UV-HPLC / APCI-MS Method for Separation and Identification of the Carotenoids Produced by *Sporobolomyces ruberrimus* H110

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ABSTRACT: *An on-line UV-HPLC / APCI-MS (atmospheric pressure chemical ionization) tandem by a platinium C_{18} column with an isocratic solvent system after optimizing was applied to the separation and the identification of different carotenoids including astaxanthin, canthaxanthin, apocarotenoic ester, torularhodin and beta carotene. This method was used to identify particular carotenoids produced by newly isolated microorganism strain of Sporobolomyces ruberrimus H110 in our laboratory. The developed method allows to distinguish torularhodin and canthaxanthin, having the same molar mass but a different chemical structure. A detection limit of 9ng /ml for torularhodin observed. CV (%) for measurement of all carotenoid concentration was always below 3 %. Torularhodin and for a smaller part beta carotene were the two pigments identified in Sporobolmyces ruberrimus H110.*

KEY WORDS*: Torularhodin, Carotenoids, UV-HPLC / APCI-MS, Sporobolmyces ruberrimus H110, Identification.*

INTRODUCTION

Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) consisting of eight isoprenoid units. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure, having a long central chain of conjugated double bands by hydrogenation, dehydrogenation, cyclization, or oxidation [1]. More than 600 different structures of carotenoids have been described among a variety of animal, plant and microbial species [2]. Carotenoids are currently produced for use as food colorants, nutritional supplements, cosmetics or health purposes [3]. In addition to their pigmenting abilities, carotenoids may

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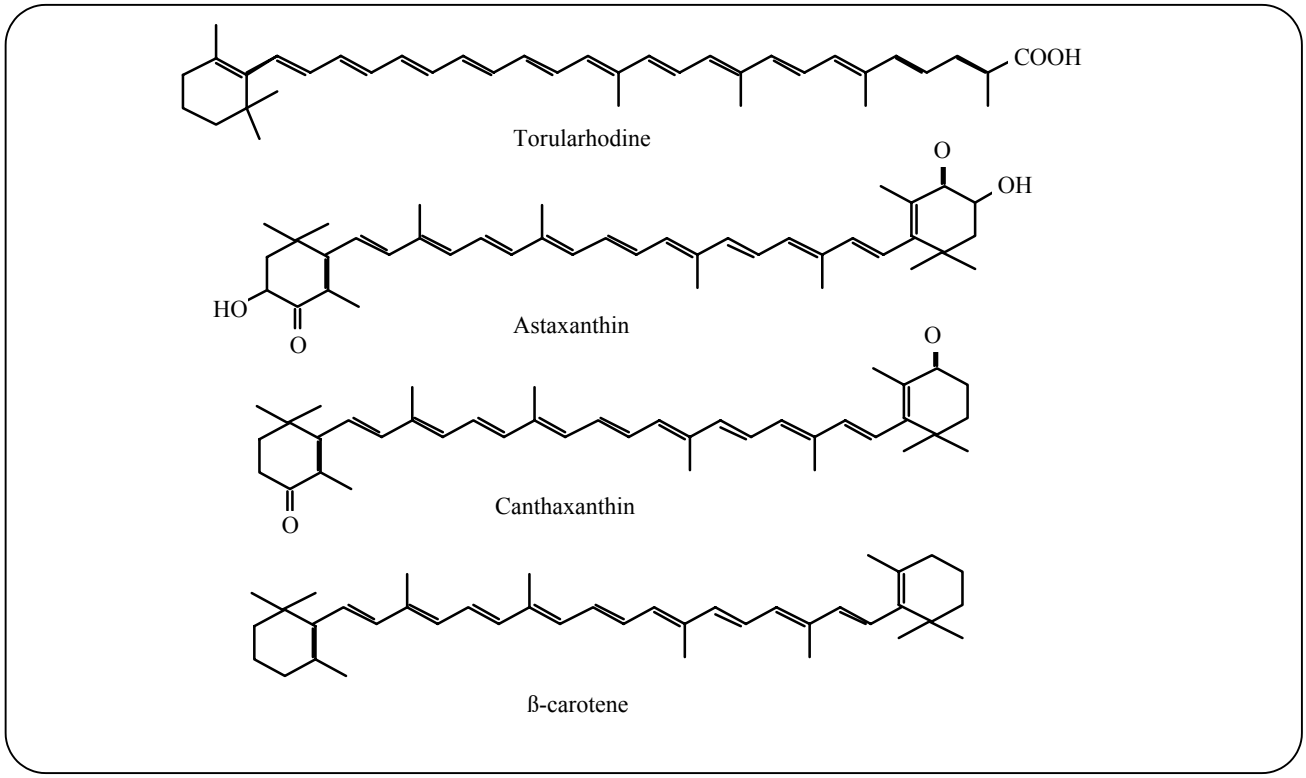

