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Beta-carotene and lycopene determination in new enriched bakery products by HPLC-DAD method

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Abstract

A fast and selective method based on high-performance liquid chromatography with diode array and atmospheric pressure chemical ionization-mass spectrometry detection (HPLC-DAD-APCI-MS) was developed and optimized to assess specific food samples content in carotenoids. Two compounds were chosen as significant carotenoids with respect to nutritional value, beta-carotene, and lycopene.

The optimum conditions suitable for samples analysis were: a mobile phase of methanol:water (95:5) (A) and acetonitrile:methylene chloride (90:10) (B), with a gradient elution: 0-4 min 0% B, 4-7 min 20% B, 7-10 min 50% B, 0-10 min flow 0.1 mL/min, 10-30 min 90% B, 10-30 min flow 0.2 mL/min. Analytes were separated within 30 min on a C18 column and a diode array detection (450 nm for beta-carotene and 470 nm for lycopene).The linear dependence between peak area and concentration ranged from $5x10^{-7}$ $molL^{-1}$ to $5x10^{-5}$ $molL^{-1}$ (r=0.9998, n=9) for beta-carotene and respectively $7.5x10^{-7}$ $molL^{-1}$ to $7.5x10^{-5}$ $molL^{-1}$ (r=0.9986, n=5) for lycopene. The optimized method was used to quantify beta-carotene and lycopene in bakery products enriched with carotenoids devoted to the nutrition of aged population, method that could be standardized and used for bakery products quality control.

Keywords: beta-carotene, lycopene, HPLC-DAD-MS, enriched food, tomato sauce

Introduction

Vitamins, compounds with various chemical structures, are significant constituents, which play an important nutritional role, the necessary amount of such nutrients being regulated by the value of recommended daily allowance (RDA). Some of the most important vitamins are the retinoids and provitamin A carotenoids, the last ones being partially converted to retinoids in the human body (S.M. LOVEDAY & al. [1]). The main sources of carotenoids are fruits and vegetables, while retinoids are found only in animal sources.

Nowadays, the major interest for the carotenoids widely found in plants is due to their antioxidant action, carotenoids acting as effective scavengers of oxygen radicals and reducing oxidative stress in the organism. Clinical studies have shown an association between carotenoid intake from food and a low risk of degenerative diseases such as cancer, cardiovascular diseases and macular degeneration (N.I. KRINSKY & al. [2]; K. NAKAGAWA & al. [3]). For example, the ratio between S-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) is very important when pre-neoplasic colorectal cancer diagnosis is performed. Beta-carotene is the most studied because it is one of the major carotenoids in our diet and in human blood and tissues. Epidemiological studies proved that, among patients who did not smoke or drink, beta-carotene supplementation was associated with a 44% decrease in the risk of colorectal adenoma recurrence (J.A. BARON & al. [4]).

Lycopene, the main pigment of tomato fruits, is effective as a natural antioxidant, especially against O_2 singlet reactive species, that are main promoters of cell injury and senescence (B. HALLIWELL & al. [5]). Tomato and tomato products are the major sources of lycopene for the human diet (F. XU & al. [6]).

Different methods were employed in carotenoids assessment, such as spectrometry (F. MASINO & al. [7]; D. MONTESANO & al. [8]; AOAC 941.15 [9], HPLC (L. HONGXIA & al. [10]; C.H. LIN & al. [11]; R. MUKHERJEE & al. [12]; D. MARINOVA & al. [13]; A.I. OLIVES BARBA & al. [14]; J.S. SEO & al. [15]) or colour evaluation (AOAC 938.04 [16]). In the recent years a number of high-performance liquid chromatography (HPLC) methods using C8, C18 and C30 bonded phase columns have been published (J.P. CHEN & al. [17]; M. HUMAYOUN & al.[18]; C.H. LIN & al. [11]; A.I. OLIVES BARBA & al. [14]; J.S. SEO & al. [15]) and the most common detector used was diode array detector (DAD). HPLC-mass spectrometric (LC-MS)- based methods with different interfaces like atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) coupled with quadruple and ion trap mass analyzers have been used for carotenoids determination in samples such as food items, plant and vegetables (D.E. BREITHAUPT [19]; D.J. HART & al. [20]; L. HONGXIA & al. [10]; D. MONTESANO & al. [8]).

