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Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 521–528

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Carotenoid composition of *Rosa canina* fruits determined by thin-layer chromatography and high-performance liquid chromatography

Teodor Hodisan [a], Carmen Socaciu [b,*], Ioana Ropan [b], Gavril Neamtu [b]

[a] *Babes-Bolyai University, Analytical Chemistry Department, 11, Arany Janos str., 3400 Cluj-Napoca, Romania*
[b] *University of Agricultural Sciences and Veterinary Medicine, Department of Chemistry and Biochemistry, 3 Mqnq.tur str., 3400 Cluj-Napoca, Romania*

Accepted 25 March 1997

Abstract

The carotenoid composition of fruits of *Rosa canina* (*Rosaceae*) was determined comparatively by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in total extracts and in three different fractions derived from previous separation of the total fruit extract on alumina columns. Both chromatographic analyses revealed as major carotenoids: β-carotene, lycopene, β-chryptoxanthin, rubixanthin, zeaxanthin and lutein. The distribution of these compounds was reproducible by TLC and by HPLC. The I—III fractions eluted successively from alumina columns by increasing the polarity of the solvents were analysed also by TLC and HPLC. In all situations, carotenoids were better separated and identified by gradient HPLC systems than by isocratic HPLC or TLC. © 1997 Elsevier Science B.V.

Keywords: *Rosa canina* (*Rosaceae*); Carotenoids; Thin-layer chromatography; High-performance liquid chromatography

1. Introduction

Carotenoids are widespread pigments in plants in which they are involved in photosynthesis and photoprotection [1–7], but they are also found in animal tissues where they may act as antioxidants or as immunomodulating, antimutagenic and tumour-preventing agents. The applications of carotenoids in medicine and cosmetics are well documented [6,8,9] as is their utilization as food additives (colorants, antioxidants) [10].

The analysis of carotenoids in plants was introduced by Tswett (1906) who first separated carotenoid mixtures on open columns; further improvements of separation protocols, identification and structure elucidation were developed by the schools of Karrer, Zechmeister, Isler and Goodwin. More than 600 carotenoid molecules have been found and characterized [1,7].

Nowadays methods for carotenoid isolation and analysis include newer separation methods

* Corresponding author.

PII S0731-7085(97)00099-X

like thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) which replace or are complementary to the classical techniques (open column chromatography) [11–14]. For identification and structure elucidation, visible spectrophotometry, NMR and mass spectrometry are the most recommended [1,5,7].

Few data about the carotenoid composition of *Rosa canina* fruits have been reported and are based exclusively upon open column chromatography for their separation [6,14].

The aim of the present studies was the characterization of carotenoid pattern in *Rosa canina* fruits using a succession of separation methods (open alumina column, TLC, HPLC) in order to reveal and identify the major components and their distribution in polar or non-polar fractions.

2. Experimental

2.1. Chemicals and standards

All solvents were of analytical grade (acetonitrile, hexane, ethyl acetate) or distilled (acetone, methanol, petroleum, ether, ethyl ether) before use for HPLC separation. They were purchased from Ammann (Switzerland). The buffers (triethylamine, TEA) and antioxidants (butylhydroxytoluene, BHT) were of chromatographic grade and were provided by Merck (Germany).

Some of the carotenoid standards (β-carotene, zeaxanthin, β-cryptoxanthin) were kindly given by Hoffmann La Roch (Basel) and others (lutein, lycopene) were purified from natural sources in the authors laboratory in the Department of Chemistry and Biochemistry, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca.

2.2. Fruit extraction and saponification

Fresh fruits of *Rosa canina* sp. were dried (in the dark, at 40°C) to 85% dried weight and were then finely ground. The powder was immediately extracted with acetone–methanol–petroleum ether (3:2:1, v/v/v) for 5 h in the dark, in order to avoid carotenoid degradation and oxidation at this stage. The crude extract was filtered, evaporated to dryness in a Rotavapor and resuspended in ethyl ether. Saponification was carried out in ethereal solution by adding 30% w/v KOH in methanol to a final concentration of 10% w/v KOH. The mixture was stirred for 8 h (overnight) with magnetic stirring and carotenoids were extracted 5–6 times successively with ethyl ether from a saline solution to neutral pH. The soaps were eliminated by these procedures and the total carotenoid extract (TE) was evaporated on a Rotavapor, brought to a fixed volume and used for quantitative evaluation of total carotenoids and also for chromatographic separation.