Fig. 1: Chemical structure of different carotenoids including, torularhodin, astaxanthin, canthaxanthin and β-carotene.

function as antioxidants by quenching photosensitizers, interacting with singlet oxygen, and scavenging peroxy radicals [4]. The species of the various taxonomic group-bacteria, fungi, and yeasts are efficient natural producers of carotenoids [5]. Thanks to the interest shown to the large ability to discover either new carotenoid-like or carotenoid producing microorganisms, a real interest is pointed out to have new well-adapted and high performance analytical methods. A few liquid chromatography-mass spectrometry (LC-MS) techniques were proposed for carotenoid analysis, including liquid chromatography electrospray (ESP) [6] or atmospheric pressure chemical ionization (APCI) sources. These devices are considered as the most robust, sensitive and versatile systems for the LC-MS analysis of low and high molar mass compounds [7].

The goal of this work is to identify the different carotenoids synthesized by *Sporobolmyces ruberrimus* H110, a new yeast recently isolated in our laboratory. The first phase was to specify the best operating conditions of an analytical method which is rather simple and rapid to separate and to quantify microbial carotenoids. Astaxanthin, canthaxanthin, torularhodin, apocarotenoid ester and beta carotene will be investigated by coupling UV-HPLC with MS via APCI (Fig. 1).

EXPERIMENTALS

Microorganism and growth medium

Sporobolmyces ruberrimus H110 yeast was isolated in GPBA (Génie des Procédés Biotechnologiques et Alimentaires - Laboratoire des Sciences du Génie Chimique, Nancy, France) laboratory. Yeast was maintained on YM agar slant at 4 °C and transferred monthly. The preculture was done in a 500 ml baffled Erlenmeyer flask containing 150 ml of medium composed of the following (g/l): glucose, 10; peptone, 5; yeast extract, 3; malt extract, 3 on a rotary shaker at 210 rpm, for 24 h at 23 °C. 150 ml of the preculture were used as inoculation in a 3-l bioreactor. Batch cultures were performed for the production of carotenoids by *Sporobolmyces ruberrimus* H110 in a 3-l bioreactor (Applikon, ADI 1030, Holland) containing 1.5 l of the following medium (g/l): $(NH_4)_2SO_4$, 20; glycerol, 67; peptone,0.5; yeast extract,1; Na_2HPO_4, 2;($MgSO_4$, $7H_20$),

1.5; KH_2PO_4, 4. The initial medium pH was adjusted with KOH 2 M and H_3PO_4 (42.5 %). Temperature, pH and agitation were maintained at 23 °C, 6.0 and 300 rpm respectively. The dissolved oxygen pressure was maintained at 50 % of air saturation by controlling agitation speed and air flow-rate.

Chemicals

Beta carotene and astaxanthin standards were obtained from sigma. Cathaxanthin and apocarotenoic esters were kindly supplied by Hoffmann-La Roche (Switzerland). acetonitrile, methanol, dichloromethane, hexane and ethyl acetate were HPLC grade (Merck). Ammonium acetate was provided by Fluka. Torularhodin was purified from microbial source in the GPBA laboratory. Approximately 1 mg of each carotenoid was dissolved in 100 ml of a hexane / ethyl acetate 1/4 (v/v) solution. The solutions of each carotenoid were kept in the dark at -20 °C under vacuum, for 3 months.

Carotenoid extraction

After four days of culture, yeast cells were harvested by centrifugation (4500×g for 10 min) and washed three times with physiological water (NaCl, 0.1 %). A physical method for disruption of yeast cells wall, using glass beads (0.4 mm, 10 % w/v) was used for 10 min with cooling (18°C). Thus, 0.3 g of centrifuged cells was resuspended in 5 ml of ethanol and vortexed for 2 min. Each suspension was separated by centrifugation and the pigment contained in the ethanol was recovered. The dried pigments were dissolved in hexane / ethyl acetate 1/4 (v/v) and finally, the samples were filtered through teflon membranes and stored in the dark, at -20 °C under vacuum.

