Journal of Chromatography, 511 (1990) 396–401
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 363

Note

Automated multiple development high-performance thin-layer chromatographic analysis of natural phenolic compounds

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(Received January 18th, 1990)

Phenolic compounds are widespread plant secondary metabolites, the flavonoids being the largest group. However, simple monocyclic phenols, phenylpropanoids and phenolic quinones are also frequently encountered[1].

Liquid chromatography, both column[2–10] and planar[11–19], has proved to be the most useful technique for the analysis of phenolic compounds. In the gradient elution mode, high-performance liquid chromatography (HPLC) has been most widely used and the intrinsic problem of gradient elution optimization in the HPLC separation of multi-component phenolic compounds mixtures has already been discussed[20]. However, once a recent plate development concept known as the automated multiple development (AMD) technique[21–24] has been properly characterized, planar chromatography may play a more important role in the analysis of complex mixtures. The most important feature that allows the successful application of AMD with high-performance thin-layer chromatography (HPTLC) in complex mixtures analysis is the possibility of carrying out the separation process using a gradient development mode. Further, multi-development is known to produce a band reconcentration effect[25] which allows spots to migrate over considerable distances without appreciable band broadening. Both gradient development and the band reconcentration effect act in the direction of improving the spot capacity, *i.e.*, allowing homogeneous spreading over a single chromatogram of many compounds which might belong to different polarity ranges[26].

An application of the AMD–HPTLC procedure to phenolic compounds separation was reported by Ebel *et al.*[27], who showed that this approach is suitable for the analysis of coumarins in multi-component extracts of medicinal plants.

In this paper a basic approach to an optimized AMD–HPTLC experimental procedure and its ability to separate several classes of natural phenolic compounds is

0021-9673/90/$03.50

presented. *Chamomilla recutita* extracts were chosen as chamomile flowers are a well known natural source of phenolic compounds (flavonoids, coumarins, phenolcarboxylic acids) spanning a wide chemical polarity range[28].

EXPERIMENTAL

Merck 5641 silica gel 60 HPTLC precoated plates, 10 × 20 cm, without fluorescence indicator, twice prewashed with methanol were used. The solvents employed were methanol, ethanol, ethyl acetate, dichloromethane and *n*-hexane of HPLC grade (Carlo Erba, Milan, Italy). Formic acid (puriss.) was purchased from Fluka (Buchs, Switzerland).

The selected phenolic compounds used as standards were obtained from Extrasyntese (Genay, France). They were used as received and dissolved in ethanol or ethanol–ethyl acetate (1:1) to give 100–200 ppm solutions. The selected standards represent the most important phenolic compounds in chamomile, *i.e.*, coumarins, phenolcarboxylic acids, flavones, flavonols and glycosides. They are listed in Table I.

TABLE I

PHENOLIC COMPOUNDS USED AS STANDARDS

No.	*Compound*	R_F[a]
1	Herniarin (7-methoxycoumarin)	0.94
2	Ferulic acid (4-hydroxy-3-methoxycinnamic acid)	0.86
3	Umbelliferone (7-hydroxycoumarin)	0.81
4	Isorhamnetin (3,4′,5,7-tetrahydroxy-3′-methoxyflavone)	0.75
5	Apigenin (4′,5,7-trihydroxyflavone)	0.69
6	Caffeic acid (3,4-dihydroxycinnamic acid)	0.60
7	Luteolin (3,4′,5,7-tetrahydroxyflavone)	0.55
8	Quercetin (3,3′,4′,5,7-pentahydroxyflavone)	0.50
9	Fisetin (3,3′,4′,7-tetrahydroxyflavone)	0.42
10	Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone)	0.38
11	Apigenin 7-O-glucoside	0.29
12	Luteolin 7-O-glucoside	0.23
13	Hyperoside (quercetin 3-α-D-galactoside)	0.18
14	Chlorogenic acid [3-(3,4-dihydroxycinnamoyl)quinic acid]	0.13
15	Rutin {quercetin-3-O-[6-(α-L-rhamnosyl)-D-glucose]}	0.09

[a] Values obtained in this work.

