Journal of Chromatography, 262 (1983) 277–284
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,760

MASS SPECTROMETRIC IDENTIFICATION OF XANTHOPHYLL FATTY ACID ESTERS FROM MARIGOLD FLOWERS (*TAGETES ERECTA*) OBTAINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CRAIG COUNTER-CURRENT DISTRIBUTION

WOLFGANG GAU*, HANS-JÜRGEN PLOSCHKE and CHRISTIAN WÜNSCHE
Pharma Analytical and Quality Control Department, Bayer AG, 5600 Wuppertal (G.F.R.)
(Received February 7th, 1983)

SUMMARY

By means of reversed-phase high-performance liquid chromatography in the system acetonitrile–dichloromethane, the xanthophyll fatty acid esters from a purified extract of marigold flower petals (*Tagetes erecta*) were isolated on a semipreparative scale. The structures of the xanthophyll fatty acid esters were elucidated by mass spectrometry. The presence of the hitherto unknown mixed esters xanthophyll palmitate stearate and xanthophyll palmitate myristate was demonstrated, in addition to the major component xanthophyll dipalmitate. The latter was isolated in larger quantities from the xanthophyll fatty acid ester mixture by Craig counter-current distribution.

INTRODUCTION

The fatty acid esters of xanthophyll[1], extracted from the petals of the marigold flower (*Tagetes erecta*), have been the subject of numerous investigations. Alam *et al.*[2] reported that xanthophyll in the marigold is esterified with lauric, myristic, palmitic and stearic acids. The quantification of some fatty acid esters of xanthophyll in an extract of marigold petals was described by Phillip and Berry[3]; the same authors also reported on the purification of xanthophyll fatty acid esters from marigold petals and the use of these esters as food dyes[4].

A purified extract of marigold petals, mainly containing xanthophyll dipalmitate (lutein dipalmitate), is marketed as an opthalmological agent under the name Adaptinol®.

The aim of this investigation was to separate the components of extracts from marigold petals using high-performance liquid chromatography (HPLC) and to elucidate the structure of hitherto unknown fatty acid esters of xanthophyll by mass spectrometry (MS). The use of a liquid–liquid distribution technique (Craig counter-current distribution, CCD) is described as an alternative to the previous purification methods and as a method for the preparation of pure xanthophyll dipalmitate in larger quantities.

0021-9673/83/$03.00

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph 1084B with automatic injection system (Type 79 842/79 841A) and variable-wavelength detector (Type 79 875A) (Hewlett-Packard, Waldbronn, G.F.R.) was used. The analytical columns (12.5 cm × 4 mm I.D.) were Hibar-Columns Batch 79 6434 (E. Merck, Darmstadt, G.F.R.) packed with RP-18 (5 μm). The preparative column (25 × 2.5 cm) was packed with LiChrosorb RP-18 (10 μm) (E. Merck) using the slurry method.

The CCD apparatus (Labortec, Basle, Switzerland) was equipped with 240m distribution tubes. The phase volume of the apparatus was 50 ml.

A Type 554 spectrophotometer (Perkin-Elmer, Überlingen, G.F.R.) was used to record the absorption spectra. The mass spectra were recorded on a MAT-CH5/DF apparatus (formerly Varian, now Finnigan-MAT, Bremen, G.F.R.): electron energy, 70 eV; source temperature, 200°C; direct inlet; probe temperature, 250°C.

Reagents and starting materials

The eluents used in HPLC, acetonitrile and dichloromethane, were of Lichrosolv® quality. Dimethylformamide, *n*-hexane and dichloromethane, used in the liquid–liquid distributions, were of p.a. quality. The UV spectral measurements were carried out in *n*-hexane (Uvasol). All solvents were supplied by E. Merck.

A pre-purified extract of *Tagetes* petals (*Tagetes*, sp. Golden Dollar, Batch 535781-89; Bayer, Wuppertal, G.F.R.) was used in these investigations.