High-Performance Liquid Chromatography

Carotenoids were separated on a C_{18} Platinium, (100 mm × 2.1 mm, 300 Å) (Altech, France) and sphere diameter 3 µm. The temperature of the column was maintained at 35 °C. The mobile phase was an isocratic solvent system consisting of acetonitrile-methanol (containing 0.1 M ammonium acetate)- dichloromethane (71:22:7, v/v/v) that was delivered by a LC pump at a flow rate of 0.2 ml/min and monitored at 480 nm. HPLC device (LC Autosampler, LC Pump and UV/VIS detector Mode 785 A Corad) was from Perkin Elmer, serie 200, Nor walk CT 06859, USA. To protect the column, a pre-column of the same material was used. The volume of solutions injected was 10 µl.

Mass Spectrometry

The device was an Applied biosystems API 150 EX (PE Sciex, Toronto, Canada) single quadrupole mass spectrometer equipped with an APCI interface and an Apple Macintosh System v.7.6.1. A Masschrom v.1.1 application version was used for data acquisition and processing. The optimum conditions of the interface were as follows: OR voltage 10 V, ring voltage 175 v, Needle Current (NC) 2 µA, curtain gas 10, nebulizing gas 12. APCI vaporization temperature was held at 400 °C. LC-MS determinations were performed by operating the Mass Spectrometer in the positive ion (PI) mode. Mass Spectra were obtained over the scan m/z 350-700 range using a step size of 0.2 amu and a dwell time of 0.6 ms.

Purification of torularhodin by semi-preparative HPLC method

Torularhodin was isolated (5 ml aliquots) by using HPLC, BIO CAD 700, perfusion chromatography work station, perseptive Biosystems, Inc (Framingham, MA, 01701, USA). Pigments were eluted by applying a 150×4.6 mm, Symmetry C_{18} (3.5 µm) column at 35 °C. The mobile phase was the same that has been used for analysis and applied with a flow rate that was set at 1 ml/min. The different fractions of torularhodin were collected and accumulated. Then solvents were evaporated under nitrogen. 5 ml of hexane / ethyl acetate 1/4 (v/v) were added to the torularhodin fraction collected and stored in the dark, under vacuum, at -18°C.

RESULTS AND DISCUSSION

Analysis of carotenoids

The separation and the identification of various carotenoids (Fig. 1) was carried out with the UV-HPLC / APCI-MS tandem. As the temperature, ring voltage and orifice plate voltage have a strong influence on the MS response, it was needed to investigate all factors. In a first step, the effect of temperature (100-450 °C) on the signal of astaxanthin using positive ion APCI-MS was determined (Fig. 2). The medium signal was obtained at 400 °C by flow injection using full-scan detection in the range of m/z 350-700 and by searching the mass (m/z)

