric evaluations were performed at the optimum wavelength which was determined by direct spectral measurement on the sorbent layer for each visualization method. For ASC and MeASC the characteristic wavelength values are as follows: λ=220 nm by evaluation with UV illumination; λ=450 nm by evaluation after visualization with ammonium molybdate; and 460 and 430 nm, respectively, for ASC and MeASC after visualization with Procházka's reagent.

3. Results and discussion

Fig. 2 represents densitograms of chromatograms of mixtures containing ASC and MeASC obtained after normal-phase developments using TLC (A), conventional OPLC (B) and personal OPLC (C) systems. The distance between peaks and their sharpness increases from TLC to personal OPLC in perfect accordance with results from Table 2. The highest efficiency and resolution were obtained using the personal OPLC system. The secondary front (β front), which is present in the case of both forced flow techniques, does not disturb the separation of ASC and MeASC. These separation systems were perfectly suitable for theoretical calculations. The

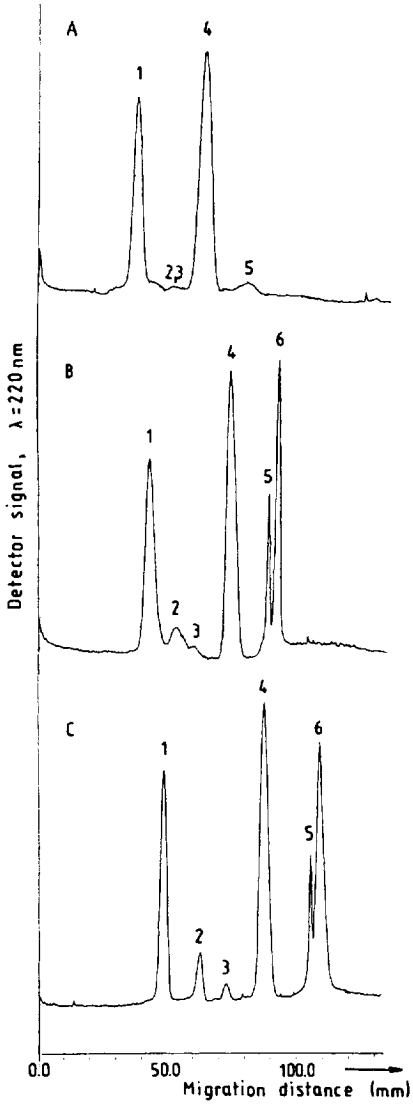

Fig. 2. Densitograms of chromatograms of mixtures containing ASC and MeASC obtained after normal-phase developments using TLC (A), conventional OPLC (B) and personal OPLC systems after visualization by illumination at $\lambda = 220$ nm. Peaks: 1 = ASC; 2,3,5 = unknown components; 4 = MeASC, 6 = secondary front (β front). Mobile phase: chloroform–methanol–acetic acid (90:10:1, v/v). Other separation conditions see Section 2.3.

peak corresponding to the β front (peak 6) can overlap with other peaks, therefore, in the case of complex mixtures (e.g., plant extracts) one has to modify the mobile phase for the elimination of the β front. The reproducibility of the eluent front in personal OPLC was as follows: $\sigma_{n-1} = 1.439$ and $\mu = 176.155 \pm 1.108$ mm in the case of repeated developments using the same chromatoplate. (Of course, solvents were evaporated between two developments).

For the characterization of results separation parameters were calculated by means of known relationships. The average plate height value, $\bar{H}$, can be calculated from the equation:

$$\bar{H}_{obs} = \bar{H} = \frac{\sigma_x^2}{z_x} = \frac{\sigma_x^2}{(z_f - z_0)R_f} \quad (1)$$

where σ_x is the standard deviation of the spot dispersion, z_x is the migration distance of the spot and $z_f - z_0$ is the development distance.