From the latest reviews on the analysis of carotenoids in vegetables (L. Feltl & al. [21]) only a few reports the simultaneous detection of retinoids and carotenoids in food products using hyphenated techniques (D. MONTESANO & al. [8]; D.B. RODRIGUEZ-AMAYA & al. [22]).

After the age of fifty, there are many metabolic and physiological changes with impact on the nutritional needs of an individual. Based on the study of biologically active compounds necessary in old people diet, some experiments were carried out to obtain food (bakery products) that will contain the necessary nutritional principles that could be easily consumed with acceptable sensory properties. Bioresources containing proper bioactive compounds for a personalized diet specific for healthy ageing and age-related pathology as preventive/curative diets were selected and included in new food recipes. For this reason we developed and optimized a high-performance liquid chromatographic method with diode array and atmospheric pressure chemical ionization-mass spectrometry detection (HPLC-DAD-ACPI-MS) for determination of beta-carotene and lycopene both in raw material (tomato sauce) and enriched bakery products (short pasta) starting from a standardized method (SR EN 12823-2 2000-07 [23]).

The food products were elaborated at pilot scale and will be clinically tested -the correlated analysis (SAM/SAH ratio) being performed according to a previous reported method (C. BIRSAN & al. [24])-and then the proper ones will be transferred to the industry for production.

Material and methods

Chemicals

Beta-carotene (Type II: Synthetic, =95%) and lycopene standard (=90%, purity) from tomato were provided from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (BIOSOLVE), methanol (BIOSOLVE), n-hexane, and dichloromethane (CHROMASOLV) were of HPLC grade. Deionized water was obtained using a Milli-Q water purification system, Elix 3 (Millipore Co.,USA).

Instrumentation

The analysis were performed using an HPLC Shimadzu system equipped with LC-20AD SP pump, a LC 20AC autosampler, CTO-20AC Column Oven thermostat, Shimadzu DGU-20A5-Degasser and SPD-M20A Diode Array Detector. The system was coupled on-line to a

LCMS-2010EV Liquid Chromatograph Mass Spectrometer. The data acquiring and processing was performed using LCMS solution software, Shimadzu. The rotary evaporator LABOROTA 4000 Heidolph was used for evaporation of the organic layer of the carotenoid extracts from real samples.

Preparation of stock solutions and samples

Stock solutions of beta-carotene and lycopene were prepared by dissolving 1mg of the standard in 1 mL acetonitrile and were stored at -25^{0}C in the dark. For the calibration, working solutions of the two compounds were freshly prepared by dilution in acetonitrile, in the concentration range $5x10^{-7}$ molL^{-1} to $5x10^{-5}$ molL^{-1} (beta-carotene) and respectively $7.5x10^{-7}$ molL^{-1} to $7.5x10^{-5}$ molL^{-1} (lycopene). The calibration curves were drawn using nine concentration levels for beta-carotene and five concentration levels for lycopene.

The method reported for the carotenoids extraction was based on a standard method (SR EN 12823-2 2000-07) and involved the saponification of 1-10 g sample with ethanol (100 mL) and KOH 60% (20 mL) in the mixture being added 1 g ascorbic acid to avoid subsequent oxidation during the extraction procedure. The resulting mixture was then heated at 30^{0}C for 30 minutes and filtered on filter paper to eliminate solid material. The obtained filtrate was repeatedly extracted (3 times) in a separating funnel with volumes of 50 mL hexane until colorless solution. The hexane layers were mixed and evaporated to dryness under reduced pressure in a Heidolph rotary evaporator. The residue was dissolved in 1 mL acetonitrile, filtered on PFTE membrane (0.22µm) and subjected immediately to LC-MS analysis. All operations were carried out in reduced light and temperature lower than 40^{0}C to avoid sample degradation.