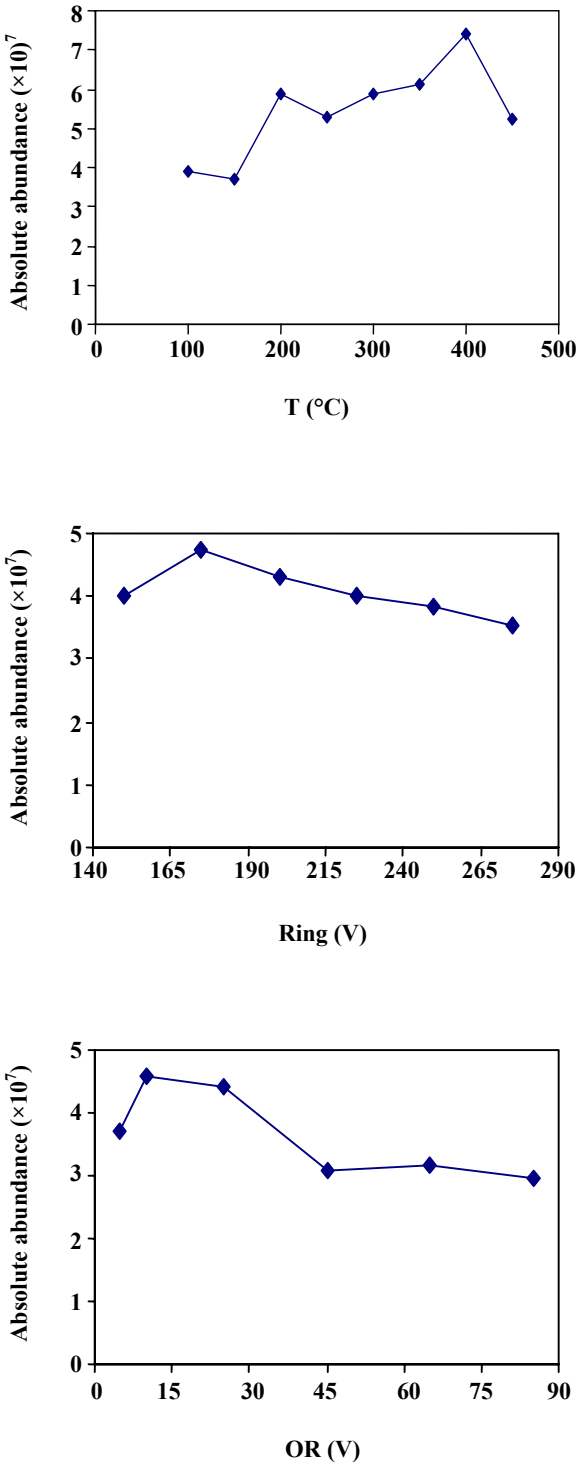

Fig. 2: Effect of temperature (A), ring voltage (B) and the orifice plate voltage (V) on the MS signal of astaxanthin in the range of m/z 350-700 and by searching of the mass m/z 597 of the protonated molecule $(M+H)^+$. Each value represents the average of three separate runs.

597 of the protonated molecule $(M+H)^+$. The minimum sensitivity for astaxanthin was obtained at 150 °C. Fig. 2 shows a sensitivy almost constant in the temperature range 200-450 °C (except at 400°C). The second step was to optimize the OR and ring voltage values by performing infusion experiments. As displayed in Fig. 2, a low orifice voltage (10 V) and a ring voltage equal to 175 were found to ensure the best sensitivy for astaxanthin. The measurements indicate too a decreasing sensitivy for astaxanthin when the ring and orifice voltage were increased. The results were approximately the same with other carotenoids. After obtaining the best sensitivity, 10 µl of astaxanthin (10 µg/ml) were injected and eluted along the LC column and detected by both UV / APCI-MS; the data (retention times and mass) obtained characterize astaxanthin (Fig. 3).

A difference appeared between the retention time of the UV trace and the total ion chromatogram (TIC). This is due to the transfer time of the eluent from the UV detector cell to the mass spectrometer. The chromatogram depicts the UV trace recorded at 480 nm (optimum value for astaxanthin) and the TIC recorded at a mass range between m/z 350 and 700 but also the recorded mass spectrum of astaxanthin (protonated molecule) (m/z 597.6). In a further step, using the operating conditions predefined, the UV-HPLC / APCI-MS tandem was employed to separate and to identify a carotenoid mixture containing astaxanthin (100 µg/l), canthaxanthin (250 µg/l), apocarotenoid ester (200 µg/l), torularhodin (750 µg/l) and β-carotene (250 µg/l). All standards were injected separately or mixed and eluted. All carotenoids were identified during 10 min as it showed in Fig 4. The upper chromatogram corresponds to the UV trace of the carotenoid mixture, which was recorded at 480 nm (mean value allowing to detect the overall allowing to detect the overall carotenoids used). Each peak is characterized by a retention time and by a $(M+H)^+$signal. The other chromatograms belong to the UV trace of the corresponding protonated molecules at m/z 597.6 for astaxanthin, m/z 565.6 for canthaxanthin, m/z 461.6 for apocarotenic ester, m/z 565.6 for torularhodin, m/z 537.6 for β-carotene. Astaxanthin and β-carotene were respectively the first and last pigment separated after 3.25 min and 7.7 min. Canthaxanthin, apocarotenoic ester and torularhodin appeared respectively after 4, 4.5 and 5.35 min.

