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QUANTITATIVE ANALYSIS OF PHENOLIC COMPOUNDS AFTER THIN-LAYER CHROMATOGRAPHIC SEPARATION

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SUMMARY

A method has been developed for the quantitative analysis of phenolic substances having non-substituted phenolic groups after their separation by thin-layer chromatography on silica gel or cellulose. After the clear detection of the phenolic substance on the chromatoplate by means of Folin–Ciocalteu reagent, the quantitative spectrophotometric determination of the substance is carried out by transferring the sorbent area containing the spot into a test-tube and using the Folin–Ciocalteu reagent, without the necessity for carrying out a prior elution for the recovery of the substance.

The method appears to have good accuracy and to be suitable for general application.

INTRODUCTION

In the course of our studies on the phenolic components of olives in waste water from treating olives and olive leaves, we encountered the problem of the quantitative analysis of certain characteristic phenolic substances[1], such as oleoeuropeine, dihydroxyphenylethanol and tyrosol, after their separation on silica gel thin-layer chromatoplates.

The UV spectrophotometric determination of these substances after elution of their spots from the adsorbent proved to be inaccurate because of the difficulty of detecting with sufficient accuracy the developed spots on the chromatoplates, as the substances are not fluorescent in UV light and at 254 nm the amounts involved in our studies do not absorb sufficiently to be detected on chromatoplates with a fluorescent indicator. In addition, detection by comparison with chromatograms simultaneously developed with chromatic reagents did not yield satisfactory results because of the inevitable irregularity that often occurs in development, with the risk of losing some of the desired spots or including nearby non-pertinent spots.

Elution of the colour obtained after treating the spots with diazotized sulpha-

nilic acid or *p*-nitroaniline yielded optical densities that were too low for the precise spectrophotometric determination of the amounts of the substances (10–100 μg) required for a satisfactory chromatogram.

The phosphomolybdotungstic acid reagent (well known as Folin–Ciocalteu's reagent)[2] gave a satisfactory detection of the phenolic substances on chromatoplates as blue spots on a white background after spraying with a sodium carbonate solution, also for a few micrograms of substance. After elution of the spots with water, the optical densities obtained were too low for determination; they were much lower than those obtained with the same amounts of phenolic compound in solution after direct reaction with the Folin–Ciocalteu reagent according to the usual method[3].

We found the eluate of the spots detected with the above reagent could react further with the reagent to yield a more intense colour, comparable with that obtained with the same amounts of phenolic substance by direct reaction in solution. It was therefore evident that the phosphomolydbotungstic acid reagent sprayed on to chromatograms was sufficient to detect the spots of phenolic compounds, but it was not sufficient to react with the whole amount of substance in the spots.

We made various attempts to accomplish a more complete reaction of the phenolic substances directly on the chromatoplate, but they all failed. In fact, by spraying the plate consecutively with the above two solutions, the reaction was satisfactory only on the surface: when the second solution was sprayed, the sorbent layer was still moist and the solution penetrated it with difficulty. The use of larger volumes of the reagents caused unacceptable expansions and smearing of the spots.

We accordingly carried out a number of tests with different amounts of oleoeuropeine, a characteristic glucoside of the olive tree[4] with an *o*-diphenolic moiety, both after thin-layer chromatography on silica gel, and when only spotted on a chromatoplate without development; the results showed the possibility of (a) detecting with the greatest accuracy the spots of the phenolic substance with Folin–Ciocalteu's reagent, even for very small amounts, by virtue of the high sensitivity of the reagent, and (b) subsequently carrying out a satisfactory spectrophotometric determination of the detected phenolic substance by transferring the sorbent area containing the spot into a test-tube and carrying out on this material the usual determination[3] with Folin–Ciocalteu's reagent.