2.3. Quantitative evaluation of total carotenoids

The concentration of total carotenoids in TE was calculated by relating the absorbance reading A (λ max = 450 nm) to the specific absorption (mean value $A^{1\%}_{1\,cm} = 2500$ of coloured carotenoids [1]:

$$x = (A \cdot y \cdot 1000)(2500 \cdot 100) = A \cdot y/250$$

where x was the weight of carotenoids in the sample (mg) and y was the volume of the sample (ml). The quantities of individual carotenoids in

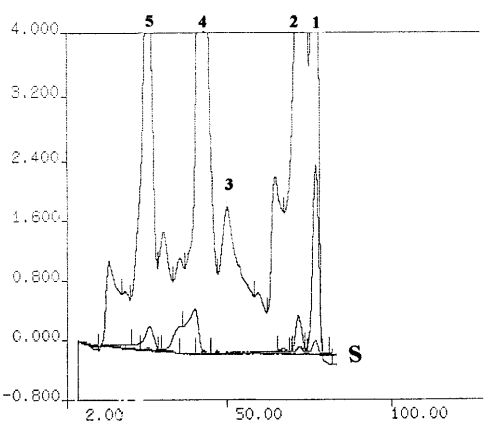

Fig. 1. The TLC chromatogram of the total extract, recorded at 450 nm. Carotenoids were identified by comparison with the standard mixture separated under the same conditions (showed at the bottom of the chromatogram). Peak identification: 1, β-carotene; 2, lycopene; 3, β-cryptoxanthin; 4, rubixanthin; 5, zeaxanthin + lutein.

Table 1
R_f values and the peak area percentages calculated from results by TLC anaysis

Peak no.	R_f	%	Identification
1	0.96	20.8	β-Carotene
2	0.90	27.8	Lycopene
3	0.62	5.5	β-Cryptoxanthin
4	0.53	23.6	Rubixanthin
5	0.32	11.3	Lutein + zeaxanthin

The identification of carotenoids was made by comparison with R_f values of the standards and spectral data (λ_{max}).

the total extract and fractions was expressed as percentages, determined from TLC and HPLC chromatograms.

2.4. Fractionation of TE on an alumina open column

An alternative way to characterise the carotenoid composition of *Rosa canina* fruits involved a preliminary separation of TE on an alumina column (Al_2O_3 grade III containing 6% water). Small volumes of TE (1–2 ml) were put on to the column and eluted successively with four solvent systems: 100% petroleum ether (fraction I); petroleum ether–ethyl ether (1:1 v/v) (fraction II), 5% v/v ethanol in ethyl ether (fraction III); and 100% ethanol (fraction IV). Each fraction was collected separately, evaporated, dissolved in ethyl ether and submitted to TLC and HPLC analysis. Because fraction IV was very poor in respect of content of coloured carotenoids, it was eliminated from further investigation.

2.5. TLC analysis

The total extract and the fractions I–III released from the alumina column were separated on silica plates (SilG, Merck, Germany) and the chromatograms were analysed on a Shimadzu CS-9000 dual-wavelength flying spot scanner. A two-step separation was used for TE: the first using 15% v/v acetone in petroleum ether (PE) and the second using 100% PE. For fractions I–III the developing systems were different: 3% v/v acetone in PE (fraction I); 7% v/v acetone in PE (fraction II); and 15% v/v acetone in PE (fraction III).

Concomitantly, individual standards or mixtures with controlled composition were developed by TLC and analysed.

2.6. HPLC analysis

The separations were performed with a HPLC system including two Altex-110 pumps, a reversed-phase Spherisorb ODS-1 column (4.4 × 25 cm) (Phase Sep.) and a Waters-PDA detector (Waters manufacturers) coupled to a computerized software HPLC-TP5 for spectra and chromatograms analysis. Isocratic and gradient eluting systems were used for TE and alumina fractions.

The mobile phase used in isocratic separation was acetonitrile–methanol–ethyl acetate (70:10:9 v/v/v) containing 1% triethylamine (TEA) solvent A) whereas in gradient separations solvent A was combined with solvent B comprising methanol–acetonitrile–hexane–dichloromethane (50:12:10:8, v/v/v/v) containing 0.5% TEA. The mixture of solvents A and B was programmed as follows: 0–6 min (100% A), 6–7.5 min (B, from 0 to 100%), 7.5–11 min (100% B) and 11–13 min (B, from 100 to 0%). The flow-rate was 1.5 ml min^{-1}.

