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Liquid chromatography–electrospray mass spectrometry of β-carotene and xanthophylls Validation of the analytical method

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Abstract

The investigation of β-carotene and the xanthophylls β-cryptoxanthin, lutein, zeaxanthin, canthaxanthin and astaxanthin using reversed-phase liquid chromatography–electrospray mass spectrometry interfaced with TurboIonspray (LC–TurboISP–MS) is described. Two narrow-bore C_{18} columns connected in series and an isocratic solvent system containing acetonitrile–methanol (0.1 *M* ammonium acetate)–dichloromethane at a flow-rate of 300 μl/min (without splitting) were used. Operating in the positive-ion mode over *m/z* 500–650, the effects on the formation of the molecular ion species or adduct ions and the MS detector response were investigated for carotenoids, varying the orifice plate voltage, the ring voltage and the ISP voltage. Both conventional ISP and TurboISP were performed; using the TurboISP–MS system, ionization efficiency increased with respect to ISP–MS, particularly at the highest temperature (500°C). Good results were particularly obtained for β-carotene, which was detectable at the low ng level, without the use of solution-phase oxidants. Using LC columns and acquiring in single-ion monitoring mode, detection limits were estimated to be in the 0.1–1 ng range; dynamic range was established between one- and two-orders of magnitude. Better sensitivity under positive-ion than negative-ion conditions was demonstrated.

Keywords: Electrospray ionization; Carotenoids; Carotenes; Xanthophylls

1. Introduction

Carotenoids are highly conjugated polyprenoids found in a variety of natural sources including fruits, vegetables and sea products with high nutritional and biological importance [1,2]. They are classified into two major groups, carotenes and xanthophylls; the former are polyene hydrocarbons, whereas the latter are usually oxygenated at the end groups. According to reports and clinical studies, carotenoids may be important in the prevention of several degenerative human heart conditions, including cancer and health disease [3]. This class of substances consists of numerous related compounds, including isomers that can be quite difficult to separate in natural samples. Due to the nutritional importance of carotenoids in human health as metabolic precursors of vitamin A and as antioxidants, the interest for new methods which can positively identify and quantify these compounds is increased [4,5]. Fixed-wavelength UV–Vis detection and UV–Vis photodiode-array detection are the common choices for high-performance liquid chromatography (HPLC), but these approaches can be inadequate for the unambiguous

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identification of carotenoids in food and biological matrices, owing to the risk of spectral interferences.

Even though only liquid chromatography–nuclear magnetic resonance (LC–NMR) makes possible the identification of structurally closely related compounds [6,7], the use of MS detection is a powerful tool for the identification of carotenoids, when it is coupled with LC, to exploit the chromatographic separation of isomers, e.g., zeaxanthin and lutein.

Recently, a critical report on carotenoid analysis using LC–MS was compiled by van Breemen [8]. Several LC–MS techniques have been used for carotenoid analysis, including continuous flow-fast atom bombardment (CF-FAB) [9], electrospray (ESP) [10] and atmospheric pressure chemical ionization (APCI) [11]; new methods based on the use of the particle beam interface under electron-capture negative-ion conditions have been recently devised for the analysis of carotenes and xanthophylls [12,13]. In the last years, atmospheric pressure ionization techniques have become accepted as the most robust, sensitive and versatile systems for the LC–MS analysis of low- and high-molecular-mass compounds [14,15]. These techniques are increasingly applied to the analysis of both xenobiotic and naturally occurring food constituents, as reported in recent reviews [16,17].

In this article we report on the use of TurboIonspray (TurboISP) ionization for LC–MS analysis of carotenoids, our primary interest being the development of a widely applicable method for the qualitative and quantitative analysis of these molecules. The TurboISP interface consists of the ISP probe used in conjunction with a heated turboprobe, which aids in the desolvation of the sprayed droplets so that high flow-rates can be handled up to 1 ml/min without split [18]. Conventional ionspray requires low LC flow-rates (50–100 μl/min) for efficient nebulization and charged droplet formation. Therefore, a post-column solvent splitting is necessary to avoid sensitivity losses when standard or narrow-bore columns are used. By coupling reversed-phase HPLC with MS via the TurboISP interface, different carotenoids were investigated: β-carotene and the xanthophylls β-cryptoxanthin, zeaxanthin, lutein, canthaxanthin and astaxanthin (Fig. 1). The following figures of merit of the LC–TurboISP–MS method were studied: dynamic range, sensitivity, limits of detection and precision. The capabilities of TurboIonspray ionization system in carotenoid characterization and quantitation were demonstrated by analyzing a microalgae sample.

2. Experimental

2.1. Chemicals

β-Carotene standard (>97% purity) was obtained from Fluka (Buchs, Switzerland); astaxanthin, canthaxanthin, β-cryptoxanthin, zeaxanthin and lutein were gifts from Hoffmann-La Roche (Basel, Switzerland). Stock standard solutions of β-carotene were prepared daily; stock standard solutions of other carotenoids were prepared monthly. Pure compounds were dissolved in tetrahydrofuran containing 1% (w/v) butylated hydroxytoluene (BHT) as an antioxidant and the solutions were stored in brown flasks at 4°C.

All organic solvents were HPLC grade. Acetonitrile, methanol, dichloromethane, tetrahydrofuran were obtained from Carlo Erba (Milan, Italy). Ammonium acetate was of analytical-reagent grade and was supplied by Sigma (Milan, Italy).

The Spirulina Pacifica (*Spirulina platensis* strain pacifica microalgae) powder samples were kindly provided by Cyanotech Corporation (Kailua-Kona, HI, USA); algae samples were extracted and prepared as previously described [13].