Plots of average plate height, $\bar{H}$, against the external pressure show a linear correlation between

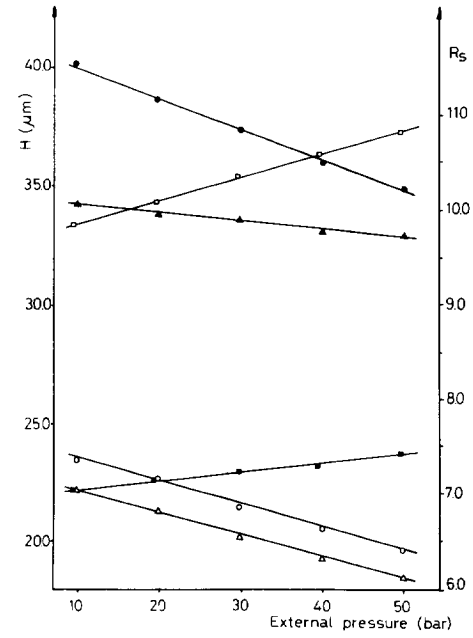

Fig. 3. Dependence of the average plate height, $\bar{H}$ and resolution, R_s on the external pressure for ASC and MeASC using personal OPLC system: —·—·= ASC, TLC sorbent layer ($\bar{H}$–P_{ext} curve); —○— = ASC, HPTLC sorbent layer ($\bar{H}$–P_{ext} curve); —▲—= MeASC, TLC sorbent layer ($\bar{H}$–P_{ext} curve); —△—= MeASC, HPTLC sorbent layer ($\bar{H}$–P_{ext} curve); —■—= TLC sorbent layer (R_s–P_{ext} curve); —□—= HPTLC sorbent layer (R_s–P_{ext} curve). For separation conditions see Section 2.3. Results of correlation calculations can be seen in Table 1.

Table 1
Correlations between efficiency parameters (H, R_s) and separation conditions (P_{ext}, u, L) calculated by regression analysis for the characterization of personal OPLC

Examined correlation	Regression method	Calculated relationship	n	r	F
H–P_{ext} curves (Fig. 3)					
ASC, TLC sorbent layer	Linear	$y = -0.13x + 41.34$	5	0.9986	1100.7***
ASC, HPTLC sorbent layer	Linear	$y = -0.09x + 24.4$	5	0.9935	457.9***
MeASC, TLC sorbent layer	Linear	$y = -0.03x + 34.53$	5	0.9920	215.1***
MeASC, HPTLC sorbent layer	Linear	$y = -0.09x + 23.08$	5	0.9987	1151.7***
R_s–P_{ext} curves (Fig. 3)					
TLC sorbent layer	Linear	$y = 0.01x + 6.96$	5	0.9929	208.8***
HPTLC sorbent layer	Linear	$y = 0.02x + 9.59$	5	0.9994	240.1***
H–u curves (Fig. 4)					
ASC, TLC sorbent layer	4th degree polynomial	$y = 0.06x^4 + 0.13x^3 + 0.18x^2 + 0.97x + 81.96$	10	0.9977	764.9***
ASC, HPTLC sorbent layer	4th degree polynomial	$y = 0.08x^4 + 0.15x^3 + 0.34x^2 + 0.92x + 42.26$	10	0.9967	527.5***
MeASC, TLC sorbent layer	4th degree polynomial	$y = 0.09x^4 + 0.10x^3 + 0.22x^2 + 0.96x + 77.90$	10	0.9970	591.9***
MeASC, HPTLC sorbent layer	4th degree polynomial	$y = 0.08x^4 + 0.17x^3 + 0.29x^2 + 0.94x + 31.71$	10	0.9980	868.4***
H–L curves (Fig. 5)					
ASC, TLC sorbent layer	Linear	$y = 0.08x + 18.65$	6	0.9955	442.5***
ASC, HPTLC sorbent layer	Linear	$y = 0.03x + 16.85$	6	0.9889	176.7***
MeASC, TLC sorbent layer	Linear	$y = 0.04x + 19.12$	6	0.9880	163.8***
MeASC, HPTLC sorbent layer	Linear	$y = 0.04x + 7.45$	6	0.9875	156.7***