For peak identification, the retention times (t_R) of samples were compared with the t_R values of standards ran under the same conditions and the spectra of each peak was compared with the spectra of the standards. The purity of the peaks was examined from a two-dimensional HPLC chromatogram, recorded concomitantly with the monodimensional one.

3. Results and discussion

3.1. Analysis of total extract by TLC and HPLC

The total extract was first submitted to quantitative analysis. The absorption at 450 nm was measured, the volume of the extract and the quantity of dried fruits used for analysis were recorded. According to the formula shown in *Materials and methods* Section 2.3, the concentra-

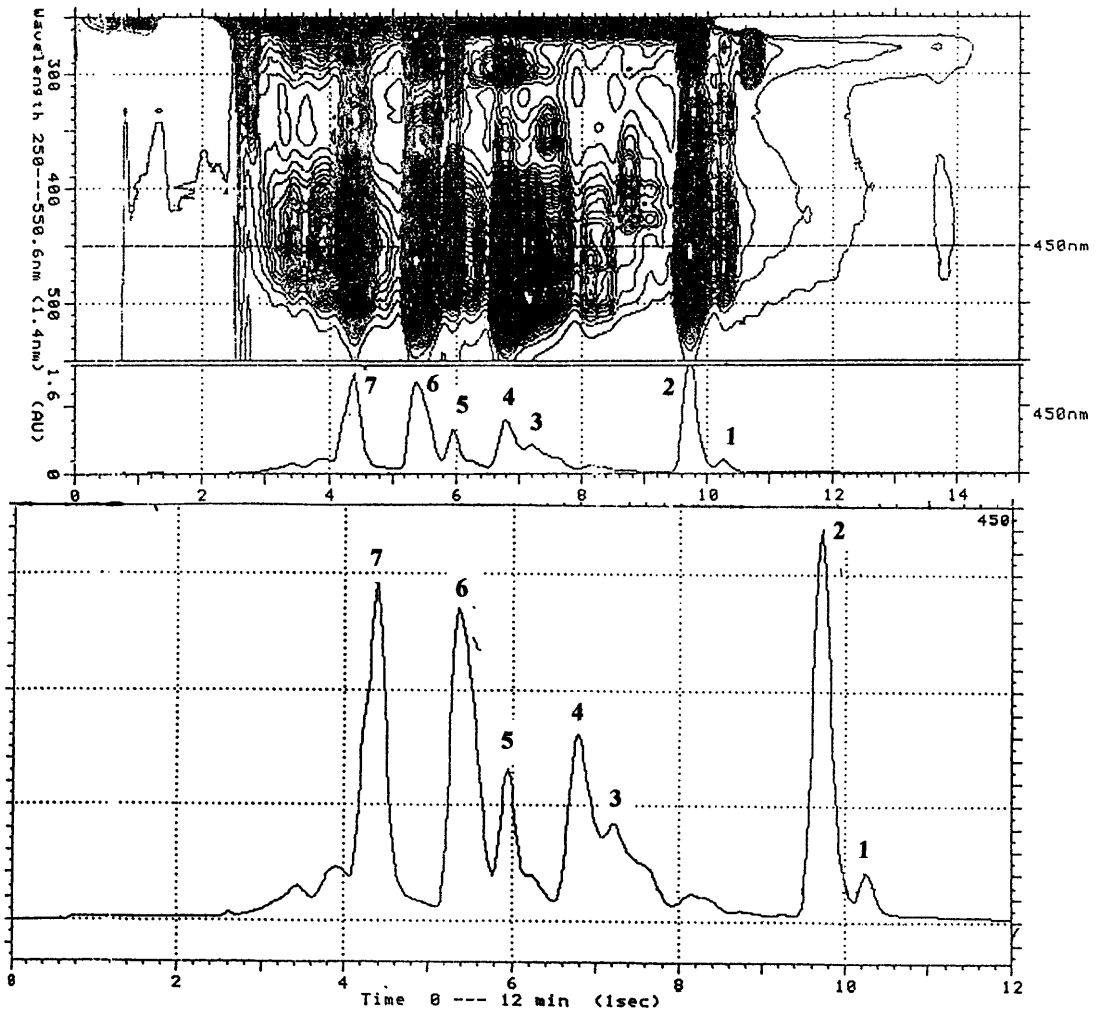

Fig. 2. Two-dimensional and monodimensional HPLC chromatograms of the total extract of *Rosa canina* fruits analysed by isocratic separation. Peak identification: 1, *cis*-β-carotene ($t_R = 10.3$ min); 2, β-carotene ($t_R = 5.96$ min); 6, rubixanthin ($t_R = 5.36$ min); 7, lutein + zeaxanthin ($t_R = 4.39$ min). Their spectral characteristics (λ_{max}) are presented in Table 2.

tion of total carotenoids was 78.5 μg g^{-1} dried weight.

The peaks obtained from TLC densitogram (Fig. 1) were identified by comparison of TE with the separation of a standard (S) under the same conditions (shown on the bottom). The R_f values and the area percentages for five peaks observed on the TLC densitogram were also calculated (Table 1). The main carotenoids identified were: β-carotene; lycopene; rubixanthin; β-cryptoxanthin; and zeaxanthin mixed with lutein. These data reveal the percentage composition of main carotenoids in the extract and not their absolute quantities. Thus lycopene represented 27.8%, rubixanthin 23.5% and β-carotene 20.8% while zeaxanthin + lutein represented 11, 3%. It was not possible to make a precise quantitative evaluation of all components because some peaks were not identified.