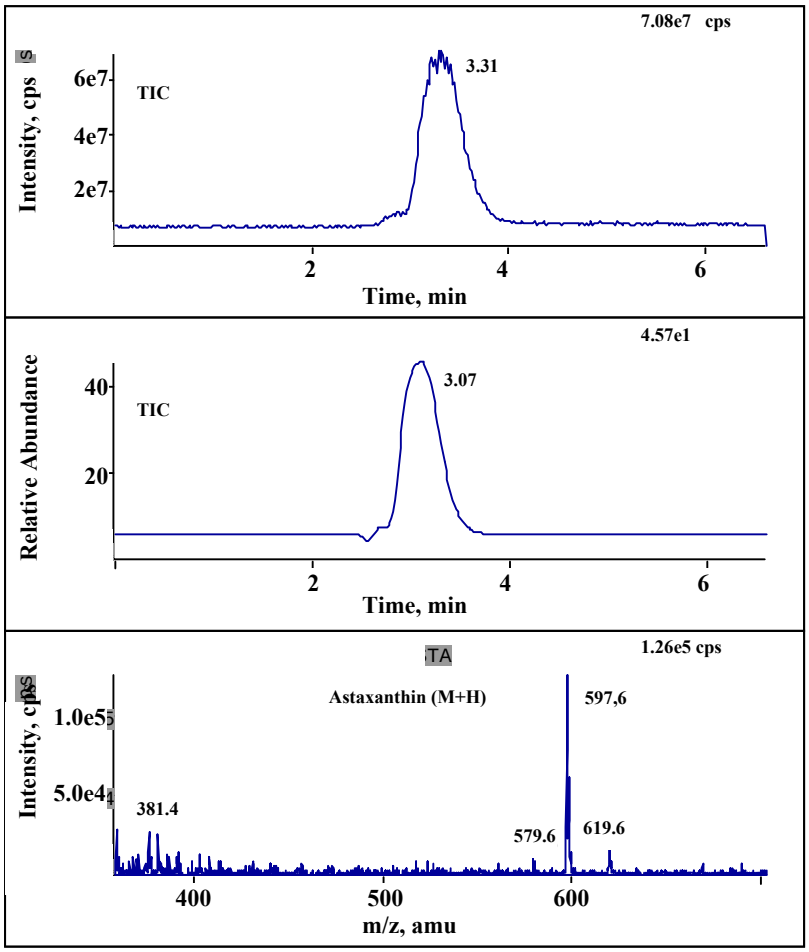

Fig. 3: Total ion current chromatogram (TIC), UV chromatogram and positive ion mass spectrum of astaxanthin using UV-HPLC/ APCI-MS. Experimental conditions: OR 10 V, ring 175 V, APCI vaporize temperature 450 °C, Needle Current (NC) 2μA.

Among the above carotenoids, canthaxanthin and torularhodin have an identical protonated molar mass (565.6). By comparing the chromatograms of each searched mass to the upper one, all substances can be easily identified. The retention times (T_R) of astaxanthin, canthaxanthin and β-carotene obtained here can be compared to those indicated in literature (Table 1). Here, the T_R of all carotenoids are lower than those previously published, particulary for β-carotene (T_R diminished by 3 to 4). Moreover, since all essential microbial carotenoids (β-carotene, torulene and torularhodin in *Rhodotorula glutinis* [8]; β-carotene in *Rhodotorula rubra* [9]; astaxanthin in *Phaffia rhodozyma* [10]; canthaxathin in *Brevibacterium* [11] are correctly separated in a short time, this method appears rather outstanding.

Among carotenoids shown in Fig. 4, astaxanthin, the most polar of them, was released early and the less polar hydrocarbon carotenoids, such as β-carotene, was held strongly, longer than xanthophylls. The addition of ammonium acetate to the mobile phase was shown to improve the recovery of carotenoids from the column (results not shown). Hart and Scott [12] reported too that the use of ammonium acetate (0.05 M added to MeOH in the mobile phase) increased the recovery from column. Previousely, Handleman et al. [13] noticed that the use of ammonium acetate reduced on –column degradation. The action of this solvent modifier is not known enough, but Hart and Scott [12] indicated that the improvement in recovery is caused by the buffering of acidity in the mobile phase, or more likely the acidity of the free