2.2. Liquid chromatography–mass spectrometry

HPLC separation was performed on two ODS Hypersil columns connected in series (200×2.1 mm, 5 μm and 100×2.1 mm, 5 μm) (Hewlett-Packard, Palo Alto, CA, USA) using an isocratic solvent system [acetonitrile–methanol (0.1 *M* ammonium acetate)–dichloromethane (71:22:7, v/v/v)] at a flow-rate of 300 μl/min; no post-column splitting was performed. The mobile phase was delivered by a Perkin-Elmer series LC 200 binary pump (Perkin-Elmer, Thornhill, Canada) equipped with an autosampler (BCS, Milan, Italy). The volume of standard solutions injected varied from 1 to 5 μl.

A Perkin-Elmer Sciex API 150EX (PE Sciex, Foster City, CA, USA) single quadrupole instrument

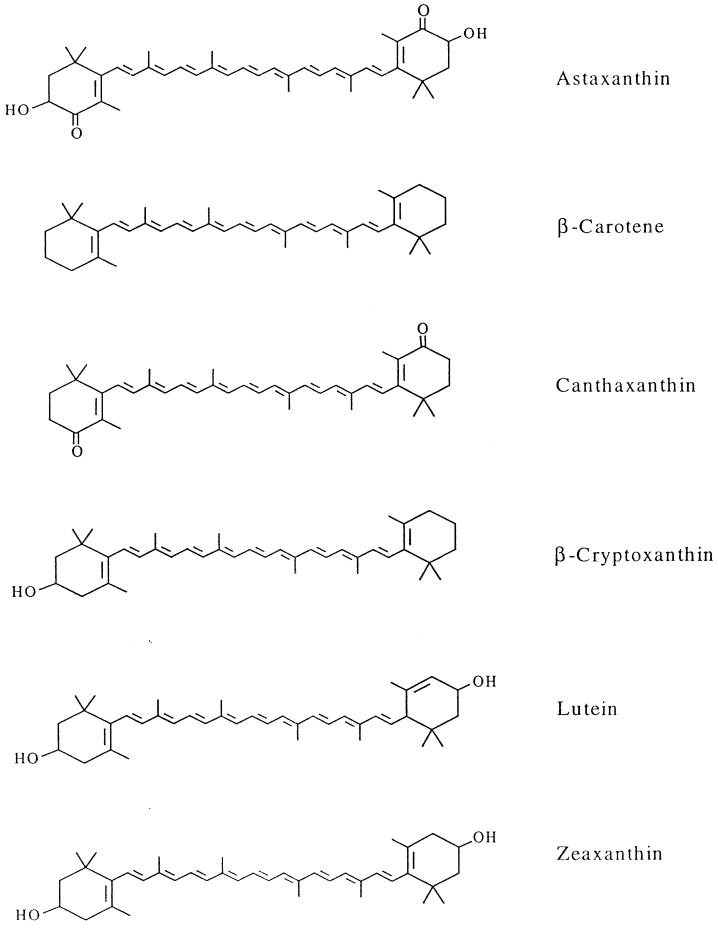

Fig. 1. Chemical structures of the carotenoids studied.

equipped with a TurboIonspray interface and an Apple Macintosh System 8.1 with a MassChrom v1.0 application version, was used for data acquisition and processing.

The nebulizing gas (zero grade air, 99.999% purity) and the curtain gas (nitrogen, 99.998% purity) were delivered at a flow-rate of 0.9 and 1.0 l/min, respectively. Using flow-injection analysis (FIA), the behavior of carotenoids was evaluated in conventional ISP (turbo off) and in TurboISP (turbo on); in the last case, the influence of the turboprobe temperature on the MS response of the analytes was investigated in the 100–500°C range. The heater gas (compressed air, technical grade) of the turboprobe was delivered at a flow-rate of 7.5 l/min. Operating parameters of the TurboISP interface were optimized by infusing standard solutions in the mobile phase at 5 μl/min, using a Harvard syringe pump (Quebec, Canada); the effects of the orifice plate voltage OR (20–180 V), the ring voltage (200–400 V) and the ISP voltage (2.5–6 kV) on the TurboISP–MS response of carotenoids were studied. The optimum conditions of the interface were as follows: OR voltage 40 V, ring voltage 260 V, ionspray voltage 3.5 kV; turboprobe temperature 500°C.

LC–MS determinations were performed by operating the mass spectrometer in the positive-ion (PI) mode. Mass spectra were acquired over the scan range m/z 500–650 using a step size of 0.1 u and a dwell time of 2 ms; the resolution of quadrupole was tuned to unit resolution. Quantitative analysis was carried out using single ion monitoring (SIM) of the

molecular ion peaks of carotenoids at m/z 568 (lutein, zeaxanthin), m/z 564 (canthaxanthin), m/z 552 (β-cryptoxanthin), m/z 536 (β-carotene) with a dwell time of 50 ms per ion; in the case of astaxanthin, the sodium adduct ion at m/z 619 was monitored. Precision was calculated in terms of intra-day and inter-day repeatability as relative standard deviation (RSD, %) at two concentration levels (50 and 100 ng for β-carotene, astaxanthin, zeaxanthin and lutein, 20 and 50 ng for canthaxanthin and 10 and 50 ng for β-cryptoxanthin) in SIM mode. Ionization was also performed in the negative-ion (NI) mode for all the analytes both in conventional ISP and TurboISP at 500°C. Full-scan NI spectra were acquired in the mass range 500–650 u at a scan rate of 0.33 scan/s.

For quantitative analysis of carotenoids in *Spirulina platensis* algae sample, calibration graphs were constructed in PI mode over the range 20–80 ng/μl for zeaxanthin, 5–50 ng/μl for β-cryptoxanthin and 60–300 ng/μl for β-carotene.

