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SEPARATION OF CROCETIN GLYCOSYL ESTERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Different methods for the separation of crocetin glycosyl esters from the ethanolic extract of saffron by high-performance liquid chromatography are discussed. After a clean-up by gel filtration on Sephadex G-50, best results were obtained with LiChrosorb SI 60 as stationary phase and ethyl acetate–isopropanol–water (56:34:10) as mobile phase.

INTRODUCTION

Earlier we reported our investigations of the carotenoid composition in the ethanol extract of saffron (stigma of *Crocus sativus* L.)[2,3]. Beside crocin, which has long been known as the digentiobiosyl ester of the polyene dicarboxylic acid crocetin (1), we isolated four additional pigments. As shown in Fig. 1, the new compounds are mono- and diglycosyl esters of crocetin (2)–(5).

For the isolation of these carotenoids, the ethanol extract was first cleaned up on a countercurrent distribution apparatus according to Signer[4], in the system *n*-butanol–water, and each fraction was purified twice by thin-layer chromatography (TLC). This procedure is very tedious, especially if large amounts of the pigments are needed for spectroscopic investigations. With the same method, we also examined the carotenoid composition of garden crocuses (*Crocus albiflorus, Crocus neapolitanus*) with regard to the elucidation of the biosynthesis of C_{20} carotenoids[5]. The investigations showed that these flowers also contain different glycosyl esters of crocetin as main pigments. Compared to saffron, new, more polar compounds were found, of which the main components seemed to be trisaccharide esters of crocetin. In this case, the separation method originally used turned out to be inadequate because of its poor performance. In the present paper, we report about a new method to separate the crocetin glycosyl esters using high-performance liquid chromatography (HPLC). Saffron was chosen for this study, because of its easy availability and the known composition of its pigments.

* Second report on separation of carotenoids by HPLC; for first report see ref. 1.
** Part of the planned Ph D. thesis of Martin Rychener.

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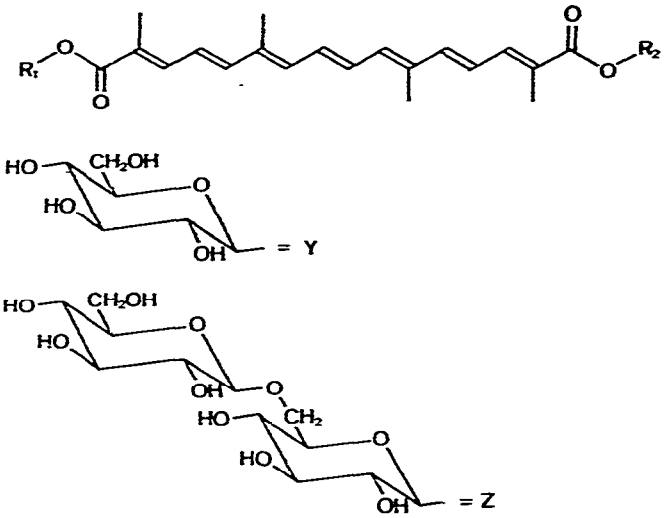

Fig. 1. Structures of crocetin glycosyl esters from saffron (*Crocus sativus* L.). 1, $R_1 = R_2 =$ Z (crocin); 2, $R_1 =$ Z, $R_2 =$ Y; 3, $R_1 =$ Z, $R_2 =$ H; 4, $R_1 = R_2 =$ Y; 5, $R_1 =$ Y, $R_2 =$ H; 6, $R_1 = R_2 =$ H (crocetin).

METHODS AND MATERIALS

General

Extraction and separation were carried out under shielding from light. Solvents were purified by standard methods[6].

Apparatus

An Altex Model 110A pump, a Rheodyne Type 7125 sample inlet system, and a Uvikon 725 detector were used. The columns were SS 316 (250 × 4.6 mm I.D.).

Clean-up

The extraction was carried out according to the details in ref. 2, followed by evaporation of ethanol. The aqueous solution was lyophilised for 24–36 h. A deep red, granular powder was obtained, in a yield of 52 g from 100 g of saffron (*Crocus naturalis pulvis*, Siegfried AG).

Gel filtration

A 5-g amount of extract dissolved in 15 ml of H_2O, was placed on a Quickfit column (80 cm × 38 mm I.D.) charged with Sephadex G-50 (Pharmacia, Uppsala, Sweden) and eluted with H_2O saturated with $CHCl_3$, at an upward flow-rate of 0.2–0.4 ml/min by hydrostatic pressure. The coloured eluate was evaporated and dried under high vacuum. The yield of the deep red powder was 800 mg.