Two bakery products (short pasta) enriched with carotenoids from tomato sauce (raw material) were chosen for preliminary analysis. Short pasta with added tomato sauce had satisfactory physical-chemical properties and sensory characteristics. The tomato sauce strongly colored the bakery products. The addition of tomato sauce aimed at enriching the final product in bioactive compounds such as carotenoids. Taking into account the fact that analyzed bakery products have to be used in nutrition of certain groups affected by age-related degenerative diseases and that lycopene and beta-carotene play as antioxidants in prevention and partial recovering after initiation of oxidative stress, we considered important to assess the quality of the bakery products with respect to those two compounds.

LC-MS analysis of beta-carotene and lycopene

The most suitable mobile phase system was established after several tests and consist in: component A methanol-water (95:5, v/v) and component B acetonitrile-dichlormethane (90:10, v/v). A C18 column (Phenomenex: Synergi 4u Max-RP 80A, 75x2.00mm) and a binary gradient was used for carotenoids analysis. The gradient changed from 0% to 90% solvent B, as follows: 0-4 min 0% solvent B, 4-7 min 20% solvent B, 7-10 min 50% solvent B, 10-30 min 90% solvent B and the column re-equilibrated with 0% solvent B for 5 minute.

The mobile phase flow rate was 0.1 mL/min for the first 10 minutes and after that 0.2 mL/min for the rest of the analysis. The column temperature was maintained at 20^{0}C and the carotenoids elution was monitored at 450 nm for beta-carotene and 470 nm for lycopene. UV spectra were recorded with a DAD detector from 200 to 700 nm.

Further, the eluent was analyzed with the Shimadzu LCMS-2010EV quadrupole mass spectrometer equipped with an APCI interface. MS analysis was carried out in the positive ion measurement mode with a detector voltage of 1.6 kV, APCI temperature of 400^{0}C, curved desolvation line (CDL) of 250^{0}C, and Block Heat temperature of 200^{0}C. The flow rate of the nebulizer gas (N_2) was 2.5 L/min. All determinations were replicated three times and the results are presented as mean values ± standard deviation. Calculations of average

concentration and standard deviation (SD) were performed by the computer program Excel 07 from Microsoft.

Results and discussions

Carotenoids identification was carried out by comparison of the corresponding HPLC retention times and spectral data between samples and standards; compounds identification was confirmed by specific molecular ions from MS spectra.

HPLC chromatogram and UV-Vis spectrum of the mixed standard solution including lycopene and beta-carotene is shown in Fig 1.

The first chromatographic peak of carotenoids, with a retention time of t_R =15.9 min was ascribed to lycopene, and corresponds to spectra characterized by the maximum wavelengths λmax: 445nm, 472nm, 498 nm. The second peak (i.e retention time, 22.4 min) was ascribed to beta-carotene and the corresponding spectrum is characterized by the three maximum wavelengths λmax: 427, 450 and 475 nm.