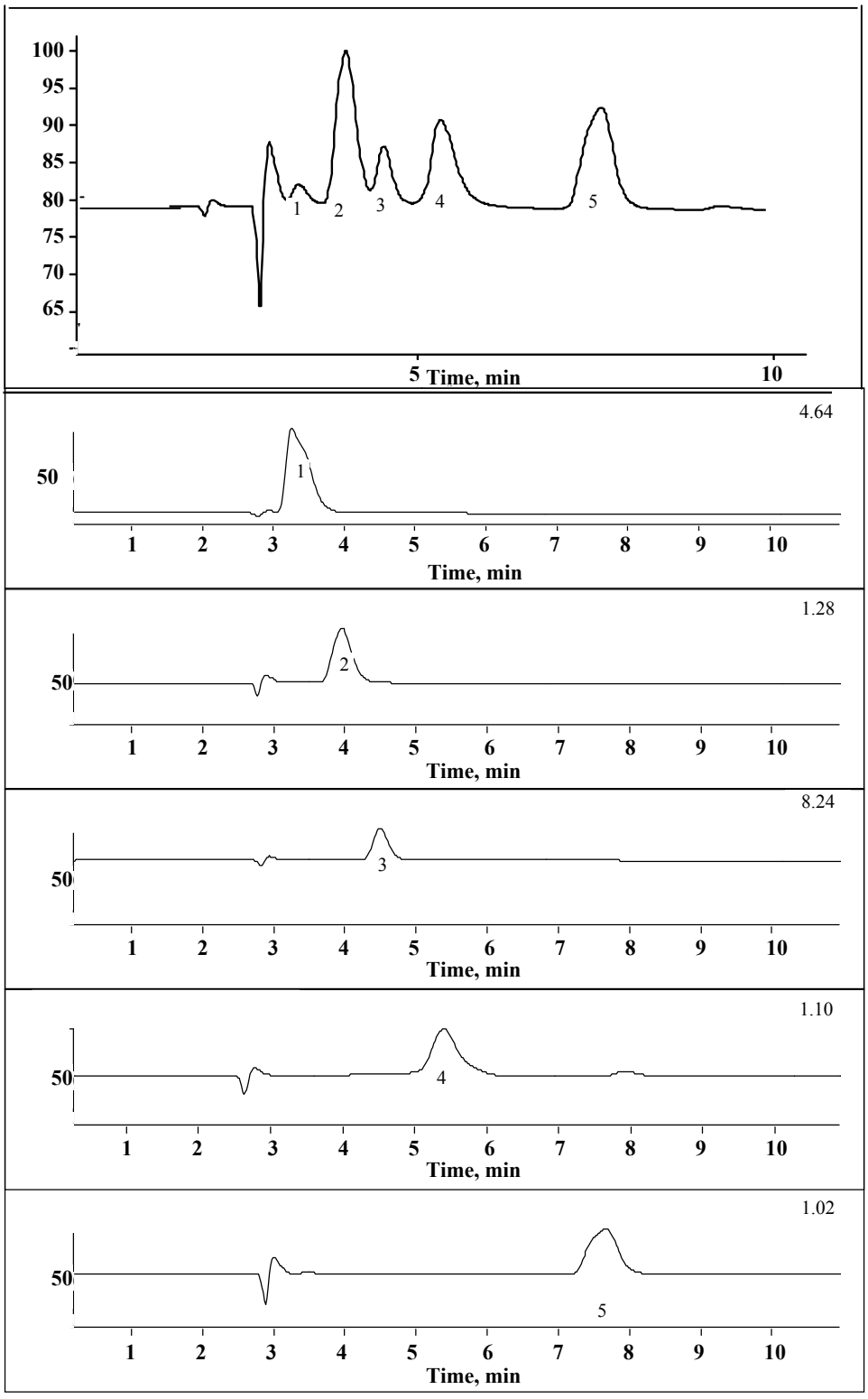

Fig. 4: Analysis of a mixture of different carotenoids by UV-HPLC / APCI-MS: 1 = astaxanthin; 2 = canthaxanthin; 3 = apocarotenoic ester; 4 = torularhodin; 5 = β-carotene.

Table 1: Technical characteristic methods used to identify carotenoids mixtures.

Carotenoid	$T_{R\ (min)}$	Analysis method	Column	Mobile phase	Reference
Astaxanthin	4	LC-TurboISP-MS	C_{18} (tow columns in serie) ODS Hypersil (200×2.1 mm, 5 µm & 100×2.1 mm, 5 µm	Acetonitrile-methanole (0.1 M ammonium acetate)-dichloromethane	(Careri et al., 1999)
Lutein	4.2				
Zeaxanthin	5				
Canthaxanthin	5.5				
Cryptoxanthin	12				
β-carotene	31				
Astaxanthin	8.7	UV-HPLC/APCI-MS	C_{30} Self-synthesized Polymeric (250×4.6 mm)	Methanol-methyl tert-butyl ether (MTBE)	(Lacker et al., 1999)
Zeaxanthin	11.4				
Canthaxanthin	12.6				
Echinenone	17.6				
Tans β-carotene	19.2				
Cis β-carotene	23.0				
Astaxanthin	3.3	UV-HPLC/APCI-MS	C_{18} Platinium (100×2.1 mm, 3 µm)	Acetonitrile-methanol (0.1 M ammonium acetate)-dichloromethane	This work
Canthaxanthin	4				
Apocarotenoic ester	4.5				
Torularhodin	5.4				
β-carotene	7.7				

Table 2: Reproducibility of the HPLC measurement (n=5) of the five pure carotenoids diluted in ethanol (% CV).

µg/ml	Astaxanthin	Canthaxanthin	Apocarotenoic ester	β-carotene	Torularhodin
0.5	1.74	3.13	2.67	2.16	2.25
1.0	1.48	1.92	2.29	2.26	1.34
1.5	1.08	2.00	2.77	1.96	2.38
% CV	1.55	2.02	2.57	2.12	2.32

silanols in the stationary phase or by preventing reactions with free metal ions. In another study, Steghens et al. [14] showed that ammonium acetate can be used to neutralize acids that can be formed from acetonitrile.