2.3. Liquid chromatography with UV–Vis detection

Chromatographic separation was performed with a Hewlett-Packard Model 1050 solvent delivery system equipped with a Hewlett-Packard HP 1050 autosampler and a Hewlett-Packard HP 1050 UV–Vis detector (2-μl flow cell) operating at 449 nm. Data were acquired by the Turbochrom 4 PE Nelson software (PE, Nelson, Cupertino, CA, USA). Chromatographic separations were carried out under the same conditions as for LC–MS. Determination of carotenoids in the alga sample was carried out by constructing the calibration graphs in the same way as for LC–TurboISP–MS.

3. Results and discussion

3.1. Evaluation of the TurboISP interface parameters

In order to investigate the performance of the LC–TurboISP–MS technique for the analysis of carotenoids, preliminary studies were carried out under both infusion and FIA conditions in full-scan mode. For all the experiments, the position of the ionspray needle was optimized horizontally and laterally with respect to the orifice to obtain the best sensitivity, a stable signal and to avoid source and detector contamination.

The main operating parameters which have an impact on performance of TurboISP are the orifice plate voltage, the ring voltage, the ionspray voltage and the turboprobe temperature, the effects of which on the MS response were investigated for all the analytes.

In a first step, TurboIonspray performance at different turboprobe temperatures (100–500°C) was compared with that of the conventional ionspray for the analysis of carotenoids. Changing the heater gas temperature from 100–500°C (Fig. 2) resulted in as much as a 15-fold increase in response for lutein, zeaxanthin and β-carotene and a six-fold increase for the other substances. High heater gas temperature values cause an easier desolvation of the sprayed droplets, without excessive heating of the dissolved analytes. In fact, from the mass spectra, no decomposition was observed even at the highest temperatures. When operating with the heater gas of TurboISP turned off, lower responses were observed for all the analytes; the difference in absolute abundance of carotenoids obtained with TurboISP with respect to conventional ISP increases with increasing temperature, as shown in Fig. 2. This behavior likely reflects the degree of desolvation achievable during passage through the interface.

The second step was to optimize the OR and ring voltage values by performing infusion experiments. As shown in Fig. 3, a low orifice voltage (40 V) was found to ensure the best sensitivity for all the carotenoids studied, whereas at higher values a signal intensity reduction was observed for all compounds. Except for canthaxanthin, for which the maximum absolute intensity was obtained at the ring voltage 260 V, less severe variation in the MS response for the other analytes was observed when this parameter was varied from 200 to 400 V. The optimum condition was therefore established at the ring voltage of 260 V (Fig. 4). The carotenoid signal did not vary with the ionspray voltage from 2.5 to 6.0 kV; this parameter was maintained at 3.5 kV.

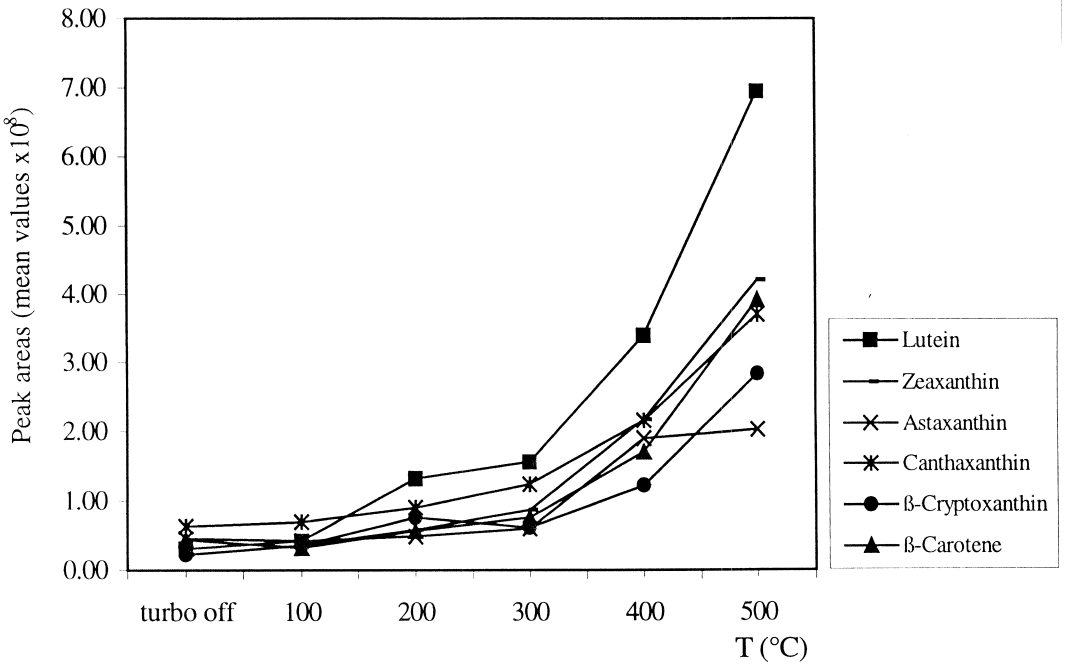

Fig. 2. Effect of turboprobe temperature on the signal of carotenoids. FIA conditions: acetonitrile–methanol (0.1 *M* ammonium acetate)–dichloromethane (71:22:7); flow-rate 300 μl/min. MS acquisition: full-scan *m/z* 500–650.