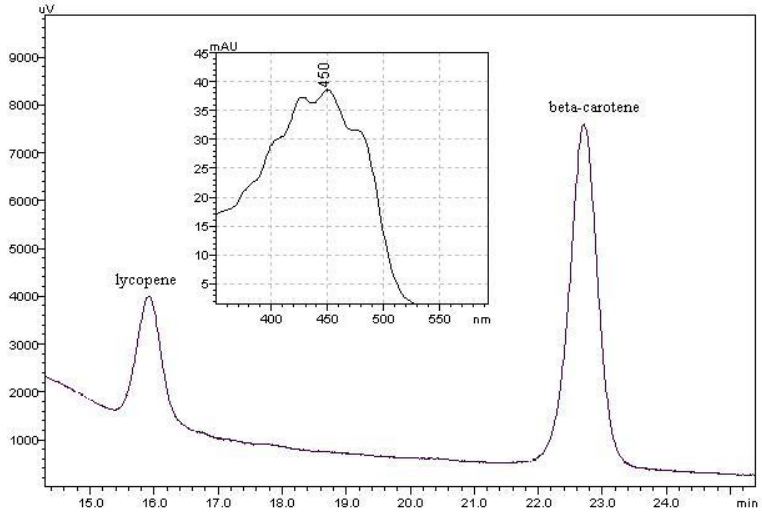

Figure 1. HPLC chromatogram and DAD UV spectrum of a standard mixture of lycopene and beta-carotene in optimal chromatographic conditions

In Fig 2, the positive ion APCI mass spectrum for beta-carotene from tomato sauce sample is shown. The principal peak at m/z 537 belongs to the protonated molecule, $[M+H]^{\oplus}$. Also other molecular radical ion with the mass m/z 538 having low intensity appears and confirms the presences of the two carotenoids in the real samples.

The equations and correlation coefficients (r^2) of the linear regression analysis for the two carotenoids for HPLC-DAD are summarized in Table 1.

The concentration of carotenoids (mg/100g) in the real samples was calculated by interpolation on the linear regression curve and applying the corresponding corrections due to extraction yield.

Table 1. The performance characteristics of HPLC-DAD carotenoids analysis.

Compound	Retention time (min)*	Equation	LoD (mol/L)	Range** (mol/L)	R^2
Beta-carotene	22.4±0.5	A = 44530c - 882	$2.9x10^{-7}$	$5x10^{-7} – 5x10^{-5}$	R = 0.9998
Lycopene	15.9±0.3	A = 173589c - 4896	$3x10^{-7}$	$7.5x10^{-7} – 7.5x10^{-5}$	R = 0.9970

* values are given as means ± standard deviations (n = 3)

** obtained with 9 standard solutions for beta-carotene and 5 for lycopene

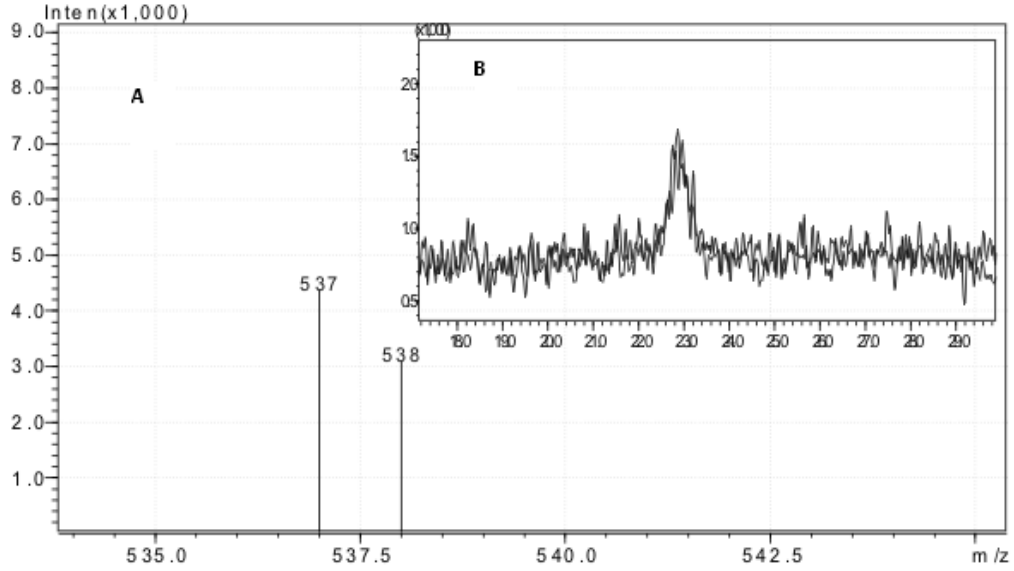

Figure 2. Mass spectrum (A) and TIC chromatogram (B) of beta-carotene in tomato sauce sample.

