Simultaneous TLC Separation of Khellin and Visnagin and Their Assay in *Ammi visnaga* Fruits, Extracts, and Formulations

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Abstract □ A rapid and sensitive two-dimensional TLC–colorimetric method for the simultaneous determination of khellin and visnagin in *Ammi visnaga* fruits, extracts, and formulations is presented. Horstmann's *m*-dinitrobenzene colorimetric method was studied; a modified method, ensuring stability of the produced color and strict adherence to Beer's law over a wide range of concentration (5–150 mcg.), is proposed.

Keyphrases □ Khellin—colorimetric determination, *m*-dinitrobenzene □ Visnagin—colorimetric determination, *m*-dinitrobenzene □ Column chromatography—separation □ TLC—separation, determination □ UV spectrometry—analysis

In 1956, Schönberg and Sidky (1) described a color test for 2-methylchromones with *m*-dinitrobenzene, in the presence of alkali, whereby a deep-violet color was produced. On the basis of this color test, Horstmann (2) later devised a quantitative method for estimating khellin and visnagin in *Ammi visnaga* fruits. He allowed the dry chromone derivative to react with *m*-dinitrobenzene ethanolic solution in the presence of ethanolic potassium hydroxide; he measured the developed dark-blue color at 570 mμ. However, Horstmann stated that the color was unstable and disappeared rather quickly. The authors of this article confirmed the instability of the produced color, and they found that the linear relationship between absorbance and concentration was poor in the range of concentration (10–70 mcg.) described by Horstmann, especially with visnagin. In addition, the ethanolic potassium hydroxide solution (12%) was unstable. It changed rapidly to a reddish-brown color, thus giving interference due to background absorption. Consequently, an attempt was made to improve the *m*-dinitrobenzene colorimetric method so as to obtain a stable, sensitive, and measurable color. It was hoped that this color could be used in a new TLC–colorimetric assay of khellin and visnagin in fruits, extracts, and pharmaceutical formulations of *Ammi visnaga*.

EXPERIMENTAL

Reagents and Apparatus—The following were used: authentic samples of khellin, visnagin, and other known constituents of *Ammi visnaga* fruits; *m*-dinitrobenzene solution, 2% w/v in ethanol; potassium hydroxide solution, 50% in water; sodium chloride solution, 10% in 25% ethanol; isobutanol–ether mixture (1:1); ethanol (aldehyde free); methanol; chloroform; aluminium oxide for adsorption chromatography (British Drug House); silica gel G (Rhône Poulenc); Beckman DU spectrophotometer; and Desaga TLC equipment.

Analytical grade reagents were used whenever possible.

Color Reaction—The factors affecting color development and stability due to the action of *m*-dinitrobenzene and potassium hydroxide on khellin or visnagin were investigated. These factors were: (*a*) temperature, (*b*) time, (*c*) medium of reaction, and (*d*) concentration of reactants. The following experimental conditions for the colorimetric estimation of khellin or visnagin were found to be optimum.

For a concentration range of 5–150 mcg. of either khellin or visnagin in 1.5 ml. of an ethanolic solution, 0.5 ml. of 2% ethanolic *m*-dinitrobenzene and 0.5 ml. of 50% aqueous potassium hydroxide were adequate, giving a final ethanolic strength of about 75% in the medium. The color was obtained best at 20 ± 2°, 30 min. after the addition of the reagents. The stability of the formed color was improved by its extraction with an isobutanol–ether mixture (1:1) in the presence of sodium chloride (10%) in ethanol (25%). The absorbance of the colored organic layer was measured at 570 mμ within 30 min. after the addition of the sodium chloride solution.