TABLE I

OPTICAL DENSITIES AT 725 nm OBTAINED WITH 50 μg OF PHENOLIC SUBSTANCE AFTER SILICA GEL THIN-LAYER CHROMATOGRAPHY, DETECTION WITH FOLIN–CIOCALTEU'S REAGENT AND ELUTION WITH WATER

Substance	*Volume of solution (ml)*	*Mean optical density of solution*			
		After elution with water alone	*Relative standard deviation (%)*	*After elution plus additional reaction*	*Relative standard deviation (%)*
Oleoeuropeine	5	0.073	± 5.3	0.239	± 1.9
β-(3,4-Dihydroxyphenyl)-ethanol	10	0.047	± 11.8	0.495	± 0.2
Tyrosol	10	0.057	± 13.4	0.378	± 1.7
Protocatechuic acid	10	0.074	± 4.1	0.627	± 1.3

As the results were very satisfactory, we have applied this method to the determination of other phenolic substances connected with olives. The optical densities at 725 nm obtained with 50 μg of these phenolic substances after chromatography on silica gel plates, detected with Folin–Ciocalteu's reagent, eluted with water and treated again in test-tubes with the same reagent, are given in Table I, and are compared with those obtained after detection and elution alone.

Furthermore, we have studied the possibility of applying the same method to various phenolic compounds of interest in pharmaceutical and food analysis. The results were always good, although in some instances, according to the particular characteristic of each individual substance, it was necessary to modify the original method.

EXPERIMENTAL

Samples

The following samples were used. (a) Phenolic substances connected with olives: oleoeuropeine, β-(3,4-dihydroxyphenyl)ethanol, tyrosol, caffeic acid; (b) catechol, hydroquinone; (c) hydroxybenzoic acids: salicylic, *p*-hydroxybenzoic, protocatechuic acid; (d) antimicrobials: chlorocresol, hexachlorophene, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate; (e) propyl gallate; (f) cynarine; (g) arbutine.

Chromatoplates

The plates which were used are: Silica Gel F_{254} pre-coated TLC plates, 20 × 20 cm, layer thickness 0.25 mm (Merck, No. 5715); Cellulose F pre-coated TLC plates, 20 × 20 cm, layer thickness 0.10 mm (Merck, No. 5718); polyamide plates for TLC (aluminium support), 20 × 20 cm, layer thickness 0.10 mm (Carlo Erba, Code 485386).

Solvent systems

(A) For silica gel plates.

I Benzene–dioxane–acetic acid (90:25:4)[5].
II Chloroform–ethyl acetate–formic acid (5:4:1)[6].
III Light petroleum–benzene–acetic acid (1:2:1)[7].
IV Ethyl acetate–methanol–water (100:16.5:13.5).
V *n*-Heptane–acetic acid (100:20)[8].
VI Light petroleum–ethyl acetate–acetic acid (8:1:1)[9].

(B) For cellulose plates.

VII Chloroform–acetic acid–water (8:2:1).
VIII *n*-Butanol–acetic acid–water (6:2:1).

(C) For polyamide plates.

IX Methanol–acetone–water (3:1:1)[10].
X Methanol–acetone–water (6:1:1)[10].

Application of the samples

Various amounts (10–100 μg) of each phenolic substance in methanolic solution (2 mg/ml) were applied on the plates as bands 2 cm wide at a distance of 3 cm from the lower edge and 2 cm from each other.

Quantitative analysis of the tested phenolic substances after thin-layer chromatography

Method A. The chromatoplates are uniformly sprayed with a 20% sodium carbonate solution, then with Folin–Ciocalteu's reagent diluted with two volumes of water. Phenolic substances appear as blue spots on a white background. The areas of sorbent containing the substances to be determined are removed from the plate with a small stainless-steel spatula, transferred into a calibrated test-tube (5 or 10 ml), then suspended in 1 ml of water by means of a glass rod. Folin–Ciocalteu's reagent (0.5 ml) is added, the suspension is mixed with the glass rod, then 20% sodium carbonate solution (3 ml) is added, mixing the suspension again. Finally, the blue liquid is diluted to an appropriate volume (5 or 10 ml) in order to obtain a solution with a colour intensity suitable for a good spectrophotometric determination. After 10 min, the liquid is centrifuged to remove both the adsorbent and the white precipitate formed with Folin–Ciocalteu's reagent. The clear blue solution is then examined spectrophotometrically at 725 nm in comparison with a blank obtained with an adsorbent area from the same chromatoplate equivalent to those with phenolic spots.

Method B. As in method A, except for use of methanol (1 ml) instead of water for the elution of the phenolic substance from the adsorbent.

Method C. The chromatoplates are uniformly sprayed only with undiluted Folin–Ciocalteu's reagent; phenolic substances appear as blue–green spots. The recovery of the spots and the subsequent operations are carried out as in method A.