The HPLC analysis of the total extract was performed under isocratic or gradient conditions. Fig. 2 shows the two-dimensional chromatogram as well as the monodimensional one, recorded under isocratic conditions, at 450 nm. The identification of the peaks was made by comparison with t_R values and λ_{max} (Table 2 and Fig. 2) of standards. Six peaks out of seven were identified, corresponding to: *cis*-β-carotene (1); β-carotene (2); lycopene (4); β-cryptoxanthin (5); rubixanthin (6); and lutein + zeaxanthin (7). Their quantitative distribution in the total extract was expressed only as percentages (Table 2), because some peaks in the chromatogram were not identified (Fig. 2). The purity of the peaks was checked from the two-dimensional chromatogram and the unpure peaks were not considered for quantitative evaluation. The data of the HPLC separation was similar to TLC data, excepting the lycopene percentage which was lower for the TLC system. The explanation of this difference could be the lower stability of lycopene compared with that of other carotenoids [9,10].

Table 2
The t_R values and identification of the HPLC peaks for the total extract by gradient separations

Sample	t_R (min)	%	λ_{max}	Identification
Standards				
1	4.55	—	451 476	Lutein
2	4.86	—	454 479	Zeaxanthin
3	7.86	—	454 477	β-Cryptoxanthin
4	8.51	—	472 504	Lycopene
5	10.59	—	454 481	β-Carotene
Total extract				
8	4.8	15.5	451 477	Lutein
7	5.1	4.1	454 479	Zeaxanthin
6	6.5	20.8	463 492	Rubixanthin
5	7.3	8.5	447 475	β-Cryptoxanthin
4	8.4	11.7	472 505	Lycopene
3	9.25	8.1	—	NI
2	10.5	19.0	453 479	β-Carotene
1	10.85	2.0	446 312	*cis*-β-Carotene

The identification was made by comparison with t_R values of the standards (under isocratic conditions) and λ_{max}.

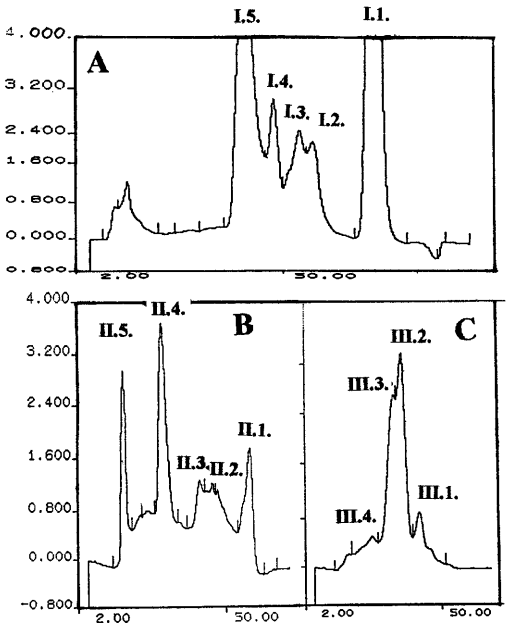

Fig. 3. TLC chromatograms (A–C) registered for fractions I–III separated on the alumina column. For peak identification (t_R values) and λ_{max}, see Table 3.