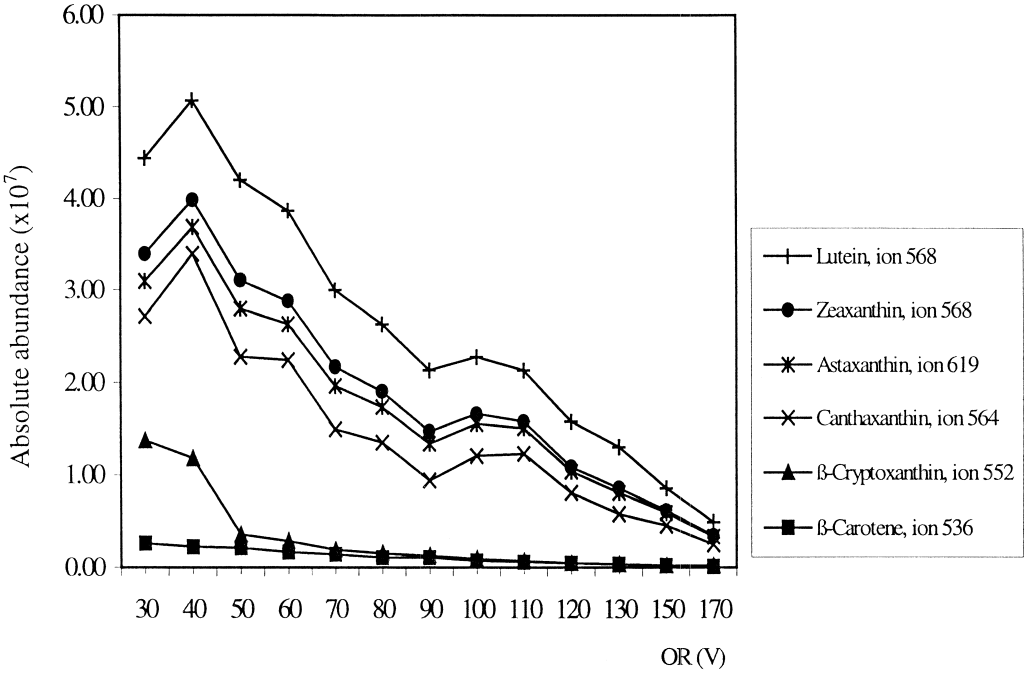

Fig. 3. Effect of orifice plate voltage on the MS signal of carotenoids. Infusion analysis: carotenoid standard solutions (10 μg/ml each) at 5 μl/min. MS acquisition: full scan *m/z* 500–650.

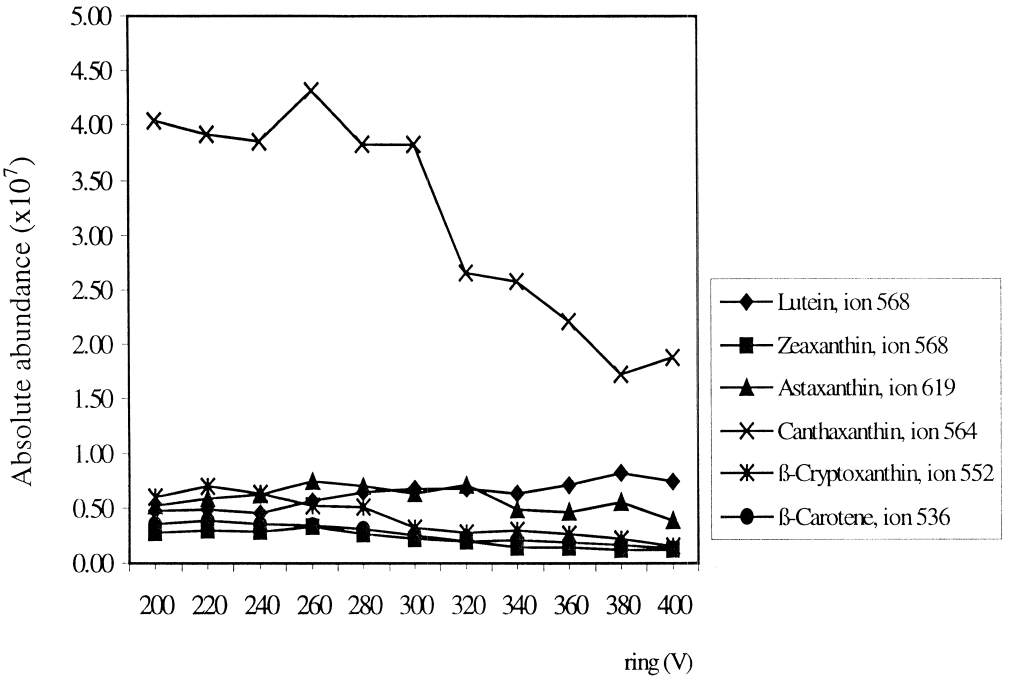

Fig. 4. Effect of ring voltage on the MS signal of carotenoids. Infusion analysis: carotenoid standard solutions (10 μg/ml each) at 5 μl/min. MS acquisition: full scan *m/z* 500–650.

3.2. TurboISP mass spectra of carotenoids and chromatographic separation

An evaluation of spectral information was carried out operating in FIA mode with the conventional ionspray and in the 100–500°C range with the TurboISP system. As shown in Fig. 5, the positive-ion TurboISP mass spectra obtained for all carotenoids except that of astaxanthin are characterized by the molecular ion $M^{+\cdot}$ due to loss of one electron and formation of a stable radical system. Using the TurboISP and increasing the temperature of the turboprobe, an increase of the molecular peak $M^{+\cdot}$ was observed for canthaxanthin, zeaxanthin, lutein, β-carotene and β-cryptoxanthin. The PI spectrum of astaxanthin exhibits the abundant sodium ion adduct (*m/z* 619) and the protonated molecule (*m/z* 597); in addition, the adduct species $[M+K]^+$ at *m/z* 635 was observed (relative intensity ca. 40%). Astaxanthin also displays the protonated molecule as the base peak in conventional ISP (Fig. 6). As attested by these results, β-carotene is also well suited to positive-ion TurboISP–MS detection; other authors described unsuccessful results for the ionspray ionization of β-carotene unless trifluoroacetic acid was added to the mobile phase post-column to carry out solution-phase oxidation [10].