HPLC

A Merck Hibar column (12.5 cm × 4 mm), packed with RP-18 (5 μm), was used for the analysis of the starting material. The mobile phase was dichloromethane–acetonitrile (25:75, v/v) and the flow-rate 3 ml/min. The detector was set at 471 nm. A 0.1% solution (20 μl) of the starting material was injected.

A preparative column (25 cm × 25 mm I.D.), packed with LiChrosorb RP-18 (10 μm), was used for the semipreparative isolation of the individual components of the Tagetes petals extract. The mobile phase was acetonitrile–dichloromethane (65:35, v/v); the flow-rate 15 ml/min. Detection was carried out at 471 nm. Each sample (11 × 25 mg dissolved in 250 μl eluent) was introduced onto the column via a sample loop. The separated substances were collected and the solvent removed under a flow of argon at 30°C on a rotary evaporator.

Preparation of xanthophyll dipalmitate standard by liquid–liquid distribution

A 25-g amount of starting material was dissolved in 375 ml of dimethylformamide–dichloromethane–hexane (8:2:10, v/v/v) and transferred to the first five tubes of the distribution train: number of distribution tubes, 240; volume of lower phase, 50 ml; upper phase, 25 ml; volume factor, 0.5; intensity of shaking, 40; number of shaking movements, 30; separation time, 2.5 min. The liquid–liquid distribution was carried out in dimethylformamide–dichloromethane–hexane (8:2:10, v/v/v) under argon with exclusion of light, using a recycling procedure. The progress of the separation was monitored by HPLC. After $n = 2025$ transfers the main fraction was found in tubes 35–65. In addition, two less pure sub-fractions were found

in tubes 25–34 and 66–75. The solvents used had previously been saturated with argon.

Fractions 35–65 were combined and the upper phase separated from the lower under exclusion of light. The lower phase was extracted four times with 500 ml *n*-hexane each time, the hexane extracts were combined with the upper phase and the combined organic phases washed twice with 500 ml water to remove the dimethylformamide. The organic phase was then dried over sodium sulphate and the solvent evaporated at 40°C on a rotary evaporator (water pump vacuum). The residue was dried for 16 h at 30°C over blue silica gel and paraffin flakes in a vacuum drying oven. The yield of pure xanthophyll dipalmitate was 710 mg.

RESULTS AND DISCUSSION

The absorption spectrum (Fig. 1) of the *Tagetes* petals extract (starting material) exhibits maxima typical of xanthophylls which, however, are shifted hypsochromically by several nanometres compared to the literature values[5] although the spectrum was recorded in the same solvent and after wavelength calibration.

Further study of the extract by HPLC showed that it was composed of five main components (Fig. 2), the structures of which were elucidated with the aid of mass spectrometry (Table I). In previous analyses of xanthophyll fatty acid esters, thin-layer chromatography (TLC) was used. The separation performance of TLC has now been surpassed by that of HPLC.

Philip and Berry[3] had postulated the presence of xanthophyll dipalmitate, dimyristate and monomyristate in a *Tagetes* extract, after comparison of R_F values using synthetic xanthophyll fatty acid esters. Upon alkaline saponification of a pu-