The limit of detection for β-carotene, astaxanthin, apocarotenic ester, canthaxanthin and torularhodin was determined by flow injection using full-scan detection and by searching the mass of the protonated molecule $(M+H)^+$. The limits of detection estimated are: astaxanthin 5 ng/ml, β-carotene 6 ng/ml, canthaxanthin 8 ng/ml, apocarotenoic ester 6 ng/ml and torularhodin 9 ng/ml.

In order to quantify the reproducibility, the measurement of five separate standard carotenoids, containing either astaxanthin, β-carotene, canthaxanthin, apocarotenoic ester or else torularhodin, at the different concentrations (0.5, 1.0 and 1.5 µg/ml), was done at the same time (intraday). Table 2 shows a very good average CV always less than 2.57 % for each pure carotenoid diluted in ethanol. The measurement of each carotenoid concentration from stock solutions, kept at -18 °C, was monitored over a period of eight weeks to assess the stability of the pure solutions and the long time reproducibility of response (intraday). All results showed good reproducibility and an excellent stability, except for torularhodin which demonstrated a noticeable decrease of the signal: about 30 %. (in ethanolic solution). Torularhodin samples were frozen (-18 °C) and then unfrozen before each analysis. As torularhodin is a strong antioxidant, the concentration was prone to decline in presence of oxygen.

This can explain the results observed. Hart & Scott [12] reported similar results for lycopen which is very unstable. Therefore, our analysis method appeared to be well-adapted to a rapid characterization and quantification of microbial carotenoids. It was performed to the

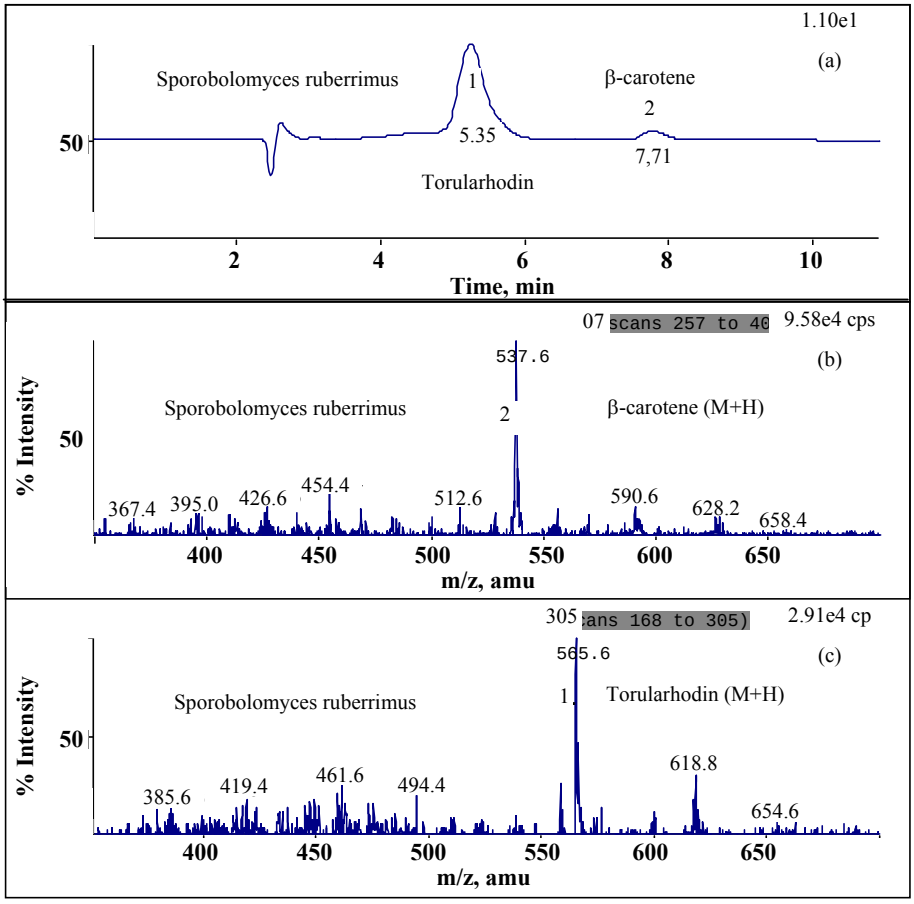

Fig. 5: UV chromatogram (a) and positive-ion mass spectra (b.c) of pigments in Sporobolomyces ruberrimus. Carotenoids identified by UV-HPLC / APCI-MS: 1 = Torularhodin; 2 = β-carotene.

identification of the unknown carotenoid content in a yeast named *Sporobolomyces ruberrimus* H 110, newly isolated.