The performed analysis was supposed to lead to modifications in fabrication line of bakery products with respect to the initial value of nutrient that occurred in the raw material (namely tomato sauce) because it was compulsory to ensure a high level of carotenoids in the final product. As consequence, it was unavoidable for the analysis chain to track the traceability of the compounds of interest between raw material and the final bakery product. In this initial step, two bakery products were produced and tested the difference between them consisting in the amount of raw material added in the technological process, namely bakery product no1: 850g tomato sauce/2000g wheat flour while bakery product no2: 700g tomato sauce/2000g wheat flour. The results for tomato sauce and the bakery products are presented in Table 2. Tomato sauce sample have the highest concentration of lycopene and beta-carotene while the bakery products (tomato sauce was added in dough) have small concentration due to the industrial process to which they were subjected. The HPLC chromatogram of the raw material (tomato sauce) is shown in Fig 3.

Table 2. Carotenoids concentration in real samples by HPLC-DAD method.

Sample	Lycopene (mg/100g) *	Beta-carotene (mg/100g) *
Tomato sauce	9.23±1.77	3.94±0.56
Bakery product no1	3.52±0.77	3.33±0.13
Bakery product no2	2.27±0.49	1.76±0.52

* values are given as means ± standard deviations (n = 3)

The HPLC chromatograms of the extracts containing lycopene and beta-carotene from samples are shown in Fig 4 and proved that temperature plays an important role in carotenoid changes in raw material and final bakery products.

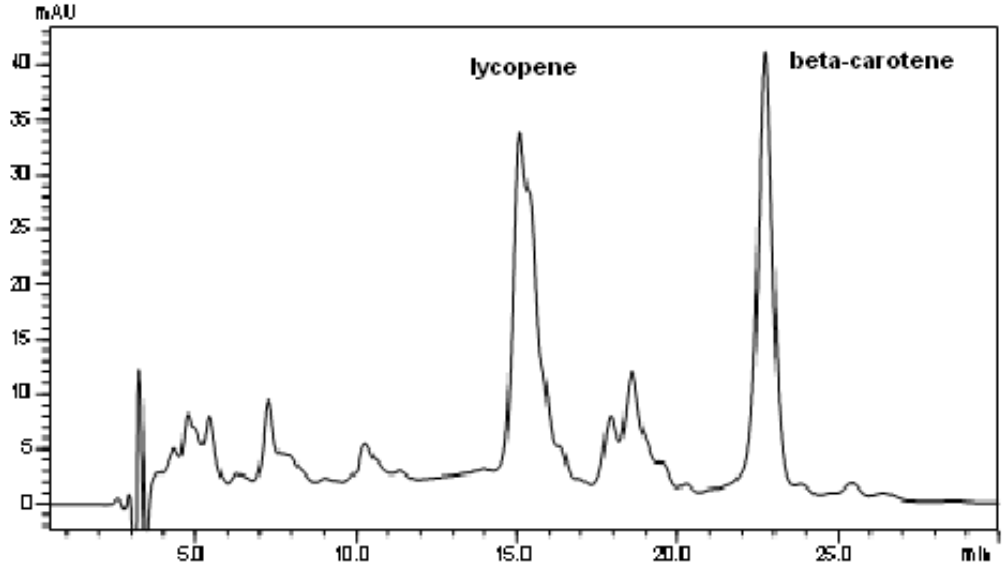

Fig 3. HPLC chromatogram of tomato sauce sample containing lycopene and beta-carotene.