When HPLC analysis was performed by gradient separation, eight main peaks were observed and identified by their visible spectra. Table 2 includes their t_R values and identification of the peaks by their spectra and comparatively with those of standards. The advantage of this system was a better separation of lutein and zeaxanthin peaks (7 and 8) compared with isocratic separation.

3.2. TLC and HPLC analysis of fractions I–III

The fractions I–III collected from the alumina column were analysed by TLC and gradient HPLC (Fig. 3A–C and Fig. 4A–C, respectively, including the spectrum of each peak). The identification of carotenoids depending on their R_f, t_R values and spectral characteristics is presented in Table 3.

Fraction I contained: two main peaks by TLC separation, representing unpolar hydrocarbons, identified as β-carotene (I.1) and lycopene (I.5)

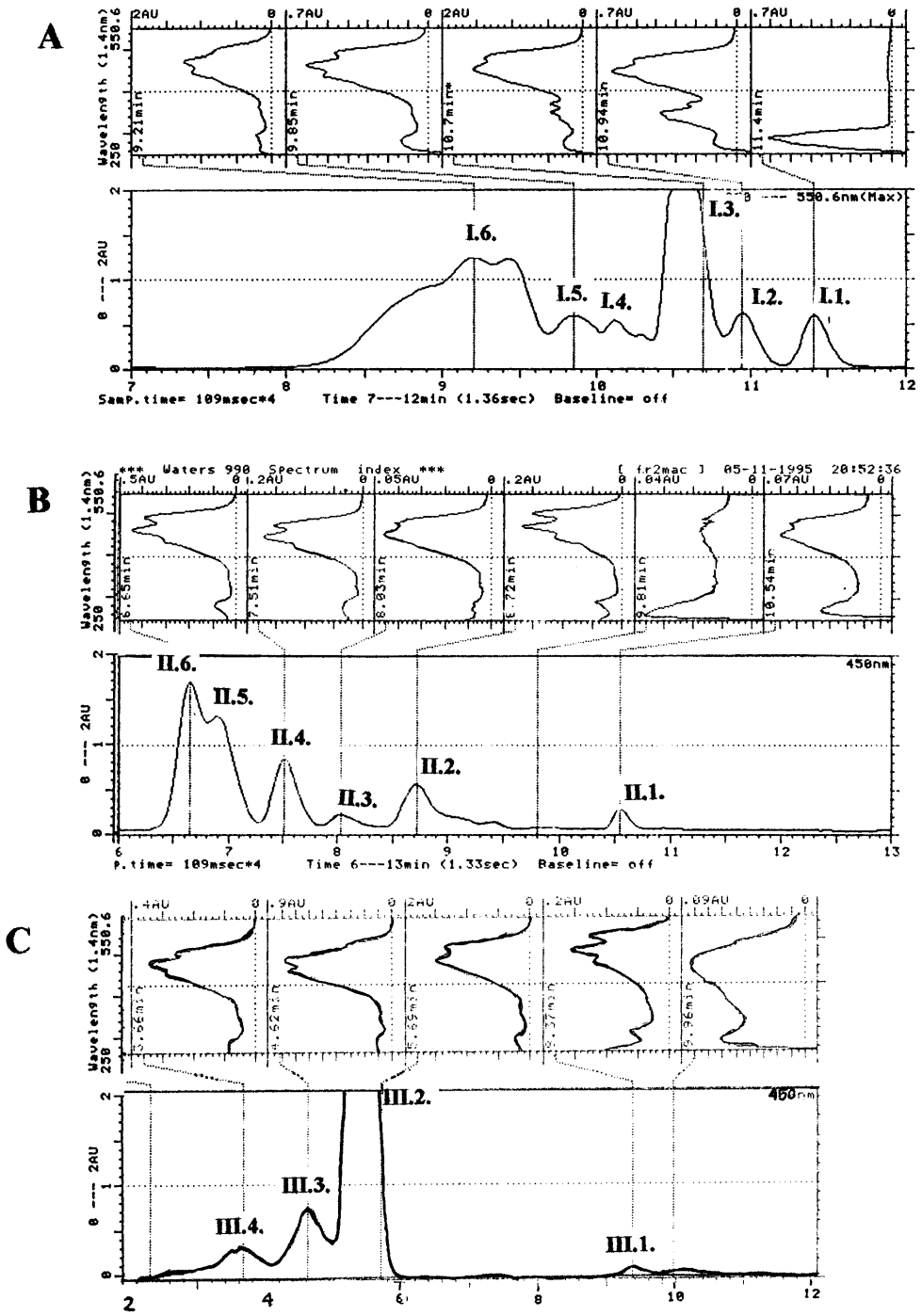

Fig. 4. Monodimensional HPLC chromatograms obtained by gradient separations of fractions I–III (A–C), including visible spectra of components by PDA detection.

Fig. 3A) or four peaks identified as phytoene (I.1), *cis*-β-carotene (I.2) and β-carotene (I.3) by HPLC (Fig. 4A).

Fraction II by TLC contained mainly β-carotene (II.1), lycopene (II.4) and rubixanthin (II.5) (Fig. 3B). Fraction II by HPLC contained β-carotene (II.1), lycopene (II.2), β-cryptoxanthin (II.4) and rubixanthin (II.6), respectively (Fig. 4B).

Fraction III by TLC was more rich in polar carotenoids: rubixanthin (III.1), zeaxanthin (III.2) and lutein (III.3) (Fig. 3C) while fraction III by HPLC contained lycopene (III.1), rubixanthin (III.2), zeaxanthin (III.3) and lutein (III.4) (Fig. 4C).

This comparative analysis of fractions I–III by TLC and HPLC demonstrated similar differences in composition but HPLC, especially in the gradient system, represented a more reliable method for the separation and identification of these components.

Table 3