Conditions for HPLC

Method A. The stationary phase was LiChrosorb SI 60, 7 μm (Merck, Darmstadt, G.F.R.). The mobile phase was ethyl acetate–isopropanol–water (56:34:10); UV detection was at 440 nm, and the flow-rate was 0.6 ml/min. The sample consisted of 20 μl of a solution in the mobile phase (crocetin chromophore

titre of 140 nmol/ml). The column was slurry-packed at 400 bar, giving a number of theoretical plates of 6000–8000 with crocetin (Hoffmann-La Roche, Basel, Switzerland) as test substance.

Method B. The stationary phase was LiChrosorb RP-18, 5 μm (Merck). The mobile phase was methanol–water (60:40). UV detection was at 440 nm, and the flow-rate was 1 ml/min. The sample consisted of 20 μl of a solution in ethanol–H_2O (titre: 220 nmol/ml). The column was packed according to the method in ref. 7, giving a number of theoretical plates of 3000–4000 with phenol as test substance.

Method C. The stationary phase was LiChrosorb SI 60, 7 μm (Merck), coated with 0.1 *M* $NaHSO_4$. The mobile phase was ethyl acetate–*n*-hexane (70:30). UV detection was at 440 nm, and the flow-rate was 0.4 ml/min. The sample consisted of 20 μl of solution in the mobile phase (titre: 220 nmol/ml). The column was packed according to the method in ref. 7, giving a number of theoretical plates of 5500–7500 with acetophenone as test substance.

Identification

The compounds were identified by mass spectrometry and UV/visible spectra, and TLC of the carbohydrates. Synthetic crocetin and crocetin-di(β-D-glucosyl) ester were used as reference compounds.

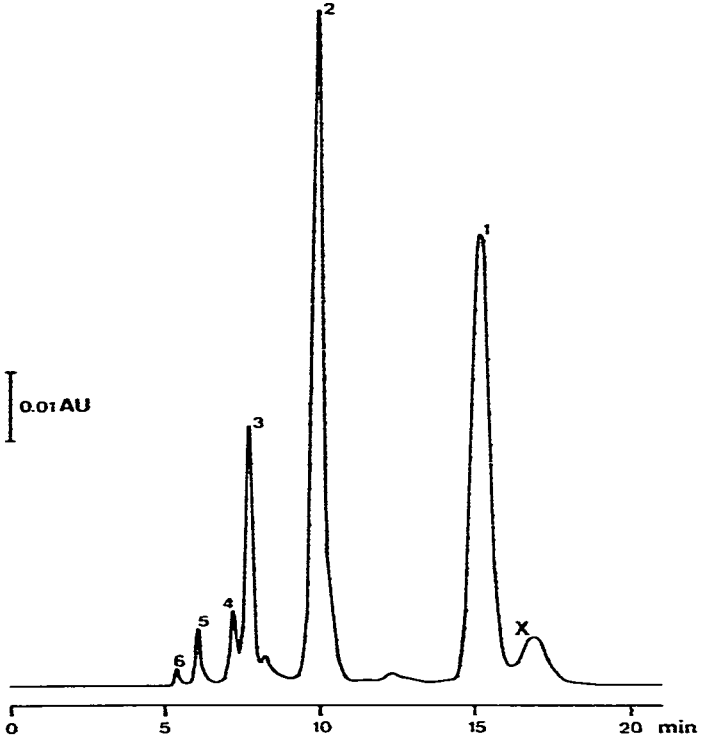

Fig. 2. Separation of precleaned saffron extract (method A).

RESULTS AND DISCUSSION

Experiments to separate the pigments of the ethanol extract of saffron without a clean up were unsuccessful. The best results were obtained after lyophilisation of the extract followed by gel filtration on Sephadex G-60. The lyophilisation of the aqueous extract yielded a deep red, granular powder and the gel filtration removed the free carbohydrates, which disturb the separation of the pigments.

HPLC on LiChrosorb SI 60 with ethyl acetate–isopropanol–water (56:34:10) (method A) proved to be successful for the separation of the carotenoid pigments. The compounds 1–6 could be separated within 20 min, as shown in Fig. 2.

As expected, the separation of the diglucosyl ester from the monogentiobiosyl ester turned out to be very difficult. The separation time could possibly be shortened using a gradient, but because of the unusual addition of water to the mobile phase, which is necessary owing to the high polarity of the glycosylesters, the reconditioning of the column would be time consuming.