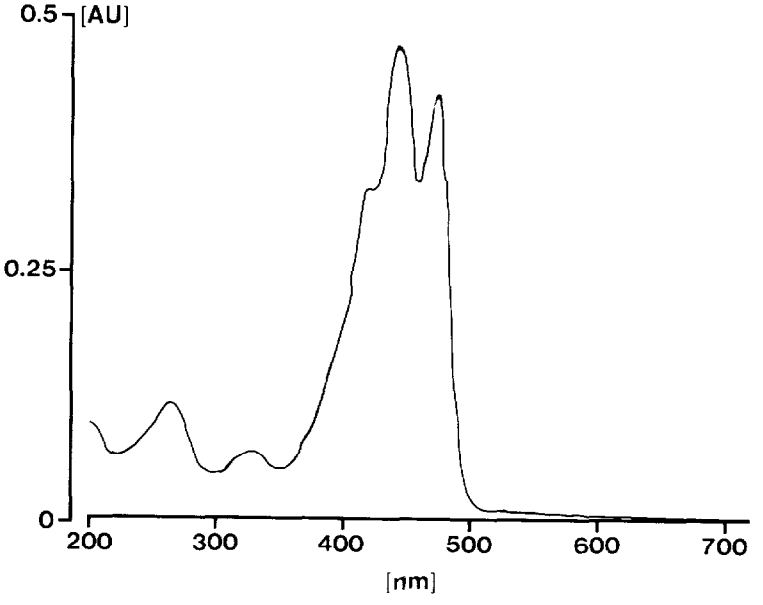


Fig. 1. Absorption spectrum of an extract of *Tagetes* petals (*Tagetes*, sp. Golden Dollar). Concentration: $8.7 \cdot 10^{-4}$% (w/v). Solvent: hexane. Cell length: 10 mm.

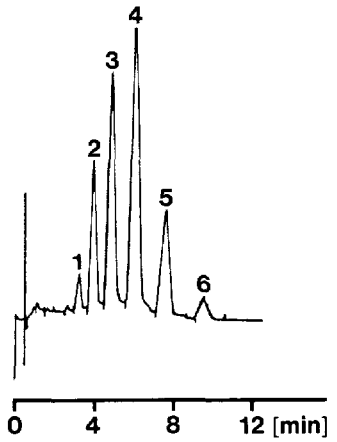


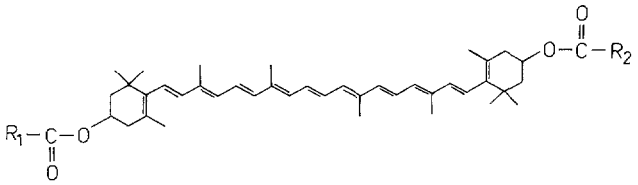
Fig. 2. HPLC of an extract of *Tagetes* petals (*Tagetes*, sp. Golden Dollar). Mobile phase: dichloromethane–acetonitrile (25:75); flow-rate: 3 ml/min. Stationary phase: RP-18, 5 μm, 12.5 cm × 4 mm. Detection at 471 nm. Injected volume: 20 μl of a 0.1% solution. Peaks: 1 = unknown; 2 = xanthophyll dimyristate; 3 = xanthophyll myristate palmitate; 4 = xanthophyll dipalmitate; 5 = xanthophyll palmitate stearate; 6 = xanthophyll distearate.

rified Tagetes extract, Alam *et al.*[2] found lauric, myristic, palmitic and stearic acids in the hydrolysate (gas–liquid chromatographic analysis of the fatty acid methyl esters).

Separation of the mixed esters from the diesters of xanthophyll was not possible by TLC under the conditions given. Although the xanthophyll fatty acid esters are highly lipophilic, we used neither the obvious choice, adsorption chromatography, nor the usual HPLC on RP-18 material with acetonitrile–water mixtures[6]. In-

TABLE I

STRUCTURES AND RETENTION TIMES OF XANTHOPHYLL FATTY ESTERS IN A PURIFIED EXTRACT OF MARIGOLD PETALS



Fraction	*Retention time (min)*	*Concentration, percent area (%)*	R_1	R_2	*Xanthophyll ester*
1	3.13	2.1			Unknown
2	3.85	12.6	$H_3C(CH_2)_{12}$	$H_3C(CH_2)_{12}$	Dimyristate
3	4.76	24.7	$H_3C(CH_2)_{14}$	$H_3C(CH_2)_{12}$	Myristate palmitate
4	5.93	35.5	$H_3C(CH_2)_{14}$	$H_3C(CH_2)_{14}$	Dipalmitate
5	7.41	14.4	$H_3C(CH_2)_{16}$	$H_3C(CH_2)_{14}$	Palmitate stearate
6	9.31	2.4	$H_3C(CH_2)_{16}$	$H_3C(CH_2)_{16}$	Distearate

stead we exploited the intensive interaction of the lipophilic C_{18} chains of the RP-18 material with the lipophilic fatty acid residues of the xanthophyll esters by using medium polar eluent mixtures (dichloromethane–acetonitrile) as mobile phases in the HPLC separation.

