ELSEVIER

Journal of Chromatography B, 670 (1995) 337–348

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Separation and identification of carotenoids in bird's plumage by high-performance liquid chromatography–diode-array detection

R. Stradi*, G. Celentano, D. Nava

Istituto di Chimica Organica, Facoltà di Farmacia, Università di Milano, Via Venezian 21, Milan 20133, Italy

First received 26 October 1994; revised manuscript received 3 April 1995; accepted 11 April 1995

Abstract

The coloured feathers of *Carduelis spinus* (Siskin), *C. flammea* (Redpoll), *Serinus serinus* (Serin), *Loxia curvirostra* (Crossbill), *Pinicola enucleator* (Grossbeak), *Carpodacus roseus* (Pallas Rosefinch) and *Pyrrhula pyrrhula* (Bullfinch) have been extracted with a new procedure using mild conditions (a few minutes at room temperature). After the separation of melanines and proteins, the extracts were analyzed by HPLC–MS and HPLC–UV-Vis. The main components of the pigments were identified in all the species examined; moreover, UV-Vis and MS data were collected also for the minor components. These data suggest that minor components are generally *cis* isomers accompanying the predominant *all-trans* isomers.

1. Introduction

Carotenoid pigments are responsible for most of the bright colours of birds. The differences in feather carotenoids are determined both by the processes responsible for their absorption and transport, as well as the metabolic capacities of the birds to modify the pigments which are taken up from the diet [1]. The carotenoid constitution provides specific plumage colour and patterns [2–5], accounts for colour polymorphism [6–8], subspecific plumage variation [9,10], and plumage variants [6,11,12]. Hence, plumage carotenoids are an important tool for understanding many aspects of the bird's biology. Unfortunately, many studies on avian coloration were done before the introduction of the advanced chromatographic techniques. Thus, a body of literature exists in which the pigments are identified only as lipochromes, complex mixtures of indefinite classes of carotenoids (e.g. oxo carotenoids, canary xanthophylls, etc.).

Regardless of the biological implication or speculation involving exogenous pigments and colours in birds, a complete and accurate profile of the constitution and distribution of the carotenoids in the plumage should be made available.

The primary aim of the present work is to report a new method which allows a complete and fast extraction of the pigments at low temperature, which excludes the formation of artifacts. This extraction method, followed by high-

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(95)00173-5

performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) and UV-Vis spectroscopy was applied to the determination of the plumage's carotenoid composition in a series of palearctic fringillidae.

2. Experimental

2.1. Chemicals and reagents

Methanol, acetonitrile and acetone were obtained from Merck (Darmstadt, Germany) and filtered through a membrane filter for small volumes of liquids, 13 mm diameter, 0.5 μm, from Millipore (Milford, MA, USA).

2.2. Extraction procedure

The carotenoids were extracted and purified as follows: ca. 5 mg of coloured barbules were carefully washed with hexane on a glass filter and finely ground for 15 min at room temperature in the presence of 3 ml methanol in a Retsch mm2 micronizer (Haan, Germany) equipped with a ZrO container. The carotenoids were completely liberated from the keratin and the white residue filtered off by a Sep-Pak C_{18} Cartridge (Waters Millipore). The filtrate containing the carotenoids was evaporated under reduced pressure at room temperature. The residue was dissolved in 200 μl of acetone. After freezing for 3 h at −78°C, the precipitate was filtered off. The clear solution containing the carotenoids was evaporated under a stream of dry nitrogen and the residue dissolved in the mobile phase.

2.3. Instrumentation and chromatography

HPLC was done with a Gynkotek A 110 instrument equipped with an isocratic Gynkotek pump Model 300 (Munich, Germany). Two sequential Lichrocart Purospher RP-18 columns (250 × 4 mm I.D.) (Merck) were employed for the analysis. The temperature of the columns was maintained at 40°C with a column block heater Model 7970, Hichrom (Reading, UK).

Peaks were measured at a wavelength of 450 nm using an HP 1050 series diode-array detector. Tridimensional chromatograms were recorded from 230 to 600 nm using a HP Chem Software. Samples were injected with a Rheodyne 7125 valve equipped with a 20-μl loop. The mobile phase was acetonitrile–methanol (70:30, v/v). All eluents were degassed with helium before use and the flow-rate was set at 0.5 ml/min.

2.4. HPLC–MS analysis

Mass spectra were recorded with an HP 5988 A particle beam instrument equipped with an HP 1050 HPLC. The same column used for HPLC analysis was employed. Samples were ionized by negative chemical ionization (CI) with methane. The ionization energy was 240 eV.

2.5. Reference carotenoids

Samples of I, II, III, IV, V, VI, VII and VIII (Table 1) were kindly supplied by Hoffmann-La Roche (Basel, Switzerland) together with the UV-Vis spectra of IX and X (acetylated).

3. Results and discussion

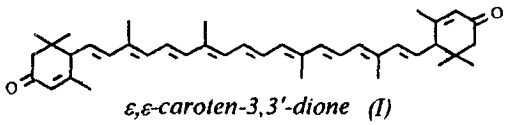
The analytical conditions employed in the present work allow the separation and the identification of the plumage carotenoids in all the species studied. Moreover, the HP 1050 series diode-array detector records the UV-Vis spectrum for any analyte (Figs. 1–3) in bidimensional or tridimensional mode. The mass spectra of each single peak were obtained, by coupling the HPLC apparatus with an HP 5988A mass spectrometer.