The concentration of lycopene and beta-carotene decreased significantly as a result of dough mixing and baking step. The use of different cooking methods leads to significant losses of carotenoids by degradation at high temperatures. According to the producer claims, after quantification of the beta-carotene and lycopene concentration it was found that the first technology should be further applied because is able to preserve the maximum amount of active principle and the bakery product no1 will further be used in daily nutrition of a number of 20 volunteers, with ages ranging between 82 and 95 years.

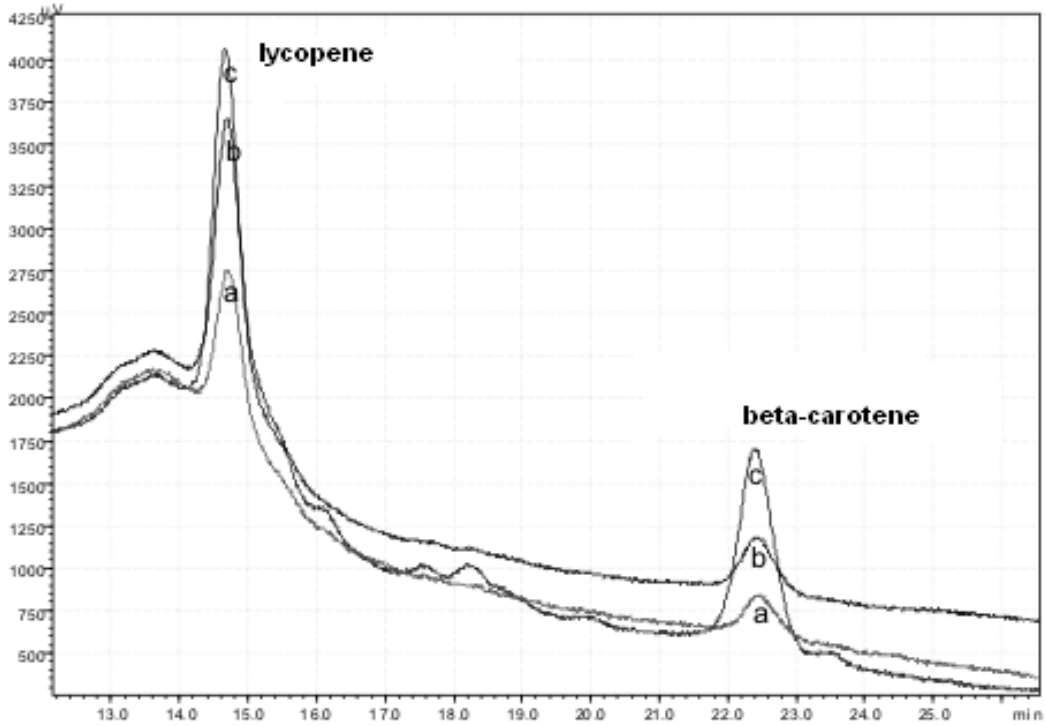

Fig 4. Overlaid HPLC chromatograms of samples containing lycopene and beta-carotene (a- bakery product no2, b-bakery product no1, c-tomato sauce).

Conclusions

In this study, a HPLC method adjusted for specific bakery products (carotenoid enriched bakery products for aged people nutrition) was optimized and proved to be sensitive (detection limits: 2.9x 10^{-7} $molL^{-1}$ for beta-carotene and 3x 10^{-7} $molL^{-1}$ for lycopene) and was applied to determine beta-carotene and lycopene. With respect to reported data, our results are better as analysis time and our method proved to have comparable sensitivity, or higher than reported (BREITHAUPT [19], SEO & al. [15], OLIVES BARBA & al. [14]). The proposed method (including extraction step) provided reliable results for the quantification of lycopene and beta-carotene from bakery products.
Our results showed that the highest carotenoid concentration in bakery products was found for bakery product no1 (beta-carotene 3.33 mg/100g and lycopene 3.52 mg/100 g), the percentage recovery lower than 80% may be the result of the processing to which the final products are subjected.

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