In a further step, the possible acquisition modes for MS determination of analytes under both ISP and TurboISP conditions was checked. For this purpose, FIA experiments were carried out to record negative-ion ISP and TurboISP mass spectra. β-Carotene and all the xanthophylls considered did not show any response under NI acquisition and conventional ISP conditions. In TurboISP mode, only the hydroxylated derivatives were detected, the NI mass spectra being characterized by the molecular ion $M^{-\cdot}$; conversely, no signals were observed for β-carotene and canthaxanthin.

The total ion chromatograms and the selected ion chromatograms for the six carotenoids under investigation (40 μg/ml each, 50 μg/ml astaxanthin) are shown in Fig. 7. Peak shapes were generally good, except for astaxanthin and β-carotene.

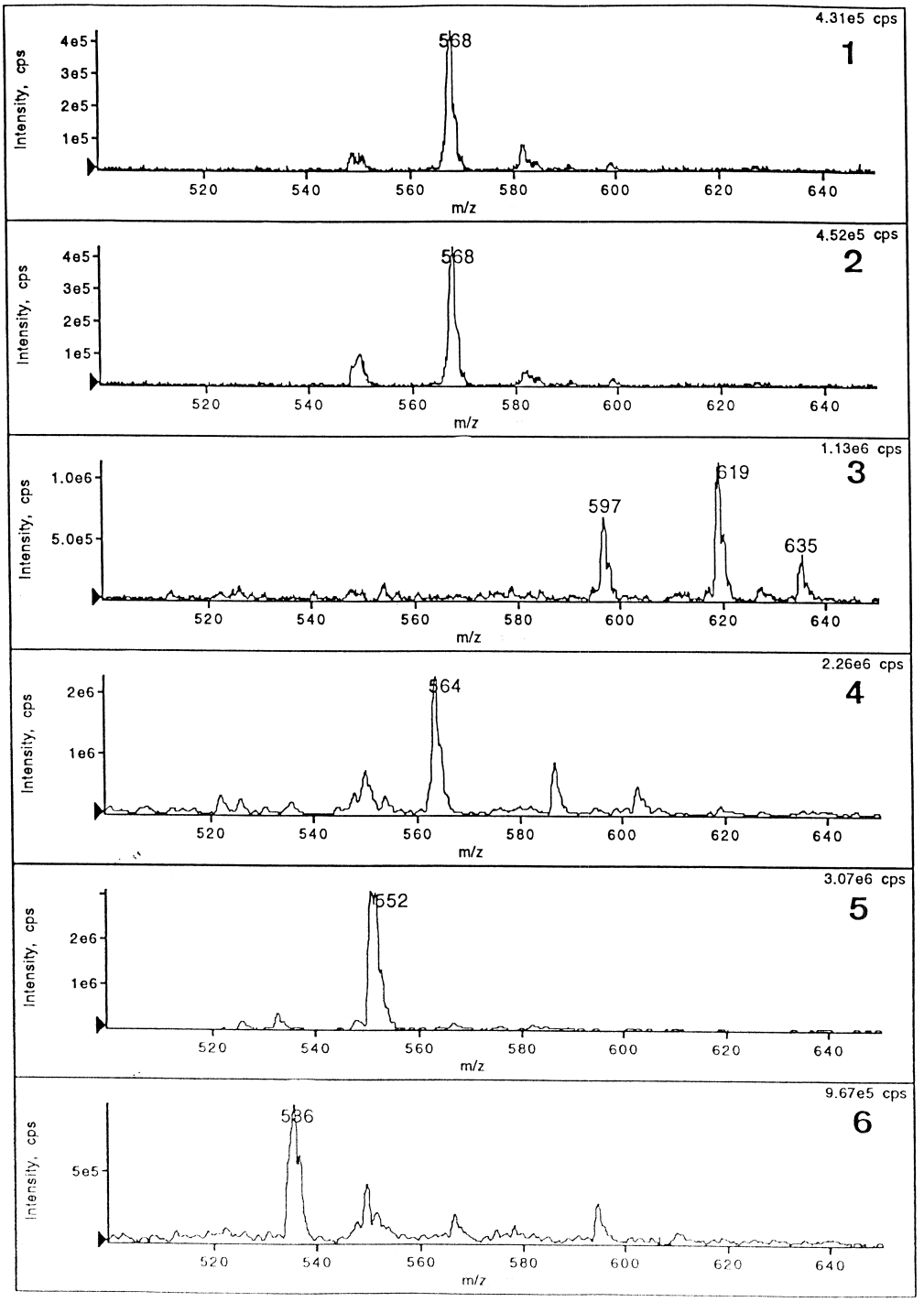

Fig. 5. Positive-ion TurboISP mass spectra of: 1, lutein; 2, zeaxanthin; 3, astaxanthin; 4, canthaxanthin; 5, β-cryptoxanthin; 6, β-carotene.