3.1. Main components

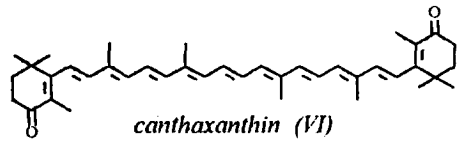
The UV-Vis and the mass spectra were compared with those described in the literature. Structural assignments were then confirmed by co-chromatography with authentic samples. Table 1 lists the main components of the pigments extracted and analyzed with the procedure described in this paper.

Table 1
Main components and structures of the pigments extracted and analyzed

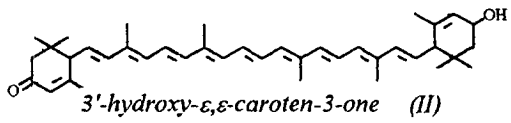
Species	Color	Main pigments	Literature data and references
Carduelis chloris (Greenfinch)	Yellow	I, II, III	Canary xanthophylls and lutein [1]
Carduelis spinus (Siskin)	Yellow	I, II	Canary xanthophylls and lutein [1]
Carduelis flammea (Redpoll)	Red	VII, X, XI	Echinenone and lutein [5]
Serinus serinus (Serin)	Yellow	I, II	Lutein [1]
Loxia curvirostra (Crossbill, male)	Yellowish red, red	I, II, III, IV, V, VI, VII	Unknown
Loxia curvirostra (Crossbill, female)	Yellowish green	I, II, III	Unknown
Carpodacus roseus (Pallas's rosefinch, male)	Reddish pink	IV, V, VI, VII	Unknown [13]
Carpodacus roseus (Pallas's rosefinch, female)	Yellowish grey	IV, V, VI, VII	Unknown [13]
Pinicola enucleator (Grossbeak, male)	Red	IV, V, VI, VII	Unknown
Pinicola enucleator (Grossbeak, female)	Yellowish brown	III, VIII	Unknown
Pyrrhula pyrrhula (Bullfinch, male)	Pink or reddish pink	IV, V, VI, IX	Canary xanthophylls and lutein [1] Unknown [13]



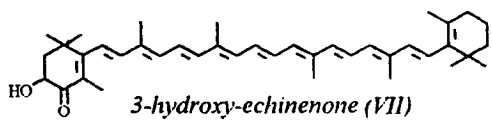
ε,ε-caroten-3,3'-dione (I)



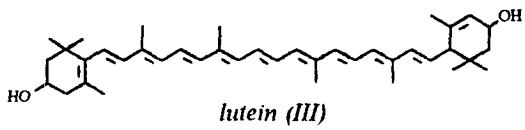
canthaxanthin (VI)



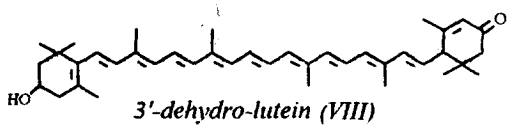
3'-hydroxy-ε,ε-caroten-3-one (II)



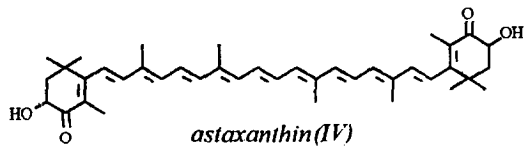
3-hydroxy-echinenone (VII)



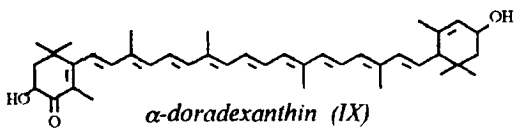
lutein (III)



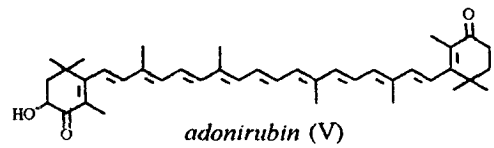
3'-dehydro-lutein (VIII)



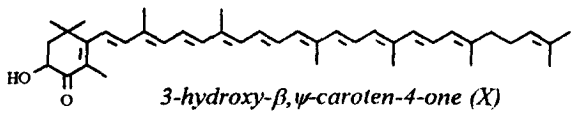
astaxanthin(IV)



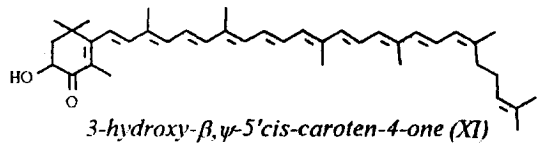
α-doradexanthin (IX)



adonirubin (V)



3-hydroxy-β,ψ-caroten-4-one (X)



3-hydroxy-β,ψ-5'cis-caroten-4-one (XI)