Standard Curve—These same conditions were employed, and absorbance–concentration curves for khellin and for visnagin were plotted using the following method. Introduce, from a 0.01% ethanolic solution of either khellin or visnagin, variable concentrations (5–150 mcg.) into dry, glass-stoppered 15-ml. test tubes and complete to 1.5 ml. with ethanol. To each tube add 0.5 ml. of *m*-dinitrobenzene solution and 0.5 ml. of potassium hydroxide solution. Mix gently and maintain the tube at 20 ± 2° for 30 min. Then add 5 ml. of sodium chloride solution and 5 ml. of isobutanol–ether (1:1). Stopper the tube, shake it for about 10 sec., and allow the phases to separate completely. Pipet a portion of the clear bluish-violet upper layer into a 1-cm. silica cell; measure the absorbance of the color at 570 mμ against a blank within 30 min. after the addition of the sodium chloride solution.

The amount of khellin or visnagin in a solution of unknown concentration can be deduced from the respective standard curve or simply by adaptation of a *K* factor:

$$K_{\text{khellin}} = \frac{\text{khellin (mcg.)}}{A_{570}} = 85.1 \qquad \text{(Eq. 1)}$$

$$K_{\text{visnagin}} = \frac{\text{visnagin (mcg.)}}{A_{570}} = 95.4 \qquad \text{(Eq. 2)}$$

where A_{570} is the absorbance value of the respective concentration at 570 mμ. These *K* values (average of six concentrations) of khellin and visnagin must be ascertained according to prevailing experimental conditions.

Quantitative TLC Recovery of Khellin and Visnagin from Their Mixtures—Silica gel G plates[1] (20 × 20 cm. and 0.25 mm. thick) were spotted with ethanolic solutions of both khellin and visnagin corresponding to a concentration range of 20–120 mcg. for each. Spotting was done, with an Agla micrometer, at the corner of each plate 2 cm. away from the lower edge and the side edge. The plates were developed with ethyl acetate until the solvent front ascended about 15 cm. The developed plates were allowed to dry and then were redeveloped with the same solvent to 15 cm. at a right angle to the former direction. The plates were then dried and examined under UV light. The well-separated yellowish-brown and greenish-blue fluorescent areas corresponding to khellin and visnagin, respectively, were removed quantitatively by means of a vacuum zone extractor and eluted with 5 ml. of chloroform. Simultaneously, similar blank areas of the silica gel G layer, free from any constituent, were treated in the same way. The chloroform was evaporated on a water bath; the residues, after cooling, were dissolved in 1.5 ml. of ethanol. The ethanolic solution was then treated with the color-developing rea-

[1] Before the layer was spread, the silica gel G was washed three times with three parts of boiling methanol and dried.

Table I—Recovery of Khellin and Visnagin from Their Mixtures by Silica Gel G Plates

Added, mcg.	Recovered, mcg.	Recovery, %
	Khellin	
20	19.0	95.0
40	39.2	98.0
60	58.5	95.0
80	77.5	96.1
100	96.5	96.5
120	118.2	98.5
	Visnagin	
120	119.0	99.1
100	98.0	98.0
80	80.1	100.1
60	59.5	96.1
40	41.4	103.9
20	19.4	97.0

Table II—Analysis of Khellin and Visnagin[a] in Samples of *Ammi visnaga* Fruits and Extracts

Khellin, %	Visnagin, %
Fruits	
1.078	0.761
1.168	0.667
1.221	0.718
1.070	0.629
1.260	0.650
Extracts	
0.599	0.372
0.627	0.392
0.601	0.437
0.559	0.417
0.530	0.401

[a] Results represent the average of at least three assays.

gents, and the color was measured following the method previously outlined. Results were deduced from previously constructed standard curves of reference khellin and of reference visnagin recovered from silica gel G plates (Table I).

Determination of Khellin and Visnagin in the Fruits and Extracts of *Ammi Visnaga* by the TLC-Colorimetric Method—*In Fruits*—Transfer 0.5 g. of powdered *Ammi visnaga* fruits to the top of 2 g. of acid alumina in the thimble of a small continuous extraction apparatus. Exhaust the powder with chloroform (Exhaustion is tested by evaporating a few milliliters of the extract to dryness and then adding a few drops each of ethanol, *m*-dinitrobenzene solution, and potassium hydroxide solution. No violet color should be obtained.) Evaporate the extract to dryness, dissolve the residue in chloroform, adjust the volume to 1.0 ml. with chloroform in a volumetric flask, and mix well. Apply aliquots of the chloroform solution containing 30–100 mcg. each of khellin and visnagin (usually about 10-30 μl.) to the corner of a silica gel G plate and proceed as described under the preceding method. See Table II.