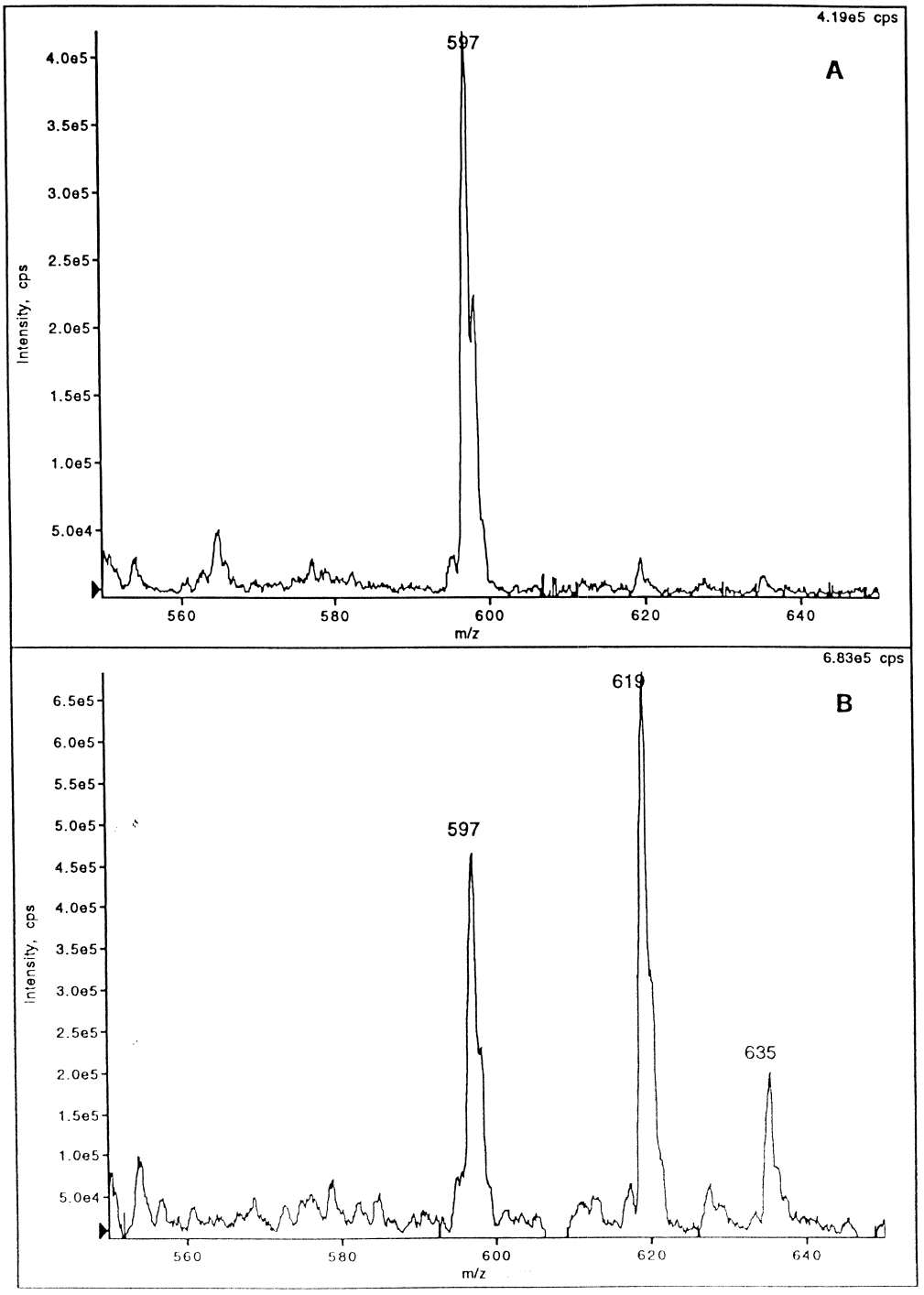

Fig. 6. Positive-ion mass spectra of astaxanthin obtained with (A) conventional ISP and (B) TurboISP at a turboprobe temperature of 500°C.