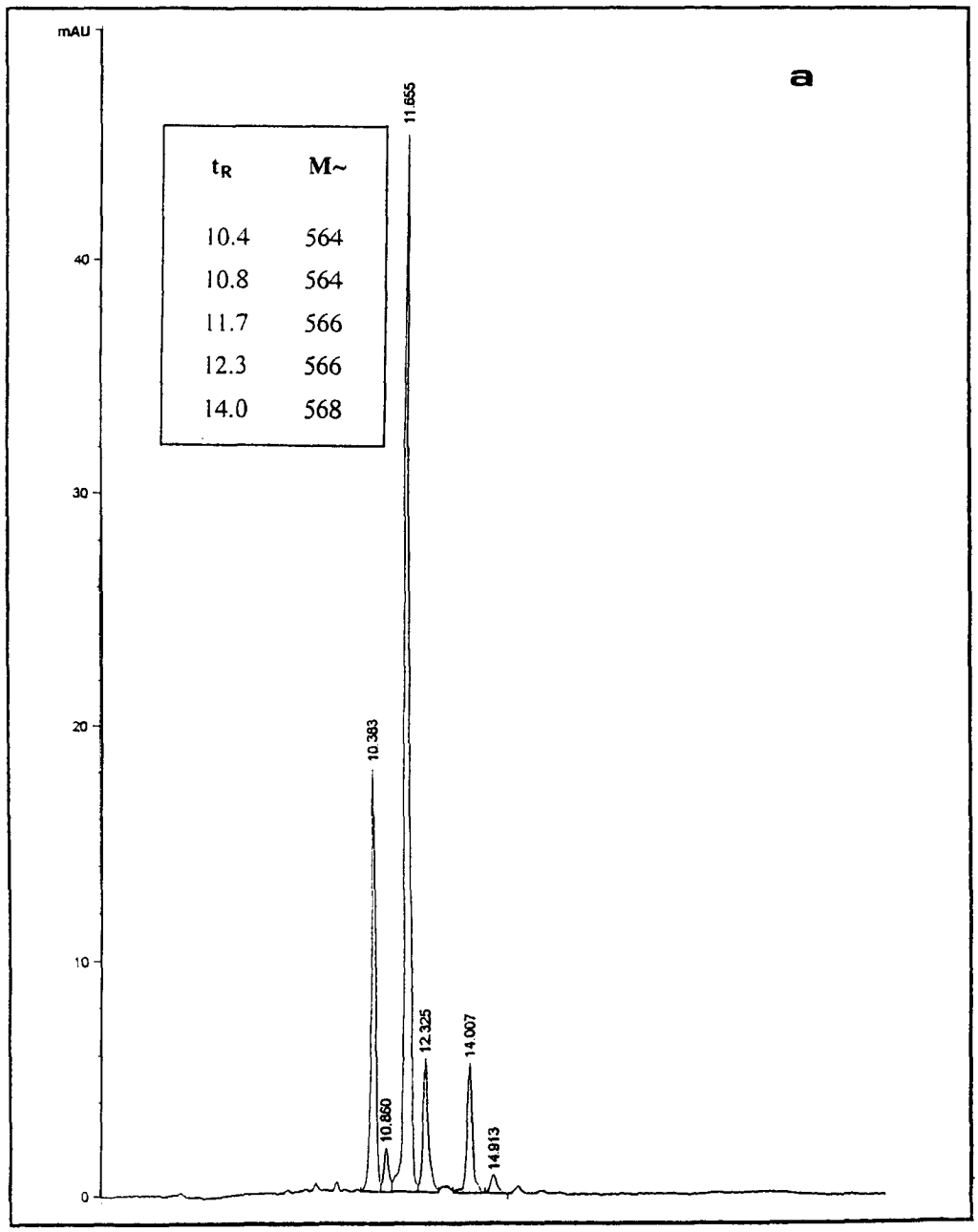
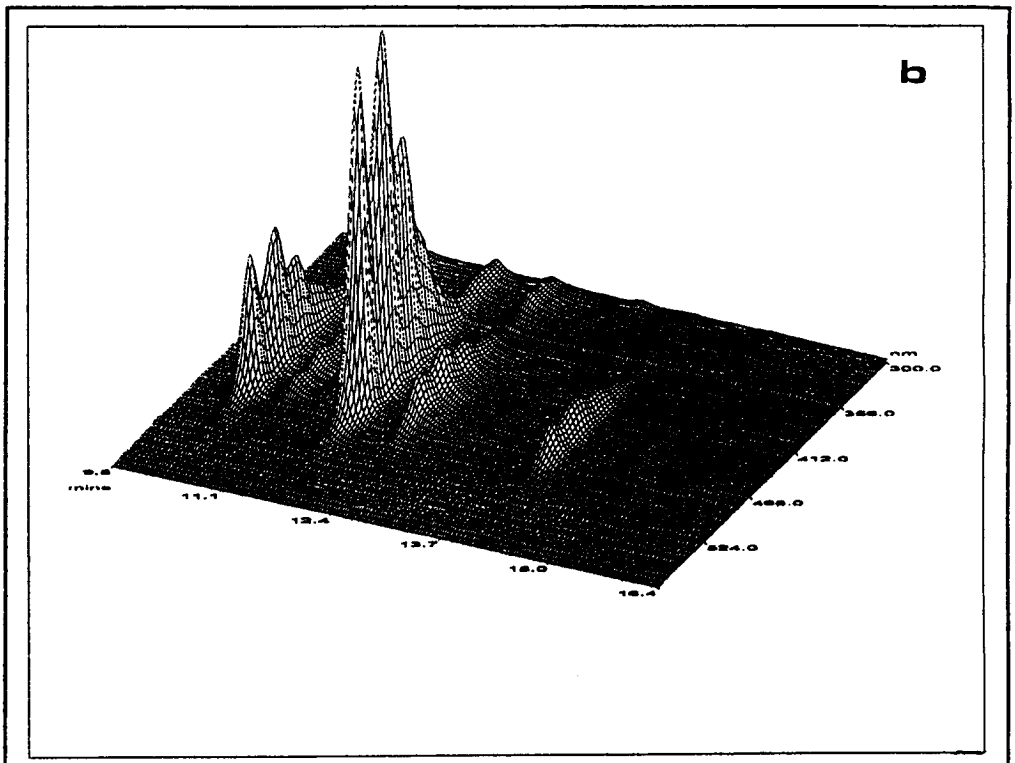

mAU
a
11.655
10.383
10.860
12.325
14.007
14.913
40
30
20
10
0
t_R M~
10.4 564
10.8 564
11.7 566
12.3 566
14.0 568