In Extracts—In the case of the liquid extract of *Ammi visnaga* fruits (Egyptian Pharmacopoeia 1963), an aliquot expected to contain 30–100 mcg. each of khellin and visnagin (corresponding to about 10–30 μl.) was applied directly to the plate and treated as previously described. See Table II.

Determination of Khellin in Pharmaceutical Formulations—*Simple Khellin Formulations*—Lynamine and Lynamine Forte tablets,[2] containing 20 and 100 mg. of khellin, respectively, were used (Table III).

Weigh and powder 20 tablets. To an amount of the powder equivalent to 40 mg. of khellin, add 40 ml. of ethanol and stir with a magnetic stirrer, equipped with a heating device, for 30 min. Filter the supernatant alcoholic solution through dry filter paper (Whatman No. 2) into a dry 100-ml. volumetric flask. Repeat extraction of the residue with 3 × 10 ml. of ethanol, filtering each time through the same filter into the same receiver. Wash, cool to room temperature, and adjust to volume with ethanol. Mix well. Dilute 10 ml. of this solution to 100 ml. with ethanol. From this dilution, pipet off 1.5 ml. (equivalent to 60 mcg. of khellin) into a dry, glass-stoppered test tube. Proceed with the *m*-dinitrobenzene colorimetric method described in the *Standard Curve* section, starting with the words, "add 0.5 ml. of *m*-dinitrobenzene solution. . . ."

Complex Khellin Formulations—Glucolynamine injections[2] were used. Each 10 ml. of the injection solution contained: khellin, 30 mg.; theophylline, 150 mg.; and glucose, 2000 mg.

Column Chromatographic Method—Measure accurately 10 ml. of the injection solution in a separator. Dilute with 30 ml. of water and extract with 4 × 20 ml. of chloroform. Filter the combined chloroform extracts, through a small layer of anhydrous sodium sulfate previously washed with chloroform, into a 100-ml. volumetric flask. Wash the filter, and adjust to volume with chloroform. Mix well. Evaporate 10 ml. of the chloroform solution (equivalent to 3 mg. of khellin) to dryness. Dissolve the residue in 2 ml. of chloroform and transfer to the top of a previously prepared 17-g. aluminium oxide column (1.4-cm. diameter). Carry out the chromatographic process in a dark cabinet equipped with UV light. Wash the column with chloroform containing 2% methanol. Reject the first nonfluorescent 8–10 ml. of effluent. Collect the yellowish-green fluorescent khellin zone into a 50-ml. volumetric flask until the eluate is free from any fluorescence. Bring to volume with chloroform and mix well. One milliliter of this solution is equivalent to 60 mcg. of khellin.

Pipet off 1 ml. of the chloroform extract, evaporate to dryness, dissolve the cooled residue in 1.5 ml. of ethanol, and complete the assay as previously described.

TLC Method—Spot directly a 20-μl. aliquot of the injection solution (equivalent to 60 mcg. of khellin) on a silica gel G (methanol-washed) plate and develop the plate with the chloroform–ethanol (98.5:1.5) solvent system (3) for a distance of 15 cm.

Dry the plate in air, elute khellin from the respective spot area, and proceed as previously described.

RESULTS AND DISCUSSION

During this investigation of factors affecting the development and stability of the studied color reaction, the authors found that the test could be conducted in ethanolic solutions of khellin or visnagin instead of the dried residues of the chromone substances mentioned by Horstmann (2). Accordingly, the evaporation step of the ethanolic solutions of the chromones could be avoided. The direct conduction of the reaction was possible on ethanolic solutions of khellin prepared by extraction or simple dilution of pharmaceutical preparations, *e.g.*, tablets and ampuls.