Fig. 3 shows the chromatogram of the semipreparative isolation of the individual components. In spite of a slight overloading of the preparative column the fractions isolated are homogeneous (Fig. 4).

The technique of liquid–liquid distribution, its possibilities and limitations have been described in a monograph by Hecker[7]. In the present work, 25 g starting material in the system dimethylformamide–hexane–dichloromethane yielded 710 mg xanthophyll dipalmitate in pure form (Fig. 5) over $n = 2025$ transfer steps (recycling procedure).

We monitored the course of the separation as a function of the number of transfer steps by HPLC and calculated the number of transfers necessary for the isolation of xanthophyll dipalmitate from our knowledge of the partition coefficients of the individual components (established after $n = 625$ transfers) according to the purity function[8]

$$n = \frac{\beta}{(\beta - 1)^2}\left[9\left(2 + \frac{\alpha + \beta}{\sqrt{\alpha\beta}}\right) + 6R\left(\frac{\alpha + 1}{\sqrt{\alpha}} + \frac{\beta + 1}{\sqrt{\beta}}\right) + R^2\left(2 + \frac{\alpha\beta + 1}{\sqrt{\alpha\beta}}\right)\right]$$

where n = number of transfer steps, β = separation factor, α = volume factor and R = purity constant. The distribution curves obtained after the corresponding number of transfer steps are shown in Fig. 6.

In CCD the course of a separation is mathematically calculable and controllable. This is not the case to that extent with other chromatographic techniques. The

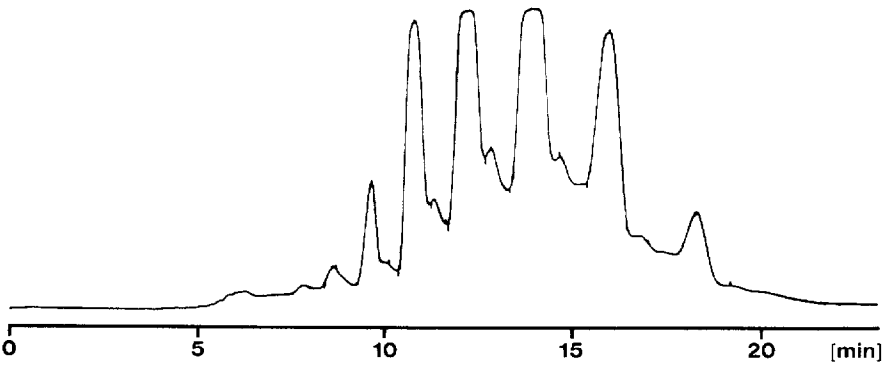
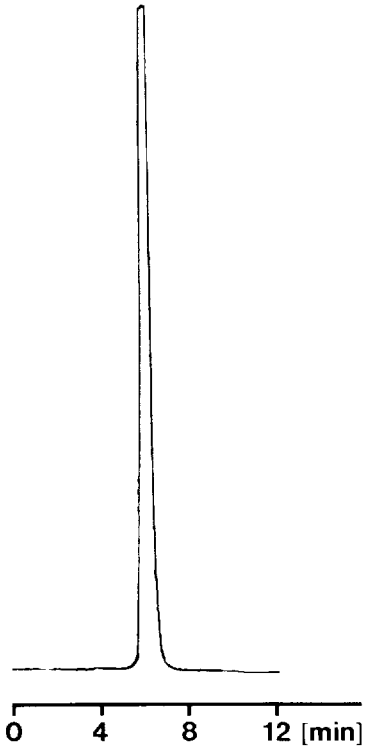


Fig. 3. Chromatogram of the semipreparative isolation of the xanthophyll fatty acid esters. Column: RP-18, 10 μm, 25 cm × 25 mm I.D. Mobile phase: acetonitrile–dichloromethane (65:35). Flow-rate: 15 ml/min. Detection at 471 nm. Injected amount: 25 mg of extract diluted in 250 μl mobile phase.