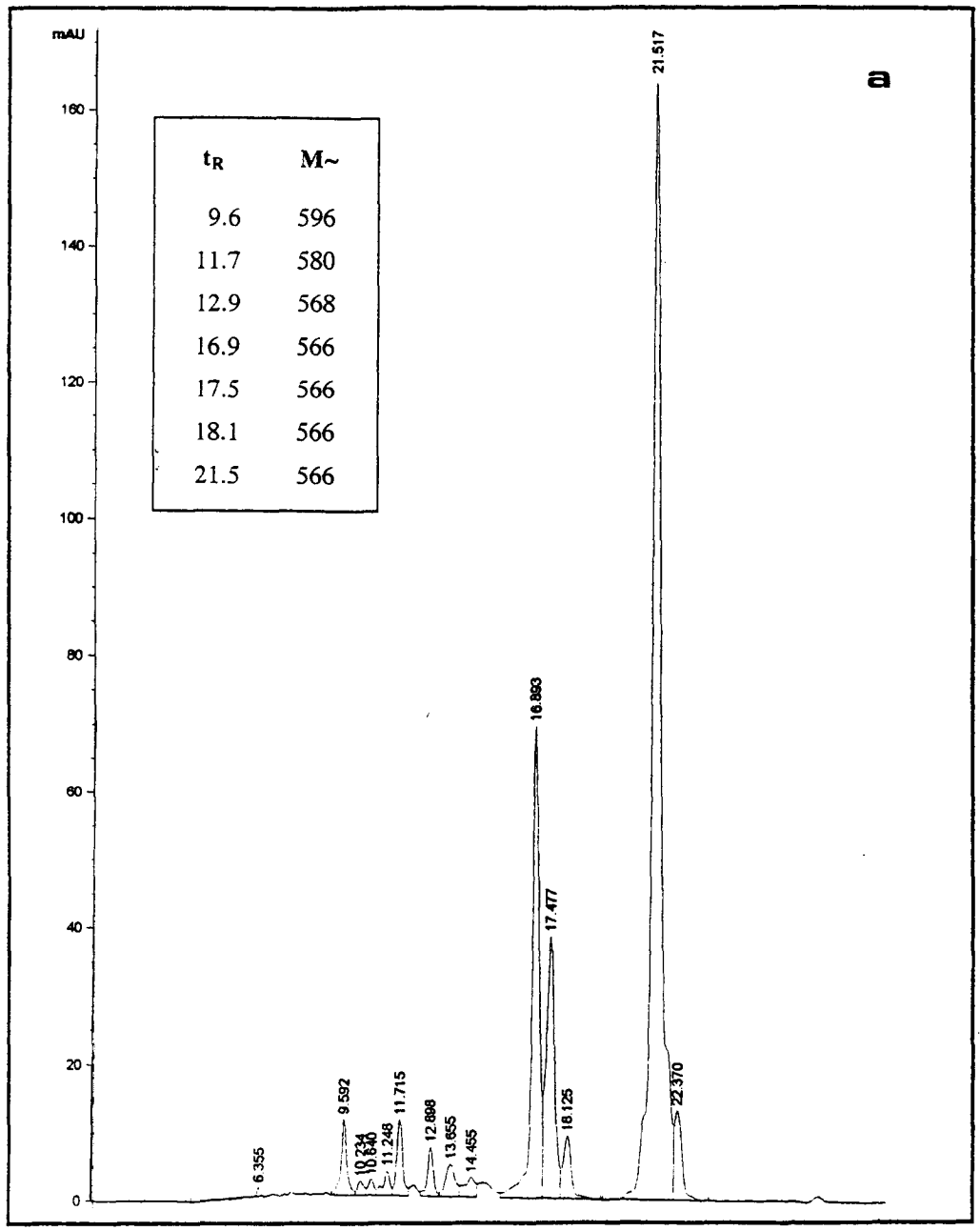
Fig. 1.