Identification of peaks recorded by TLC and HPLC—gradient analysis of fractions I–III collected from the alumina column

Fraction	TLC peaks (R_f)	HPLC peaks (t_R)
I	I.1 β-Carotene (0.75)	I.1 Phytoene (11.4 min)
	I.2–I.4 NI	I.2 *Cis*-β-Carotene (10.9 min)
		I.3 β-Carotene (10.7 min)
	I.5 Lycopene (0.45)	I.4–I.5 NI (10.1–9.8 min)
		I.6 NI (9.2 min)
II	II.1 β-Carotene (0.8)	II.1 β-Carotene (10.5 min)
	II.2–II.3 NI	II.2 Lycopene (8.7 min)
		II.3 NI (8.0 min)
	II.4 Lycopene (0.38)	II.4 β-Cryptoxanthin (7.5 min)
	II.5 Rubixanthin (0.2)	II.5 NI (6.9 min)
		II.6 Rubixanthin (6.6 min)
III	III.1 Rubixanthin (0.54)	III.1 Lycopene (9.4 min)
	III.2 Zeaxanthin (0.44)	III.2 Rubixanthin (5.6 min)
	III.3 Lutein (0.40)	III.3 Zeaxanthin (4.6 min)
	III.4 NI	III.4 Lutein (3.6 min)

4. Conclusions

The quantity of total carotenoids in *Rosa canina* fruits, evaluated by visible spectroscopy of the total extract was 78.5 μg g^{-1} dried weight. The chromatographic analysis (TLC and HPLC) revealed as major carotenoids: β-carotene, lycopene, rubixanthin, lutein and zeaxanthin in total extracts, differently distributed in three (I–III) different fractions eluted from alumina columns. The percentage of carotenoids determined by HPLC analysis, showed almost equal contributions (around 20%) of rubixanthin, β-carotene, lycopene and lutein to the fruit colour. Thin-layer chromatography proved to be a useful and rapid screening method for carotenoid separation but high-performance liquid chromatography allowed a higher resolution of peaks, especially by the gradient system and better identification by mono- and two-dimensional chromatograms through combined PDA-detection and spectral analysis.

Acknowledgements

We thank Hoffman La Roche (Basel, Switzerland) for their kind gift of carotenoid standards and also to members of Swiss carotenoid research group (University of Berne) lead by Professor dr.dr.h.c. Hanspeter Pfander, for their help and suggestions regarding the HPLC analysis of carotenoids.

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