The appropriate concentration of *m*-dinitrobenzene was found to be 2% ethanolic solution as described by Horstmann (2). Lower concentrations, *e.g.*, 1%, gave lower intensities of the developed color, while higher concentrations were unsuitable because crystals separated on standing. The 2% ethanolic solution was reasonably stable when kept at room temperature (25–30°) for about 2 weeks.

The color produced by reacting khellin or visnagin with *m*-dinitrobenzene in 95% ethanol was more intense than with the 50% ethanolic medium. However, the bluish-violet color obtained in 95% ethanol, as described by Horstmann, was highly unstable and thus difficult to measure. The reddish-brown color obtained in the 50% ethanolic medium had a different wavelength of maximam absorption (480 mμ) and did not obey Beer's law. On further trials, 75% ethanol was found to be a suitable medium for obtaining a sensitive and stable color. This was achieved by the addition of 0.5 ml. of 50% aqueous potassium hydroxide solution to a mixture of ethanolic solution of the sample (1.5 ml.) and 2% *m*-dinitrobenzene solution in 95% ethanol (0.5 ml.).

The optimum temperature for this color reaction was found to be 20 ± 2°. Raising the temperature to 30 or 40° caused a diminution in the color intensity; the absorbances decreased by 20 and 15% at 30° for khellin and visnagin, respectively, although linearity between concentration and absorbance was still maintained.

The time necessary for maximum color intensity was found to be 30–40 min. Dilution of the reaction mixture after full development of the color with 50% ethanol, as described by Horstmann, led to a rapid fading of the color and diminution of the absorbance values. To increase the stability and sensitivity of the formed color after full development, the authors attempted its isolation by extraction of the reaction mixture with suitable organic solvents—*viz.*,

[2] Memphis Chemical Co., Cairo, U.A.R.

Table III—Analysis of Pharmaceutical Formulations of Khellin[a]

Samples	Labeled Khellin Content, mg.	Khellin Found, mg.	Recovery, %
Lynamine tablets	20	20.3	101.5
Lynamine Forte tablets	100	100.3	100.3
Glucolynamine injection	30		
Column		29.44	98.1
TLC		29.98	99.9

[a] Results represent the average of at least three assays.

chloroform, ether, isopropyl ether, and isobutanol. The color could not be extracted with less polar solvents such as *n*-hexane, cyclohexane, and benzene. With chloroform and isopropyl ether, the clarity of the colored solution was impaired by the presence of interfering opalescence which could not be removed by either filtration or centrifugation. Ether gave a clear solution which was stable for about 110 min., but its volatility rendered it unsuitable for spectrophotometric measurements. Extraction of the colored product with isobutanol resulted in a clear solution, but the color was unstable. An isobutanol–ether mixture (1:1) proved to be the most suitable solvent, being less volatile and giving sharp and quick separation of the phases during extraction of the developed color, which was aided by the addition of 10% sodium chloride in 25% ethanol. The separated organic layer was clear and suitable for direct spectrophotometric measurement. The color thus obtained was found to be stable for at least 45 min. and obeyed Beer's law over a concentration range of 5–150 mcg. for either khellin or visnagin, which is a much wider range than that described by Horstmann (10–70 mcg.).

3,5-Dinitrobenzoic acid was also tried as a color-developing reagent, using similar conditions as described for the *m*-dinitrobenzene method. It gave a bluish-violet colored product with khellin and visnagin and was also soluble in an isobutanol–ether mixture (1:1).

Khellol and khellol-glucoside reacted also with *m*-dinitrobenzene and 3,5-dinitrobenzoic acid in the same manner as did khellin and visnagin, although their colored products with 3,5-dinitrobenzoic acid were not extractable with the organic solvent mixture, isobutanol–ether (1:1).