c

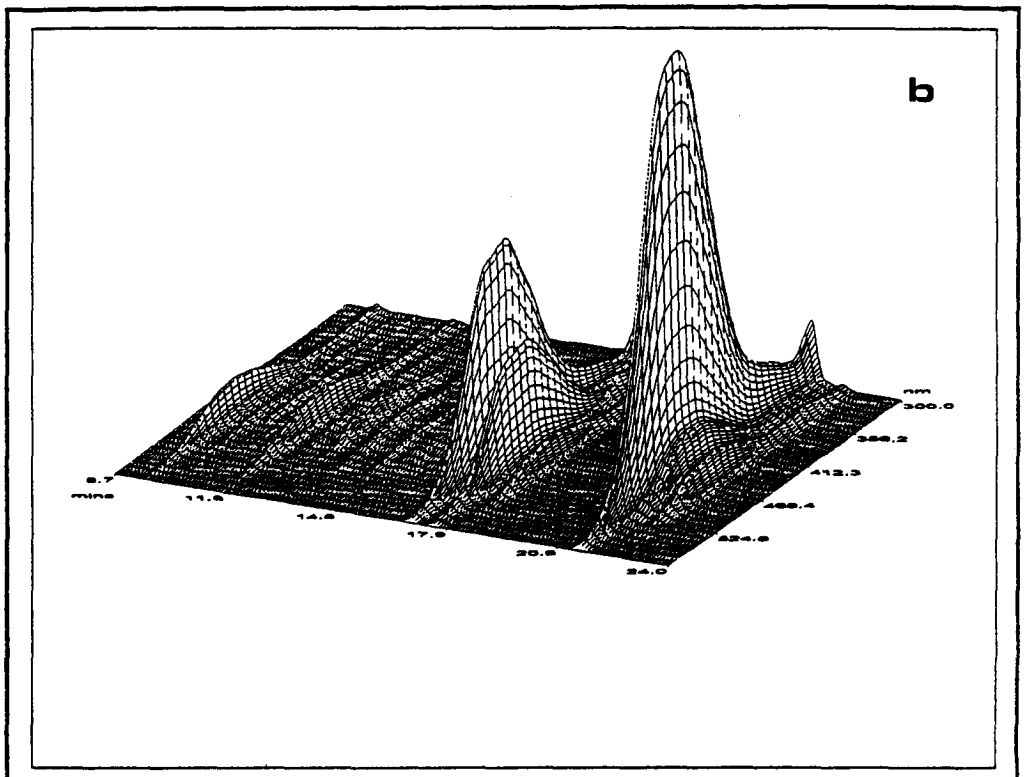
t_R (min)	λ max (nm)	STRUCTURE
10.4	416, 440, 470	I
10.8	330, 411, 435, 463	I cis
11.7	416, 440, 470	II
12.3	329, 411, 434, 462	II cis
14.0	421, 448, 463	III

Fig. 1. Pigment composition of *Carduelis chloris*' plumage: (a) bidimensional HPLC chromatogram and MS; (b) tridimensional HPLC chromatogram; (c) Vis data and structural assignment.



t_R	M~
9.6	596
11.7	580
12.9	568
16.9	566
17.5	566
18.1	566
21.5	566

Fig. 2.



c

t_R (min)	λ max (nm)	STRUCTURE
9.6	474	IV
11.7	474	V
12.9	421, 448, 473	III
16.9	467, 475, 484	X
17.5	467, 475, 484	XI
21.5	465	VII

Fig. 2. Pigment composition of *Carduelis flammea's* plumage: (a) bidimensional HPLC chromatogram and MS; (b) tridimensional HPLC chromatogram; (c) Vis data and structural assignment.