P_{ext}: external pressure; u: front velocity; $L = z_f - z_0$: development distance.

these parameters for both compounds (Fig. 3 Table 1). It should be noted that $\bar{H}$ decreases with the external pressure. Values of $\bar{H}$ are higher on TLC sorbent layer than on HPTLC sorbent layer. The lowest $\bar{H}$ value which corresponds to the highest efficiency is obtained for each case at 5.0 MPa external pressure.

The resolution, R_s, was calculated using the following equation [24]:

$$R_s = \frac{(z_x)_2 - (z_x)_1}{2(\sigma_2 + \sigma_1)} = \frac{(R_f)_2 - (R_f)_1}{2(\sigma_2 + \sigma_1)}(z_f - z_0) \qquad (2)$$

Also plotting the resolution, R_s, versus external pressure results in increasing linear relationship (Fig. 3, Table 1). Using HPTLC sorbent layer higher resolutions are achieved than on TLC sorbent layer. The best resolution is obtained at 5.0 MPa external pressure. Personal OPLC is superior to the Chrompres family which is able to provide only 1.2 MPa external pressure with Chrompres 10 and 2.5 MPa with Chrompres 25, respectively.

Plots of $\bar{H}$ as function of front velocity give the well-known form of curves in chromatography (Fig. 4, Table 1). These convex curves have a minimum value for $\bar{H}$ and the corresponding front velocity value is the optimum for development. This value is shifted slightly to higher value in the case of the HPTLC sorbent layer in comparison to the TLC sorbent layer. In the immediate vicinity of the optimum a useable front velocity range can be determined which is broader for the HPTLC sorbent layer than for the TLC sorbent layer. The ascending branch of the curves obtained on the TLC sorbent layer for both compounds is more abrupt than those obtained on the HPTLC sorbent layer.

According to Fig. 5 and Table 1 there is a linear correlation between $\bar{H}$ and development distance. The most abrupt line is that obtained for ASC on the TLC sorbent layer (highest $\bar{H}$ and slope value), the others have smaller slope. $\bar{H}$ increases by a small degree with the development distance. In the case of the HPTLC sorbent layer the diameter of the spot is very narrow, so $\bar{H}$ is practically constant along the plate [18]. This means that the theoretical plate number, N increases linearly with the development distance contrary to TLC. Because of higher efficiency parameters it is recommendable to use a HPTLC sorbent layer for OPLC.

Separation parameters of ASC and MeASC ob-

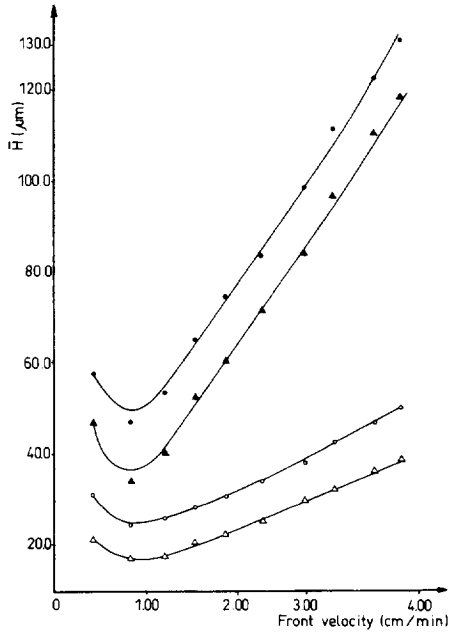

Fig. 4. Relationship between the average plate height, $\bar{H}$ and the front velocity for ASC and MeASC using personal OPLC system: —●— = ASC, TLC sorbent layer; —○— = ASC, HPTLC sorbent layer; —▲— = MeASC, TLC sorbent layer; —△— = MeASC, HPTLC sorbent layer For separation conditions see Section 2.3. Results of correlation calculations can be seen in Table 1.