Extraction of the active principles from the powdered fruits was carried out by using chloroform according to a previously described method (4). Horstmann used boiling water, a nonselective solvent leading to the extraction of water-soluble impurities, which necessitated a preliminary purification of the aqueous extract before paper chromatography. The presence of acid aluminium oxide during the extraction step was beneficial; it retained most of the extractive matter other than the chromone constituents (4). In the proposed method, the chloroformic extract was chromatographed (TLC) directly without any need for preliminary purification.

Horstmann effected the separation of khellin and visnagin from *Ammi visnaga* fruit extracts by paper chromatography using filter paper impregnated with polyamide and water-saturated butanol as the developing solvent. In view of the well-known advantages, especially rapidity, of TLC over paper chromatography, the authors of this study resorted to the former technique. Successful separation of khellin and visnagin from the other constituents of *Ammi visnaga* fruits has been achieved on silica gel G plates (3). Contrary to previous findings (5, 6), the authors were not able to achieve complete separation of khellin from visnagin using the solvent systems described. Solvent systems other than those reported in the literature, *e.g.*, chloroform–ethanol–methanol (97:1:2), chloroform–methanol–formamide (90:5:5), and chloroform–methanol–water (90:9:1), were tried and found unsatisfactory. However, two-dimensional TLC on silica gel G plates (Fig. 1), using ethyl acetate as the developing system, offered a better separation of khellin from visnagin than the unidimensional multiple-run technique. This method was used in the quantitative recovery of these two constituents and their subsequent colorimetric estimation. The results cited in Table I indicate the efficient recovery of khellin (95–98.5%) and visnagin (96.1–103.5%). The ratio of khellin content to visnagin content in the examined fruits and extracts of *Ammi visnaga* was found to range from 1.4:1 to 1.9:1 and from 1.3:1 to 1.6:1, respectively.

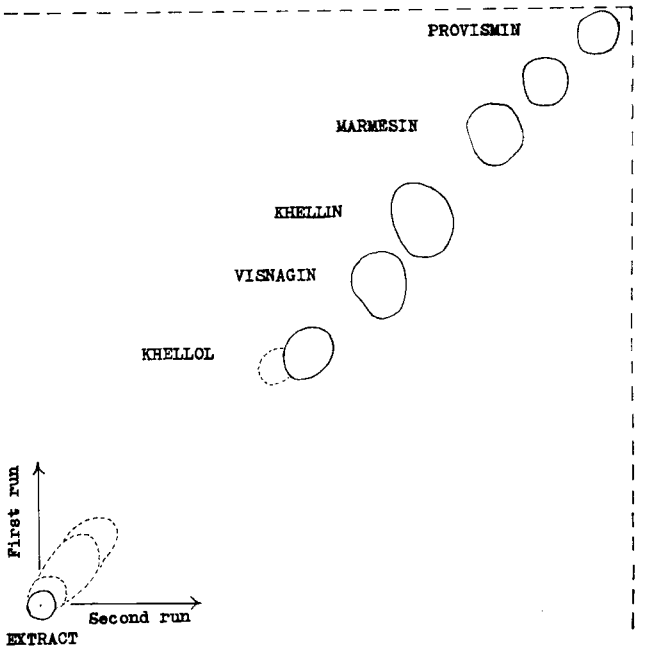

Figure 1—*Two-dimensional TLC using ethyl acetate as the developing system.*

Quantitative extraction of khellin from tablets was achieved efficiently by hot ethanol. The *m*-dinitrobenzene colorimetric method could then be applied directly to the suitably diluted ethanolic extract. On the other hand, direct application of this color reaction to complex khellin formulations (*e.g.*, glucolynamine injection) after suitable dilution with ethanol (1:100) was unsuccessful. The results obtained were about 70% of the labeled khellin concentration. Thus the separation of khellin from the other interfering ingredients (theophylline and glucose) was deemed necessary. This was achieved by either chromatographing the chloroform extract on aluminium oxide or recovery of khellin by TLC prior to its colorimetric determination.

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