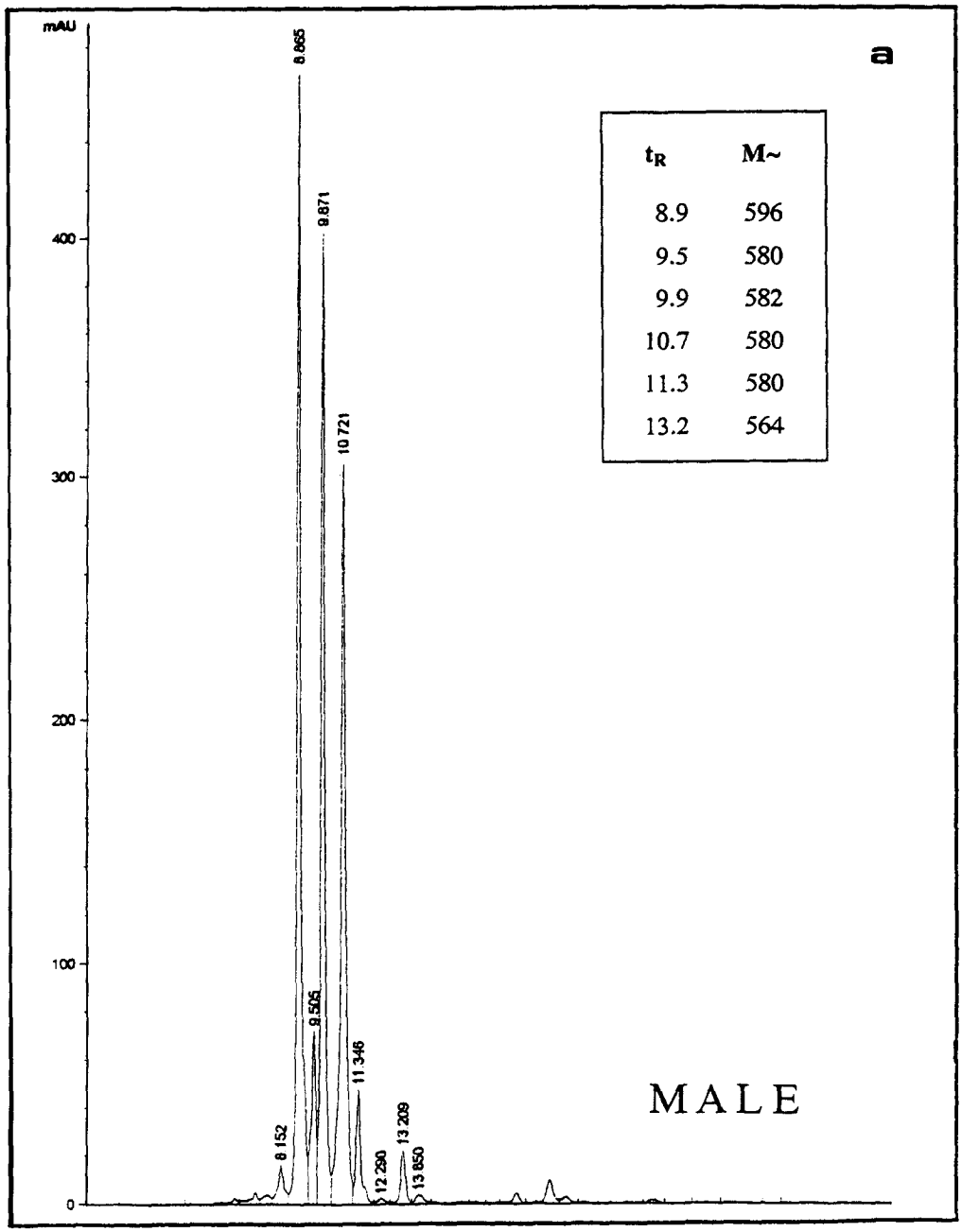
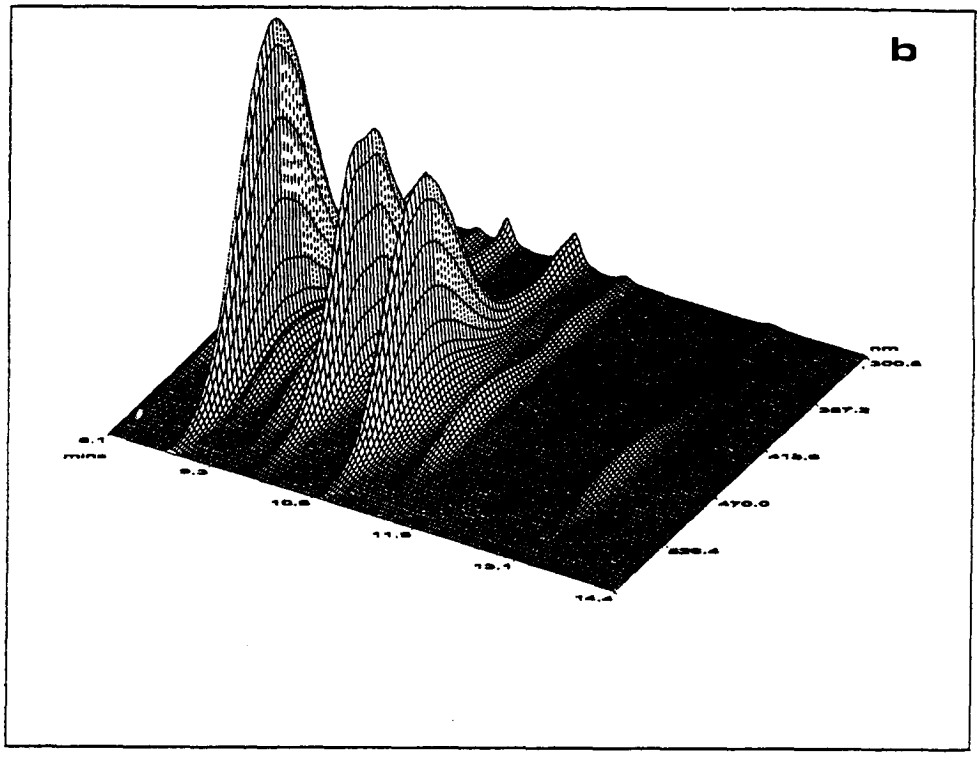

mAU
400
300
200
100
0
8.865
9.871
10.721
8.152
9.505
11.346
12.290
13.209
13.850
a
t_R M~
8.9 596
9.5 580
9.9 582
10.7 580
11.3 580
13.2 564
MALE

Fig. 3.



c

t_R (min)	λ max (nm)	STRUCTURE
8.9	475	**IV**
9.5	465	XIII
9.9	453, 473	**IX**
10.7	474	**V**
11.3	355, 467	V cis
13.2	473	VI

Fig. 3. Pigment composition of *Pyrrhula pyrrhula's* plumage: (a) bidimensional HPLC chromatogram and MS; (b) tridimensional HPLC chromatogram; (c) VIs data and structural assignment.