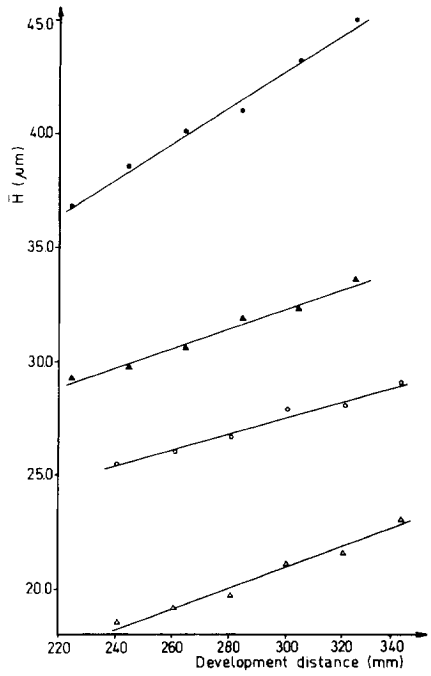

Fig. 5. Dependence of the average plate height, $\bar{H}$ on the development distance for ASC and MeASC using personal OPLC system: —●— = ASC, TLC sorbent layer, —○— = MeASC, HPTLC sorbent layer; —▲— = MeASC, TLC sorbent layer; —△— = MeASC, HPTLC sorbent layer. For separation conditions see Section 2.3. Results of correlation calculations can be seen in Table 1.

tained by TLC and OPLC using Chrompres 10 and personal OPLC systems are presented in Table 2. The lowest R_F values are obtained in the case of TLC, the highest in the case of personal OPLC. The maximum value of $\Delta R_F = R_F^{MeASC} - R_F^{ASC} = 0.21$ also is achieved with personal OPLC, the minimum value (0.12) was with TLC.

The capacity factor, k' [24] decreases from TLC to personal OPLC. According to the calculations α [24] decreases from 0.48 (TLC) to 0.40 (personal OPLC), and N increases for both compounds from TLC to personal OPLC. In complete concordance with the above-mentioned separation parameters the lowest resolution value is obtained using TLC followed by that obtained in the case of Chrompres 10 and finally the best resolution was achieved with personal OPLC. The comparison of TLC and OPLC performed by Chrompres 10 and personal OPLC for separation of ASC and MeASC proved that the best separation (highest efficiency and resolution) can be obtained using the new OPLC system.

4. Conclusions

Simple methods are described for the efficient separation of ASC and MeASC by normal-phase TLC, conventional OPLC and personal OPLC systems. These methods can be used routinely to search for these biologically active substances from different plant species.

The comparative study of TLC and OPLC (Chrompres 10 and personal OPLC) demonstrates that the highest efficiency and resolution can be achieved by means of personal OPLC.

These preliminary experiences with personal OPLC as automated layer liquid chromatographic technique show that the sophistication of the original layer liquid chromatographic technique increases dramatically the efficiency of the simple and flexible TLC and conventional OPLC. The result is a high

Table 2
Comparison of separation parameters (R_F, k', α, N, R_s) obtained by TLC, conventional OPLC as well as personal OPLC systems using HPTLC sorbent layer for ASC and MeASC

Planar layer liquid chromatographic technique		Compound	R_F	k'	α	N	R_s
TLC		ASC	0.15	5.67	0.48	1 058	2.68
		MeASC	0.27	2.70		2 442	
OPLC	Chrompres 10 instrument	ASC	0.21	3.76	0.41	4 767	7.52
		MeASC	0.39	1.56		5 949	
	Personal OPLC instrument	ASC	0.28	2.57	0.40	6 743	8.27
		MeASC	0.49	1.04		10 173	

level of layer liquid chromatography which is applicable for analytical and preparative separations alike.

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