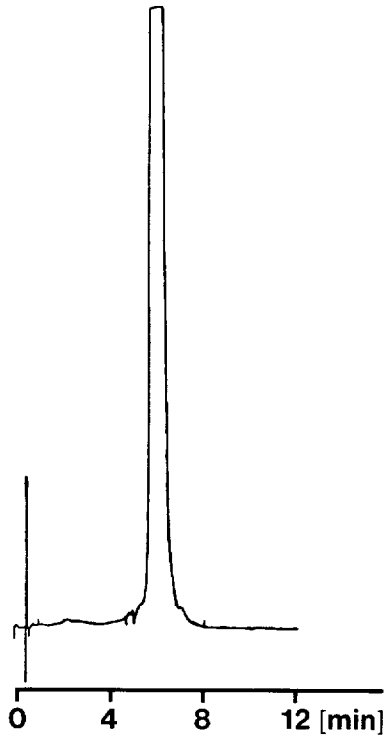
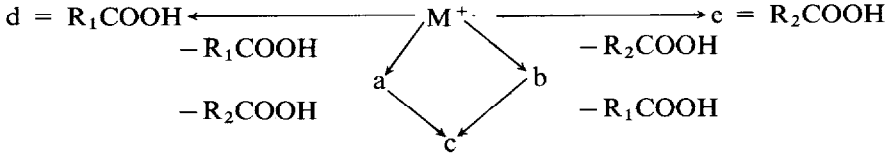


Fig. 4. Xanthophyll dipalmitate purified by semipreparative HPLC (for conditions see Fig. 2).

Fig. 5. Xanthophyll dipalmitate purified by Craig CCD (for chromatographic conditions see Fig. 2).

absorption data for the pure xanthophyll dipalmitate obtained by Craig distribution are shown in Table II (*cf.*, ref. 5).

The mass spectra of the xanthophyll fatty acid esters are characterized by their simplicity. Only six characteristic types of ions appear apart from the very intense alkyl and alkylene fragments below *m/e* 200:



The molecular ions have a relative intensity of 5–10%; the peak heights of a, b and c are about those of the fatty acid ions which form the base peak above *m/e* 200 (Table III).

The typical ions a–e are missing in the mass spectrum of fraction 1; the absence of ion c in particular shows that this fraction cannot be a xanthophyll derivative.