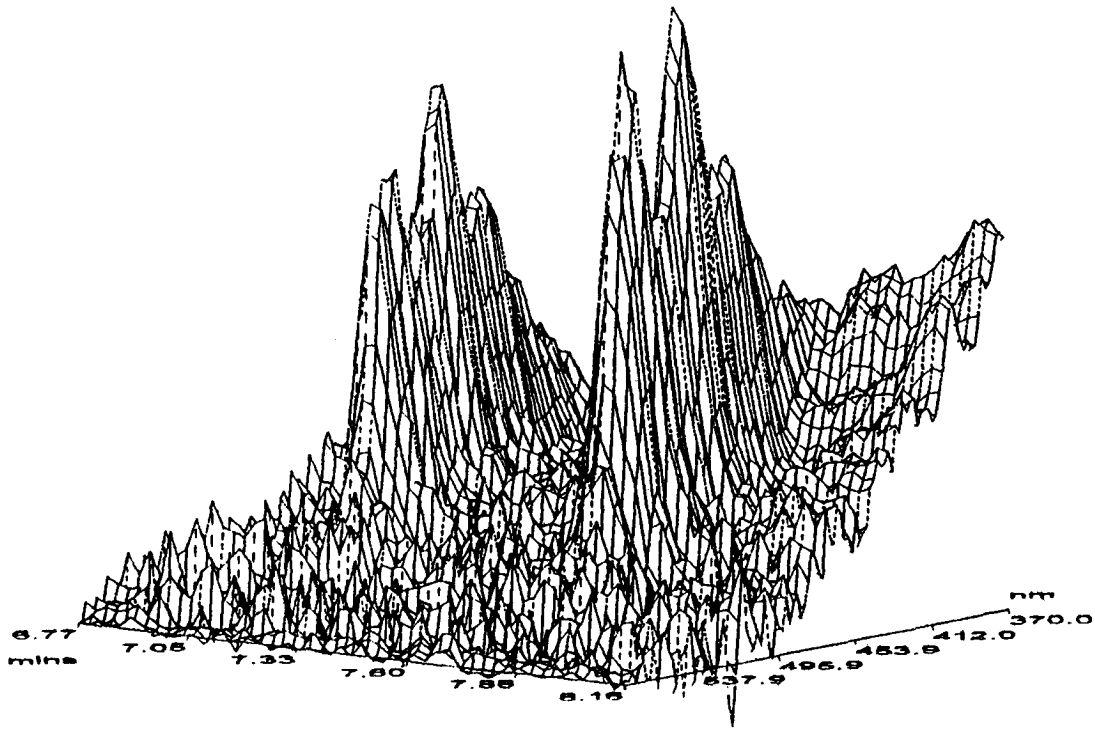


Fig. 4. Tridimensional HPLC chromatogram of two components (<1%) in the *C. chloris*' plumage.

3.2. Minor components

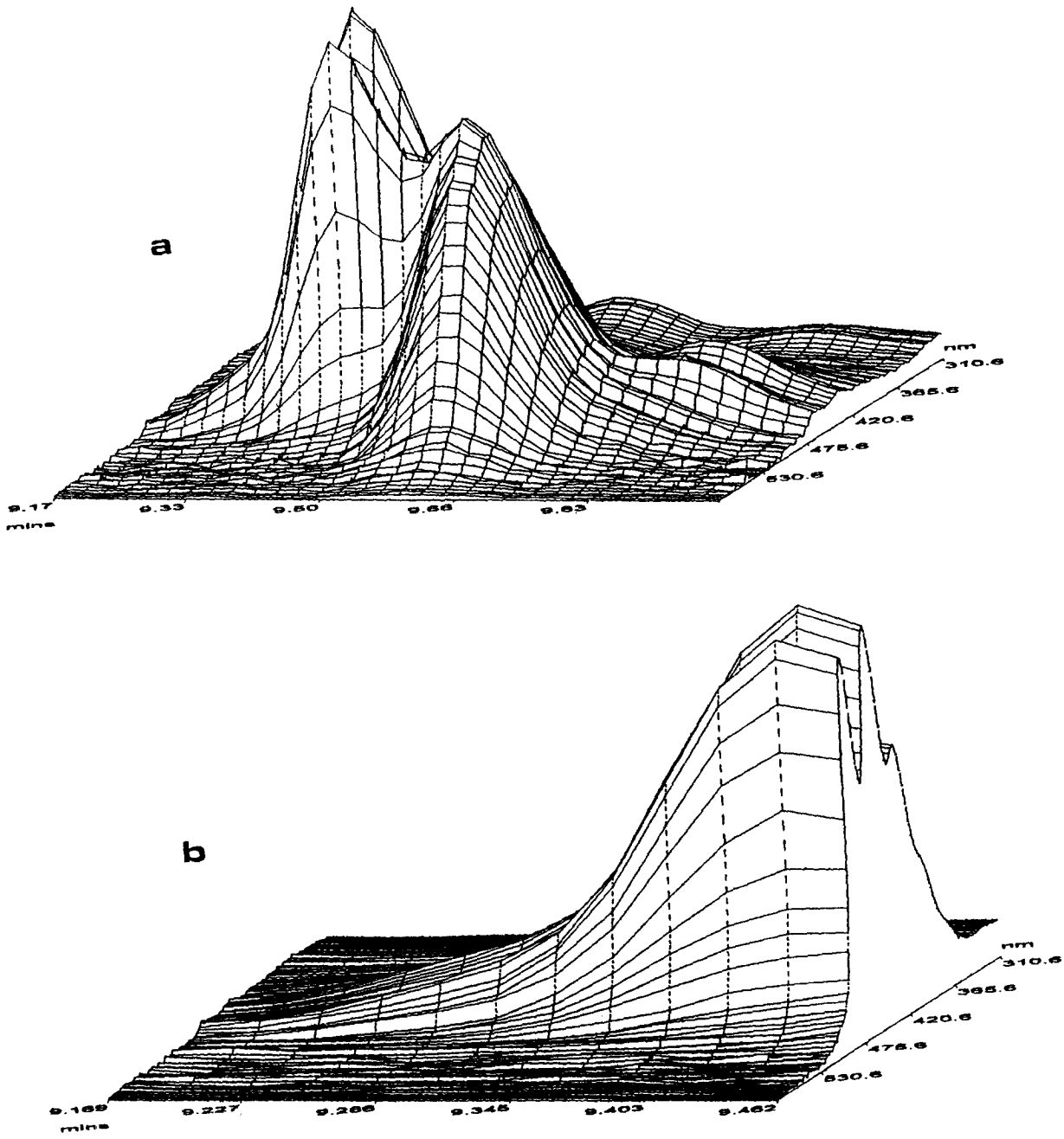
The minor components of the pigments have not been completely elucidated. However, the information obtained from the diode-array detector showed that the majority were *cis* isomers of the main carotenoids which exist in *all-trans* configuration. It is noteworthy that employing the HP 1050 series diode-array detector coupled with HPLC, we obtained detailed visible spectra even when the analyte concentration was less than 1%, or when two analytes co-eluted.