Identification of carotenoids present in Sporobolomyces ruberrimus H110 yeast strain

Carotenoids were identified by their retention time and by their $(M+H)^+$ signal described in Fig. 5. the upper chromatogram corresponds to the UV trace which was recorded at 480 nm. Torularhodin and β-carotene are the two pigments identified in *Sporobolomyces ruberrimus.* Two other traces belong to the computer reconstructed mass spectrum of the corresponding protonated molecules at m/z 565.6 for torularhodin and m/z 537.6 for β-carotene. The UV chromatogram shows that the yeast strain contains a large amount of torularhodin and a few β-carotene. Torularhodin has been identified also in a few microorganisms including *Rhodotorula* [15,8,16] but in less proportion.

Applying the UV-HPLC method to the monitoring of a carotenoid production run by Sporobolomyces ruberrimus H110

The UV-HPLC method was applied to the quantitative assay of carotenoids in real microbial producing conditions. The volumetric carotenoid concentration were calculated after breaking the cells (*SP. ruberrimus* H110) and extraction of all pigments using ethanol.

Fig. 6 shows the kinetic evolution curve in time of the torularhodin and β-carotene concentration over the fermentation run of technical glycerol-containing medium in an aerated batch reactor.

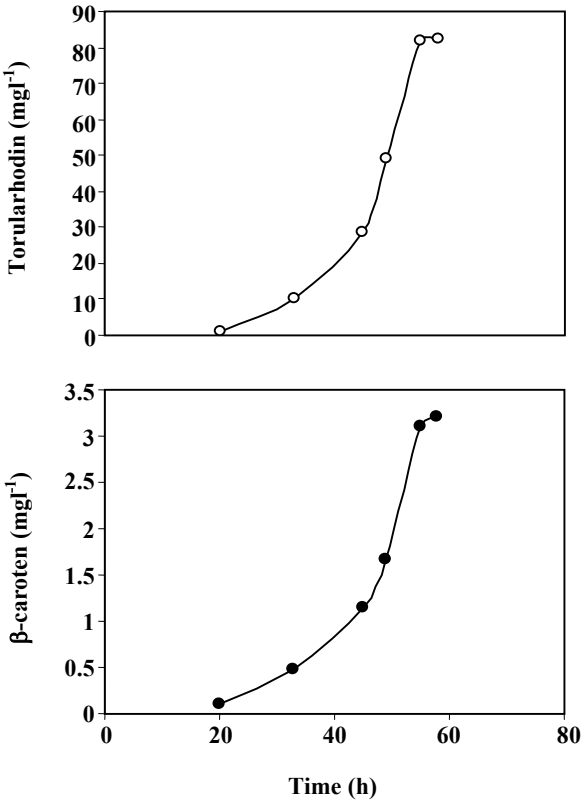

Fig. 6: Kinetics of carotenoid production by Sporobolomyces ruberimus on technical glycerol. ○ Torularhodin, ● β-carotene.

CONCLUSIONS

To study the different carotenoids produced by *Sporobolomyces ruberrimus* H110 that newly isolated, it was needed to set up an analytical method based on a UV-HPLC / APCI-MS tandem. A previous study has allowed to define the best separation conditions of different carotenoids by HPLC using a Symmetry C_{18} column and then, the LC analytical conditions had to be adapted to the on-line APCI-MS. The first part of the present work described the following step which consisted in optimizing the operating conditions using the positive ion detection mode of the APCI-MS device, leading to an effective identification of microbial carotenoids.

The analytical results revealed that the method was fast, relatively simple and really useful to distinguish between two carotenoids having an identical molar mass but a different retention time. A high sensitivity provides an efficient method for the quantification of carotenoid pigments for all microorganisms and natural products.

To our knowledge, no previous article on the pigment produced by *Sporobolomyces ruberrimus* H110 has been published. The results indicated that *Sporobolomyces ruberrimus* H110 produced essentially torularhodin and a small amount of β-carotene. Torularhodin has a very important antioxidant activity [16]; [17]; [18] and is reported to be a provitamin A [17]; [18]. It is abundantly biosynthesized by this strain.

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Nomenclature

APCI	Atomospheric pressure chemical ionization
Amu	Atomic mass unit
CF-FAB	Continous flow-fast atom bombardment
ESI	Electro-spray ionization
ESP	Electro-spray
LC	Liquid chromatography
NC	Needle current
OR	Orifice plate voltage
PI	Positive ion
TIC	Total-ion chromatography

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