Method D. As in method C, except for the use of methanol (1 ml) instead of water for the elution of the phenolic substance from the adsorbent.

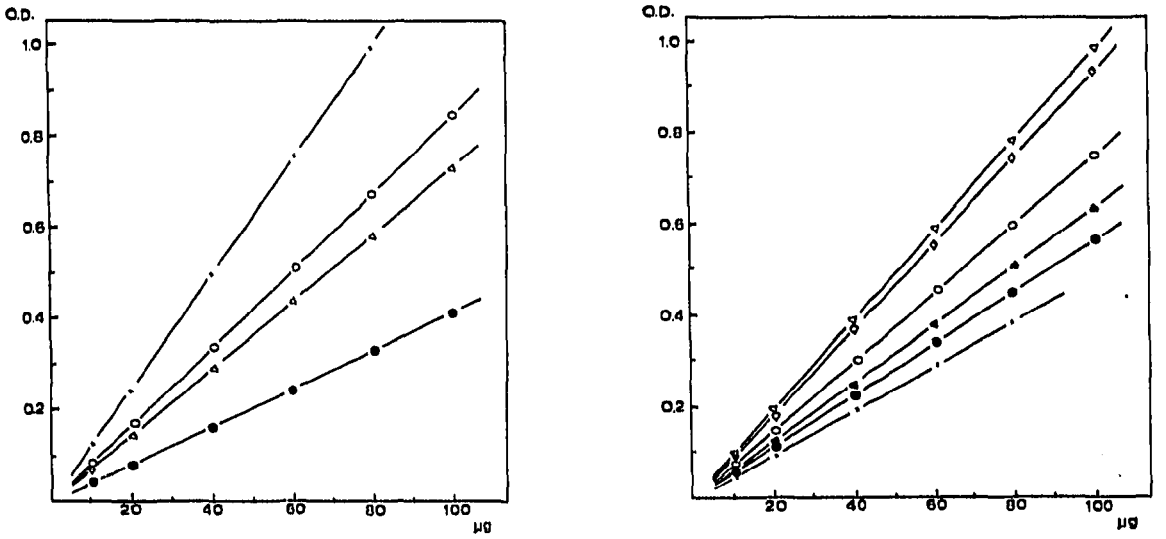

Fig. 1. Optical density *versus* concentration curves for the determination after silica gel thin-layer chromatography of oleoeuropeine (●—●), β-(3,4-dihydroxyphenyl)ethanol (○—○), tyrosol (△—△) and caffeic acid (×—×).

Fig. 2. Optical density *versus* concentration curves for the determination after silica gel thin-layer chromatography of salicylic acid (●—●), *p*-hydroxybenzoic acid (○—○), protocatechuic acid (△—△), chlorocresol (▲—▲), propyl gallate (◇—◇) and hexachlorophene (×—×).

RESULTS AND DISCUSSION

Determinations after silica gel thin-layer chromatography

For the four phenolic substances connected with olives, oleoeuropeine (R_F = 0.60 with solvent I), β-(3,4-dihydroxyphenyl)ethanol, tyrosol and caffeic acid (R_F = 0.52, 0.72 and 0.68, respectively, with solvent II), good proportionality was obtained between concentration and optical density within the experimental limits of 10 and 100 μg by method A (Fig. 1).

With method A, good determinations were also obtained for salicylic acid, *p*-hydroxybenzoic acid, protocatechuic acid, chlorocresol (R_F = 0.61, 0.59, 0.38 and 0.81, respectively, with solvent I) and propyl gallate (R_F = 0.28 with solvent III) (Fig. 2).

Methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate (R_F = 0.86 and 0.92, respectively, with solvent VI) were detected as weak spots, but sufficiently visible for their clear localization on the chromatoplate. Treatment according to method A yielded solutions with optical densities too low for accurate determinations, owing to the weak reactivity of both substances with Folin–Ciocalteu's reagent. However, good results were obtained by suspending the sorbent area containing the spot in 1 *N* sodium hydroxide and heating it in a boiling water bath for 20 min, in order to hydrolyse the ester. Following method A, satisfactorily coloured solutions were obtained, comparable with those obtained from the free *p*-hydroxybenzoic acid (see Fig. 2).

For the slightly soluble hexachlorophene (R_F = 0.16 with solvent V), it was necessary to carry out the elution of the detected spots of the substance with methanol instead of water (method B). The results were satisfactory (Fig. 2).