Preparation of extracts

The source material was a commercial aqueous alcoholic extract of chamomile flowers. A 1-g amount of this sample was suspended in 10 ml of water acidified with hydrochloric acid to pH 2–3. It was extracted successively with three 20-ml portions of ethyl acetate. The aqueous phase was discarded and the organic phase filtered through anhydrous sodium sulphate and evaporated to dryness under reduced pressure (at 40–50°C). The residue was dissolved in 2 ml of ethanol–ethyl acetate (1:1) and the resulting solution was used for AMD analysis.

Sample application

Standards and sample solutions were applied to the plates either as spots with a Pt–Ir capillary or as 10-mm wide bands with a Linomat IV (Camag, Muttenz, Switzerland) (3 μl; delivery speed 4 s/μl).

Detection

Fluorescence detection was used at an excitation wavelength of 360 nm. Coumarins and phenolcarboxylic acids give an inherent bluish fluorescence. The flavonoids were derivatized by dipping the developed plates in a 4% aqueous aluminium sulphate solution[15]. After 10-min exposure to UV light they showed yellow fluorescent spots that were stable for several weeks. The derivatized plates were scanned with a Camag Scanner II equipped with a Merck–Hitachi Chromato-Integrator. Fluorescence was induced at 360 nm with a mercury vapour lamp. Emission was measured through a cut-off filter (400 nm).

Development

The AMD system (Camag) and its operating conditions have been described in detail elsewhere[21–24]. Briefly, an HPTLC plate is subjected to a stepwise gradient development in an enclosed chamber. The developing solvent mixture is prepared in a gradient mixer from the solvent components in separate bottles. Between developments, the mobile phase is removed from the developing chamber and the plate is dried under vacuum and then conditioned in an atmosphere of controlled composition. The parameters to be specified for an AMD run are the solvent composition in each feeding bottle, the number of development steps, the drying times between steps, the development times (determining the run distance) for each step and the preconditioning conditions.

The experimental AMD parameters used in this study were as follows: number of steps, 15; drying times, 6 min for the first four steps, then 4 min for the remaining steps, with the exception of the last step (10 min); preconditioning, nitrogen bubbled through water was the conditioning atmosphere; and preconditioning time, 15 s for each step. The filling of the feeding bottles and the development times are reported in Table II and Fig. 1, respectively.

TABLE II

OPTIMIZED SOLVENT COMPOSITION USED IN THE AMD EXPERIMENTS

Obtained using the instructions for the Camag AMD system (1986).

Component	*Starting with step No.*			
	1	*2*	*6*	*11*
	Use bottle No.			
	1	*2*	*3*	*4*
Methanol	70.5	23.5	9.4	100
Dichloromethane	25	74	90	–
Water	4.5	1.5	0.6	–
Formic acid	1	1	1	–

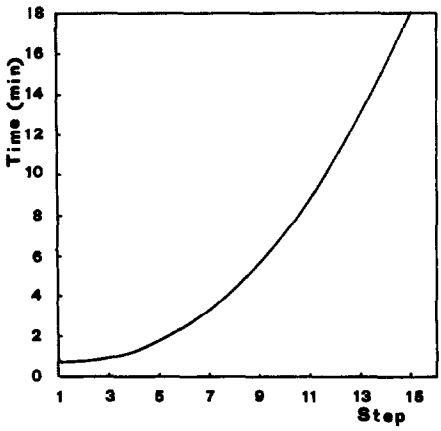

Fig. 1. Development time determining the running distance for each step.

RESULTS

The AMD gradient optimization was carried out on mixtures of the selected standards listed in Table I. As a starting point of the optimization, a "universal gradient" was chosen, *i.e.*, an AMD gradient that started with a very polar solvent and changed its composition, passing through a central or "basic" solvent of medium polarity and ending with a non-polar solvent[24]. Among the possible eluents spanning the overall polarity range, methanol was chosen as the strongest (most-polar) starting solvent and *n*-hexane as the weakest (non-polar), final solvent. As dichloromethane showed the best selectivity with respect to other medium-polarity solvents, *e.g.*, ethyl acetate or diethyl ether, it was used as a basis component. These general gradient conditions were gradually modified on the basis of trial-and-error experiments. First, formic acid was added to minimize the ionization of the weakly acidic compounds under examination. Water was added afterwards as a tailing suppressor for the most polar compounds. This last gradient-adjustment step required mixing of the starting, high-polarity solvent (methanol) with the medium-polarity eluent (dichloromethane) as water and formic acid actually acted as high solvent strength components as well. Finally, in the optimizing procedure, the non-polar gradient component (*n*-hexane) was eliminated because, under our conditions, it caused only a broadening of the developed bands. Hence, the final optimized gradient composition started (see Table II) from methanol–dichloromethane–water–formic acid (70.5:25:4.5:1) and proceeded gradually through 15 steps ending with pure dichloromethane (Fig. 2).