Besides the pigments 1–6, which were already known, a new compound X appeared in the chromatogram. This pigment, which is more polar than crocin, is identical with the trisaccharide ester that occurs as main component in garden crocuses. Because of its low concentration in saffron, X was not detected using TLC. The chromatogram also showed the quantitative composition of the pigments in saffron. As expected, the main pigment is crocin (*ca.* 40–45 %), followed by the mixed ester 2 (*ca.* 35 %), the diglucosyl ester 3 (*ca.* 10 %) and the two monoesters 4 and 5 (each *ca.* 2 %).

The reversed-phase systems, often used to separate polar compounds, proved to be useful only for the separation of diglycosyl esters. Fig. 3 shows a perfect separa-

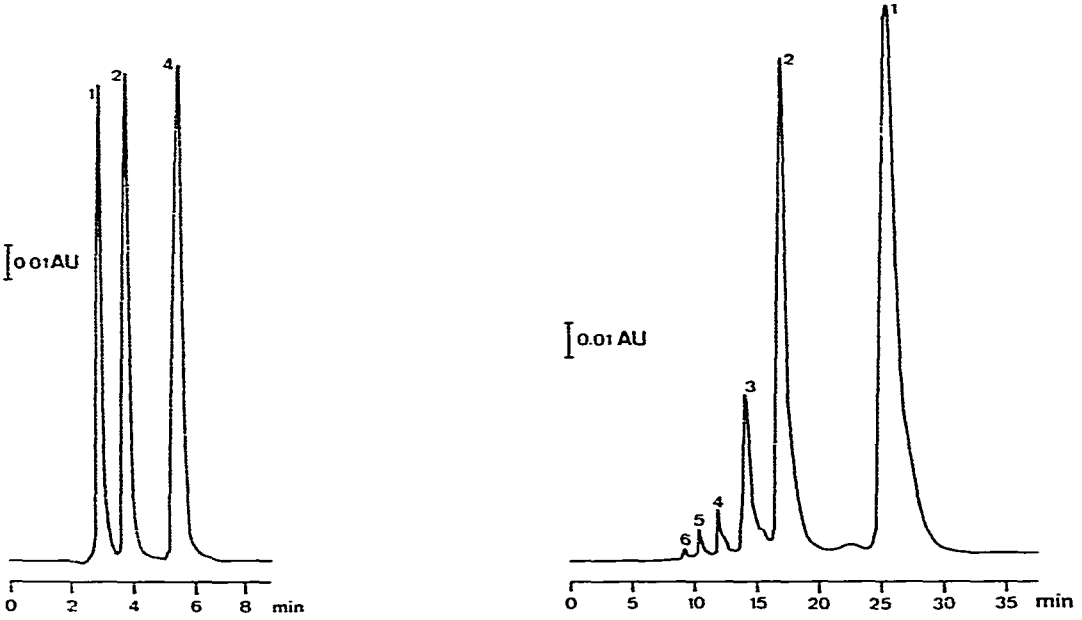

Fig. 3. Separation of diglycosyl esters from saffron (method B).

Fig. 4. Separation of precleaned and acetylated saffron extract (method C).

tion of the digentiobiosyl (1), the diglucosyl (4) and the mixed ester (2) within 6 min, using a LiChrosorb RP-18 column (method B).

For the separation of compounds with a free carboxyl group, *i.e.* the monoglycosyl esters and crocetin, method B was unsuitable because of a partial deprotonation of the carboxyl groups under these conditions. This resulted in strong tailing and extremely long retention times. The addition of an acid to the mobile phase is not appropriate owing to the lability of the glycosyl esters towards hydrolysis.

Because peracetates of the glycosyl esters are generally better separable than the non-acetylated ones, the cleaned up extract was peracetylated with acetic anhydride in pyridine. To avoid chemical tailing, a LiChrosorb SI 60 column was coated with 0.1 *M* $NaHSO_4$ (ref. 8) and ethyl acetate–*n*-hexane (70:30) was used as mobile phase (method C) (Fig. 4). A relatively slow flow-rate (0.4 ml/min) was needed to obtain a satisfactory separation. The separation time, however, was longer compared with that of the non-acetylated pigments, and the polar pigment corresponding to X was not separated from the crocin under these conditions. Moreover, the quality of the coated column decreased quickly.

Our investigations have shown that the best results in the separation of glycosyl esters from saffron can be obtained by using HPLC on silica gel after cleaning up the extract by lyophilisation and gel filtration, because of the higher number of theoretical plates of silica gel columns compared with reversed-phase systems. In comparison with TLC, HPLC gives shorter separation times under very mild conditions. The performance of the system is shown by the fact that for the first time more polar crocetin derivatives than crocin were detected in saffron.

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