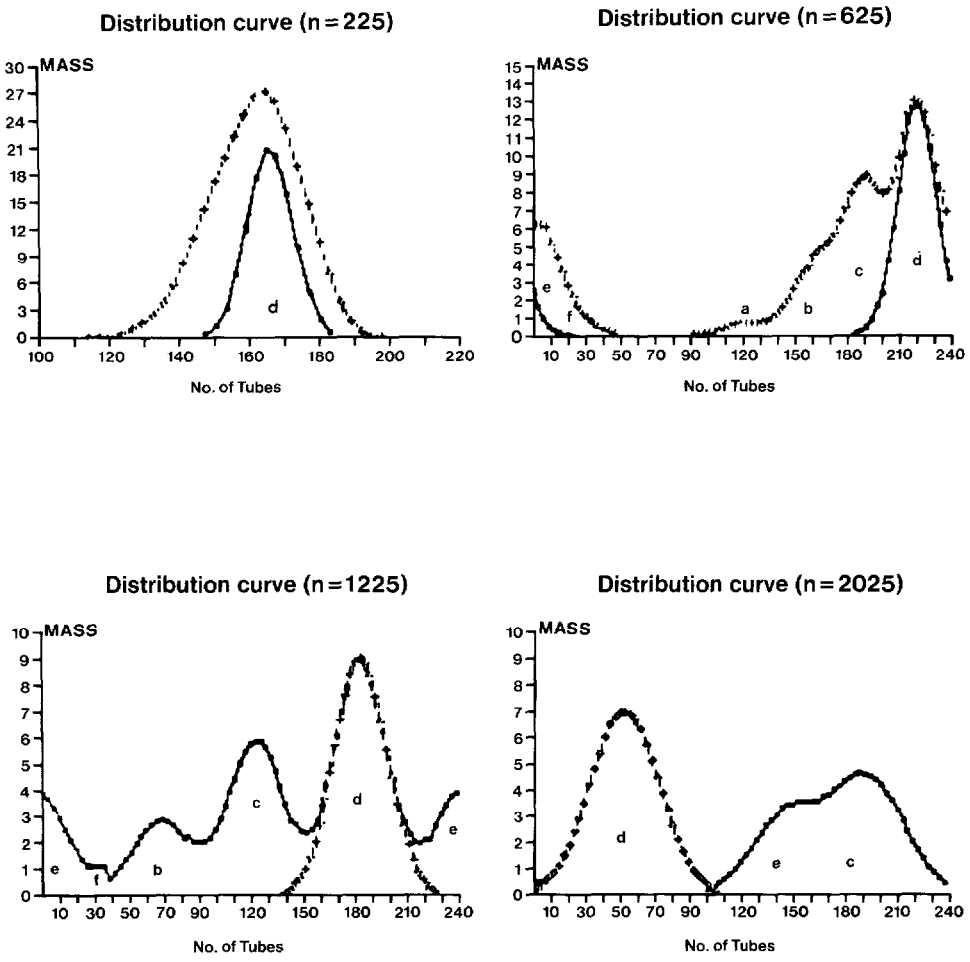


Fig. 6. Distribution curves (theoretical) after the corresponding number of transfers. a, Unknown substance, k = 1.4; b, xanthophyll dimyristate, k = 1.8; c, xanthophyll palmitate myristate, k = 2.2; d, xanthophyll dipalmitate, k = 2.8; e, xanthophyll palmitate stearate, k = 3.6; f, xanthophyll distearate, k = 4.4. k = Partition coefficient; n = number of transfers.

TABLE II

ABSORPTION DATA FOR XANTHOPHYLL DIPALMITATE PURIFIED BY COUNTER CURRENT DISTRIBUTION

Solvent: *n*-hexane.

$\lambda(nm)$	ε	$C(mol/l)$	$E^{1\%}_{1\,cm}$
471	113,152		1082
442	127,091		1215.4
419	89,647	$3.658 \cdot 10^{-6}$	857.3

TABLE III

MASS NUMBERS OF THE SIGNIFICANT IONS IN THE MASS SPECTRA OF THE XANTHOPHYLL FATTY ACID ESTERS

	Fraction				
	6	*5*	*4*	*3*	*2*
M	1100	1072	1044	1016	988
a	816	788	788	760	760
b	816	816	788	788	760
c	532	532	532	532	532
d	284	284	256	256	228
e	284	256	256	228	228

CONCLUSIONS

The separation of the xanthophyll fatty acid esters present in a purified extract of marigold flower petals was successfully accomplished by HPLC. These xanthophyll fatty acid esters were also prepared on a semipreparative scale by HPLC. It was shown that, for the preparation of larger quantities of pure xanthophyll dipalmitate, Craig CCD is the method of choice.

ACKNOWLEDGEMENTS

We are indebted to Mr. Klaus Schmidt for technical assistance and to Dr. B. Weber for supplying the spectroscopic data.

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