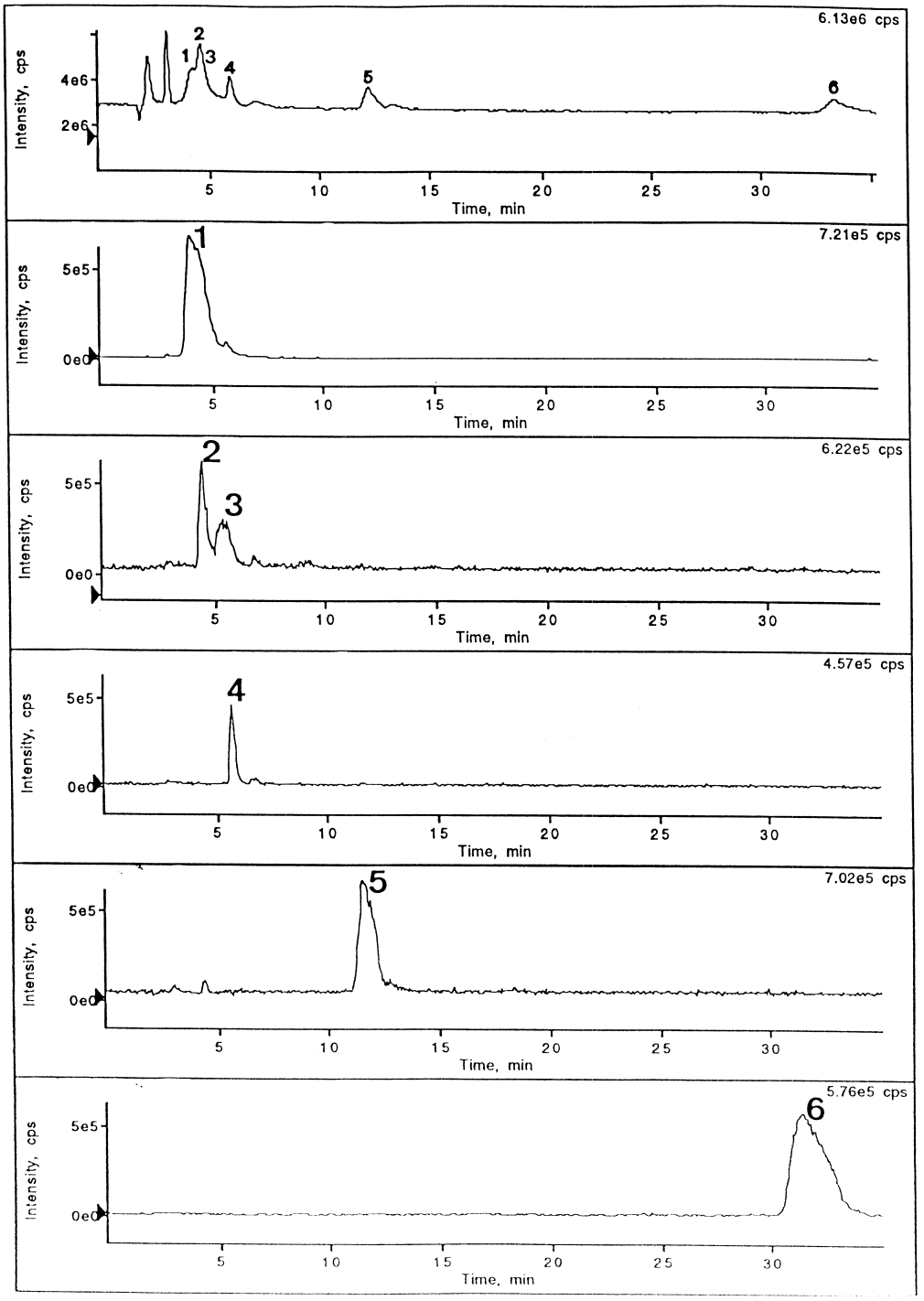

Fig. 7. Total ion current chromatogram (A) and extract ion current profiles relative to a mixture of carotenoids obtained by TurboISP in full-scan PI mode. For chromatographic conditions see Experimental. TurboISP conditions: OR 40 V, ring 260 V, ISP 3.5 kV, heater gas temperature 500°C. Peaks: 1=astaxanthin; 2=lutein; 3=zeaxanthin; 4=canthaxanthin; 5=β-cryptoxanthin; 6=β-carotene.

3.3. Linearity, detection limits and precision of the LC–TurboISP–MS system

The results obtained using the LC–TurboISP–MS method attest to good performance for carotenoid analysis, so further studies on linearity, detection limits and precision were performed. In order to achieve optimum sensitivity, all experiments were carried out under SIM conditions.

Table 1 summarizes the data concerning the dynamic range and the detection limits of the analytes. The dynamic range was established between one- and two-orders of magnitude; non-linearity effects of the LC–TurboISP–MS system were observed for all analytes as concentration levels were increased further, as shown in Table 1. Comparing the slope values of the calibration curves, the highest sensitivity was observed for β-carotene.

The limits of detection ($S/N=3$) were determined under chromatographic conditions in the 0.1–1 ng range; the lowest value was obtained for canthaxanthin (0.1 ng, 0.2 pmol) followed by β-cryptoxanthin (0.4 ng, 0.7 pmol), β-carotene (0.5 ng, 0.9 pmol) and lutein (0.6 ng, 1 pmol) (Table 1). Van Breemen reported detection limits for lutein and β-carotene between 1 and 2 pmol each, using flow-injection under ESP-SIM conditions [10]. Using positive-ion APCI in FIA mode, limits of detection were estimated to be ca. 3 and 13 pmol for α-carotene and lutein, respectively [11]. The results achieved in this work attest to the improved detection limits obtained with the TurboISP–MS technique with respect to APCI.

As for the intra-day instrumental precision of the analysis, the RSDs ($n=5$) are between 1 and 6.4%; also the inter-day repeatability was good, RSDs ($n=15$) being in the range 1.3–10% (Table 2).

Table 2
Intra-day and inter-day repeatability of the LC–TurboISP–MS technique[a]

Analyte	ng	RSD (%)	
		Intra-day[b]	Inter-day[c]
Lutein	50	0.8	6.8
	100	4.5	5.9
Zeaxanthin	50	6.4	6.4
	100	3.8	5.8
Astaxanthin	50	2.6	2.2
	100	1.2	1.6
Canthaxanthin	50	3.5	1.3
	20	4.1	3.6
β-Cryptoxanthin	10	2.6	12
	50	4.3	3
β-Carotene	50	2.2	5.4
	100	1.0	10

[a] Mobile phase: CH_3CN–MeOH with 0.1 *M* CH_3COONH_4–CH_2Cl_2 (71:22:7, v/v/v).
[b] Calculated from mean values ($n=5$).
[c] Calculated from mean values ($n=15$).

3.4. LC–UV–Vis and LC–TurboISP–MS determination of carotenoids in Spirulina platensis microalgae

The method developed was applied to the qualitative and quantitative assay of carotenoids in a carotenoid-rich product such as the *Spirulina platensis* strain pacifica microalgae. The TIC chromato-

Table 1
Calibration graph results for LC–TurboISP–MS analysis of carotenoids[a,b]

Analyte	Range (ng)	$a\cdot10^{-6}$	$b\cdot10^{-6}$[c]	n	r^2	DL[d]
Lutein	2.5–40	1.60±0.05	2.0±0.5	21	0.995	0.6
Zeaxanthin	2.5–40	0.67±0.07	3.5±1.1	19	0.992	0.6
Astaxanthin	5–200	1.93±0.02	–	27	0.998	1
Canthaxanthin	0.5–50	0.78±0.01	1.8±0.3	26	0.997	0.1
β-Cryptoxanthin	2.5–100	1.06±0.08	2.3±1.8	24	0.988	0.4
β-Carotene	2.5–80	3.4±0.3	9±6	21	0.989	0.5

[a] Calibration fitting: $y=ax+b$.
[b] ± values are confidence intervals for 95% probability level.
[c] $P<0.05$.
[d] Limit of detection ($S/N=3$) in ng under SIM conditions.