Owing to the relatively high concentration of non-volatile modifiers in the solvent, careful adjustment of plate drying times between each development step was carried out. It was observed that dryings shorter than the optimized ones (see Experimental) produced more diffuse, broadened bands, while longer drying times caused marked tailing.

In Table I the average R_F values of the standards obtained by means of three optimized AMD runs are reported. The selected phenolic compounds are separated

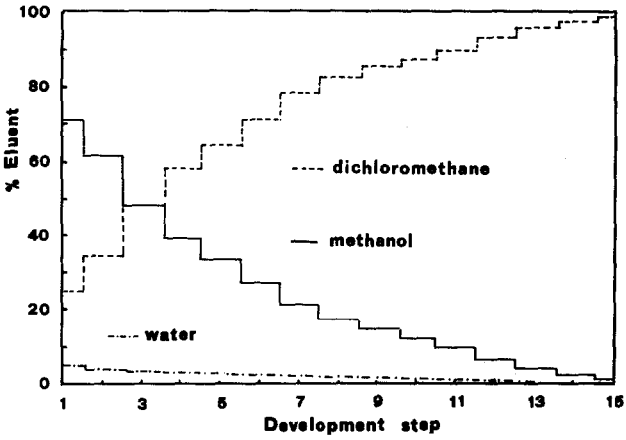

Fig. 2. Solvent composition obtained for each step for the optimized gradient.

and well distributed on the layer. The flavonoids isorhamnetin, luteolin and quercetin, for which poor separations have been obtained using various TLC systems[13], are well separated here. The polarity range of the standards spans from the low/–medium-polarity herniarin to highly polar rutin. It can be seen that inside each compound class the group contribution to the retention[11] follows trends that have been already observed in both silica gel and paper chromatography[11,13]. Hence the order of R_F values reported in previous work is still valid, *i.e.*, $ROCH_3 > ROH > R(OH)_n >$ RO–sugar.

Once the gradient for the selected standards had been optimized, it was employed for the analysis of the chamomile extract. The results are reported in Fig. 3. A regular distribution of the bands is observed, making further gradient optimization no longer necessary. Some components of the sample were identified by comparison

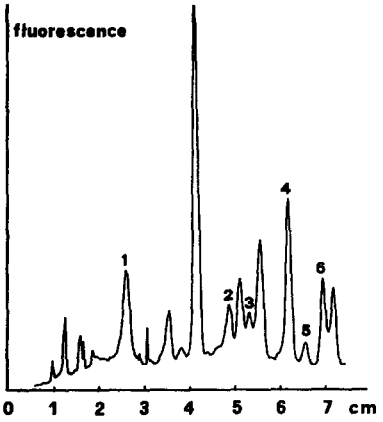

Fig. 3. Densitogram of the chamomile sample. The separation was obtained with the optimized gradient shown in Fig. 2. Peaks: 1 = apigenin 7-O-glucoside; 2 = caffeic acid; 3 = apigenin; 4 = umbelliferone; 5 = ferulic acid; 6 = herniarin.

between their retentions and the R_F values of the reference compounds and by standard addition. However, control of their purity has not yet been achieved. This aspect together with quantitative implications will be subject of further studies.

In conclusion, the AMD–HPTLC approach appears to be appropriate for the analysis of phenolic compounds because of its enhanced screening possibilities with respect to traditional TLC techniques. Further, gradient development on silica gel plates may be useful for the analysis of natural extracts as it often allows simpler clean-up procedures.

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