Fig. 4 shows the tridimensional chromatogram of the *C. Chloris* pigments recorded between 6.77 and 8.15 min. In this range, two carotenoids (t_R = 7.05, 0.68% and t_R = 7.6, 0.95%) are eluted. The tridimensional and bidimensional UV-Vis spectra permit to assign the structure of ε,ε-carotenoids to the compounds corresponding to these peaks.

Fig. 5 shows the tridimensional peak of astaxanthin (IV) and ε,ε-caroten-3,3'-dione (I) which practically co-eluted. The computer elaboration of this signal clearly reveals the visible spectra of IV and I, allowing their identification.

4. Conclusions

The main carotenoids giving rise to the colour of the plumage have been identified; only X and XI need further investigation to confirm their structures. Moreover, useful structural information has been obtained even for carotenoids present at very low concentration. Thus, the nature of canary xanthophylls in *Carduelis spinus* has been elucidated as 3'-hydroxy-ε,ε-caroten-3-one (II) and ε,ε-caroten-3,3'-dione (I). I and II [not lutein (III), as previously claimed] are also major components of the *Serinus* plumage. In contrast to the findings of Troy and Brush [5]



(Continued on p. 348)

Fig. 5.

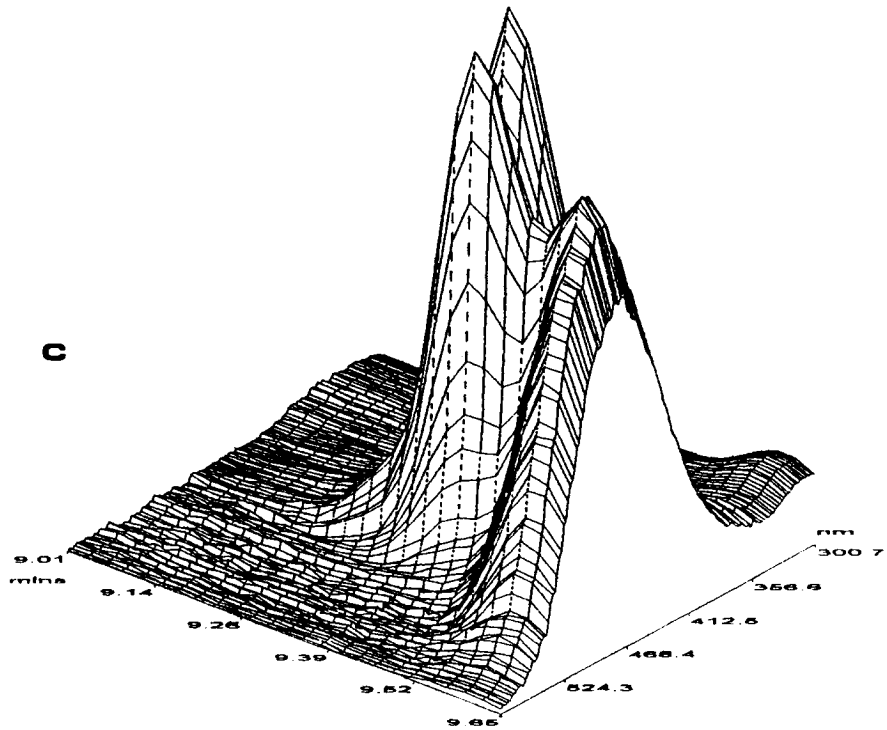


Fig. 5. Tridimensional HPLC chromatogram of astaxanthin (IV) and ε,ε-caroten-3,3′-dione (I) in Loxia male's plumage (a); computer elaboration showing Vis spectra of I (b) and IV (c).

Carduelis flammea red plumage contains 3-hydroxyechinenone besides X and XI. Carotenoid patterns of several other species have been elucidated for the first time.

In conclusion, we consider that because of its rapidity and selectivity, the analytical method described in the present paper is suitable for the molecular reinvestigation of the exogenous pigments in bird's plumage.

References

[1] T.W. Goodwin, The Biochemistry of the Carotenoids in Animals, Vol. II, Chapman and Hall, London, 1984.
[2] D.L. Fox and T.S. Hopkins, Comp. Biochem. Physiol., 17 (1966) 841.
[3] D.L. Fox, V.E. Smith and A.A. Wolfson, Comp. Biochem. Physiol., 23 (1967) 225.
[4] A.H. Brush and N.K. Johnson, Condor, 78 (1976) 412.
[5] D.M. Troy and A.H. Brush, Condor, 85 (1983) 443.
[6] O. Völker, J. Ornithol., 105 (1964) 186.
[7] A.H. Brush and H. Seifried, Poephila Gouldiac, Auk, 85 (1968) 416.
[8] N.K. Johnson and A.H. Brush, Syst. Zool, 21 (1972) 245.
[9] F.H. Test, Univ. Calif. Publ. Zool., 46 (1942) 371.
[10] J. Ford and I.W. Simpson, Emu, 87 (1987) 53.
[11] A.H. Brush, Comp. Biochem. Physiol., 36 (1970) 785.
[12] J. Hudon and A.H. Brush, Condor, 92 (1990) 798.
[13] J. Hudon and A.H. Brush, Methods Enzymol., 213 (1992) 312.