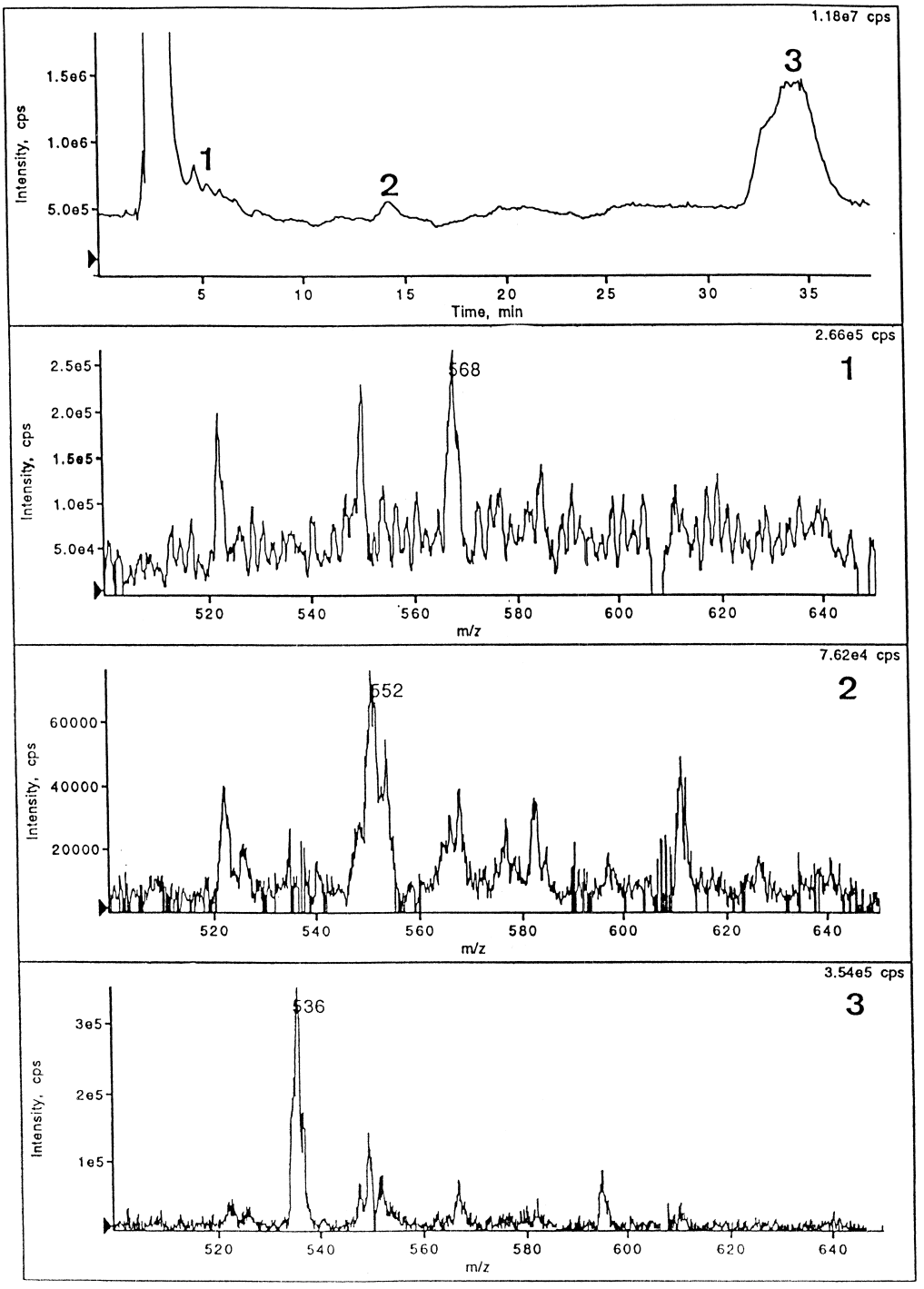

Fig. 8. Total ion current chromatogram of alga *Spirulina Platensis* algae sample by LC–TurboISP–MS and positive-ion mass spectra of the carotenoids identified: 1=zeaxanthin; 2=β-cryptoxanthin; 3=β-carotene.

Table 3
Quantitative analysis (mg/100 g) of carotenoids in *Spirulina platensis* algae sample

Analyte	LC–UV–Vis[a]	LC–TurboISP–MS[b]
Zeaxanthin	119.0±1.0	98.4±1.3
β-Cryptoxanthin	10.5±0.3	12±2
β-Carotene	228±7	225±4

[a] UV–Vis detection at 449 nm.
[b] SIM acquisition: m/z 568 for zeaxanthin; m/z 552 for β-cryptoxanthin; m/z 536 for β-carotene, (dwell time, 50 ms per ion).

gram and the positive-ion mass spectra obtained (Fig. 8) allowed us to identify zeaxanthin, β-cryptoxanthin and β-carotene among the analytes studied.

The quantitative assay of the compounds identified was carried out under SIM conditions and the results were compared with those obtained by performing UV–Vis detection at 449 nm (Table 3).

A good agreement between the results obtained with the two detection techniques is observed for β-cryptoxanthin and β-carotene, whereas a significantly higher concentration value of zeaxanthin was determined using LC–UV–Vis. This can be explained on the basis of a spectral interference in the spectrophotometric measurement, which could be responsible for overestimation of the zeaxanthin peak area.

4. Conclusions

This work demonstrates that using the positive-ion mode of detection, TurboISP–MS may be a valuable tool in analyses of both carotenes and xanthophylls. Particularly valuable is the sensitivity achieved, so that it can be inferred that among all the LC–API-MS methods used for carotenoid determination, LC–TurboISP–MS is the most sensitive technique developed. Sensitivity proved to be adequate for the qualitative and quantitative assay of carotenoids in complex matrices such as natural products.

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