Determinations after cellulose thin-layer chromatography

The results were as good as those obtained by silica gel chromatography. Tyrosol (R_F = 0.69 with solvent VII) and the glucoside arbutine (R_F = 0.42 with solvent VIII) were determined by method A.

Hydroquinone, catechol (R_F = 0.16 and 0.62, respectively, with solvent VII) and caffeic acid (R_F = 0.80 with solvent VIII) required a modified method for detecting the spots, because of the instability of these substances on cellulose in the presence of alkali, which causes serious interference in the subsequent determination. Undiluted Folin–Ciocalteu's reagent was used alone, without spraying with sodium carbonate solution (method C). The same conditions of detection were used for propyl gallate (R_F = 0.47 with solvent VII) and cynarine (R_F = 0.49 with solvent VIII); for both substances, the use of methanol was necessary for eluting the spots before adding the reagent (method D).

Some of the optical density–concentration curves obtained at 725 nm are shown in Fig. 3.

Determinations after polyamide thin-layer chromatography

In spite of the satisfactory detection of the tested phenolic substances, the subsequent reaction with Folin–Ciocalteu's reagent of the recovered spots in the test-tube failed. It was concluded that polyamide strongly inhibited the reaction between phenols and phosphomolybdotungstic acid.

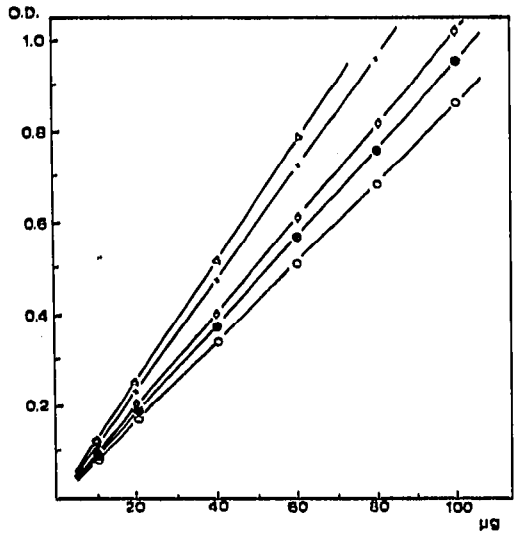

Fig. 3. Optical density *versus* concentration curves for the determination after cellulose thin-layer chromatography of tyrosol (●—●), arbutine (○—○), catechol (△—△), hydroquinone (×—×) and cynarine (◇—◇).

Accuracy of the methods

The relative standard deviations in the spectrophotometric determination of the phenolic substances are given in Table II for both silica gel and cellulose chromatoplates.

TABLE II

ACCURACY OF THE METHODS

Substance	*After silica gel TLC*		*After cellulose TLC*	
	Method	*Relative standard deviation (%)*	*Method*	*Relative standard deviation (%)*
Oleoeuropeine	A	± 1.9		
β-(3,4-Dihydroxyphenyl)ethanol	A	± 0.2		
Tyrosol	A	± 1.7	A	± 1.6
Caffeic acid	A	± 1.3	C	± 1.4
Catechol			C	± 1.3
Hydroquinone			C	± 1.8
Salicylic acid	A	± 2.6		
p-Hydroxybenzoic acid	A	± 1.5		
Protocatechuic acid	A	± 1.3		
Chlorocresol	A	± 1.8		
Hexachlorophene	B	± 1.5		
Propyl gallate	A	± 1.6	D	± 1.5
Cynarine			D	± 1.7
Arbutine			A	± 1.2

CONCLUSIONS

The original objective of this study was to develop a rapid and sensitive method for the quantitative analysis of some phenolic substances present in olives after their thin-layer chromatographic separation. The satisfactory results obtained after a large number of determinations encouraged us to extend the method to other substances containing unsubstituted phenolic groups; this method appeared to be suitable for general application, but modifications were required for some substances according to their solubility and their reactivity with Folin–Ciocalteu's reagent, as stated above.

After the unequivocal detection of the phenolic substances separated on silica gel or cellulose thin-layer chromatoplates, the method offers the possibility of the determination of the substances by carrying out the chromatic reaction directly on the sorbent area recovered with the spot from the plate, without the necessity for carrying out a prior elution for the recovery of the substance.

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