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On-line coupling of automatic solid-phase extraction and HPLC for determination of carotenoids in serum

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ABSTRACT

The automated method developed for the determination of carotenoids uses 200 μL of serum, which was mixed with 400 μL of tetrahydrofuran, vortexed for 1 min, settled for 10 min, centrifuged for 6 min and the supernatant injected into an automatic solid-phase extraction (SPE) system for cleanup–preconcentration. A 10% water–acetonitrile mobile phase at 1.5 mL min^{-1} eluted the retained compounds and transferred them on-line to a reversed-phase analytical column for individual separation of the target analytes. Visible detection was performed at 450 and 460 nm. The detection limits for the target analytes were between 3 and 30 ng mL^{-1}; the precision (expressed as relative standard deviation) ranged between 2.83 and 5.06% for repeatability and between 3.80 and 7.40% for within laboratory reproducibility. The total analysis time was 18 min. The proposed method is reliable, robust, and has an excellent potential for high-throughput use in both clinical and research laboratories.

1. Introduction

Carotenoids are fat-soluble pigments naturally present in plant chloroplasts and other photosynthetic organisms like algae, some types of fungi and bacteria. In the human organism, carotenoids, which are exclusively provided from the diet either free as esters or linked to proteins, are found in tissues and blood [1]. It is generally accepted that serum carotenoid concentrations are markers of recent fruit and vegetable intake [2], whereas tissue carotenoid levels are indicators of longer-term carotenoid consumption patterns [3].

In addition to provitamin A activity displayed by some carotenoids, several other biological activities of these compounds have been reported, including antioxidant capacity [4], which allows them to prevent chronic diseases by protecting against free radical damage [1]. Lycopene exhibits the highest antioxidant activity, followed by β-cryptoxanthin, β-carotene, lutein and zeaxanthin [5]. Oxidative stress has been related with diseases ranging from cancer [6] to age-related macular degeneration [7], even to the aging process itself [8]. Carotenoids have been reported to protect against oxidative stress and prevent cardiovascular diseases [4].

Epidemiological studies suggest that diet rich in vegetables containing β-carotene and other carotenoids decreases human lung cancer risk [9]; meanwhile low levels of carotenoids in placental tissue and maternal serum of women with preeclampsia suggest that oxidative stress or a dietary antioxidant influence might affect the pathophysiology of preeclampsia [10]. Also, carotenoids display other biological activities such as blue light filtering [4]. In fact, β-carotene is prescribed to ameliorate photosensitivity associated to erythropoietic protoporphyria and other light-sensitive diseases [11].

Individual separation and determination of carotenoids in biological samples is mainly carried out by HPLC with different types of detectors. A number of normal- and reversed-phase chromatographic methods have been described for individual separation of carotenes in serum using both isocratic [12] and gradient elution [13], the latter providing better resolution than the former. Concerning detection, the most widely employed technique has been ultraviolet–visible absorption, either conventional or photodiode-array detectors (DAD) [14]. More sensitive detectors (e.g. mass spectrometry – with atmospheric pressure ionization interfaces (APCI) or electrospray ionization interfaces (ESI) – and nuclear magnetic resonance spectroscopy) have also been used for carotenoids analysis. Nevertheless, these detectors are more expensive than absorption-based detectors and their use for routine analysis is more complicated [15,16].

The most common sample preparation procedure for subsequent determination of carotenoids in human serum involves a

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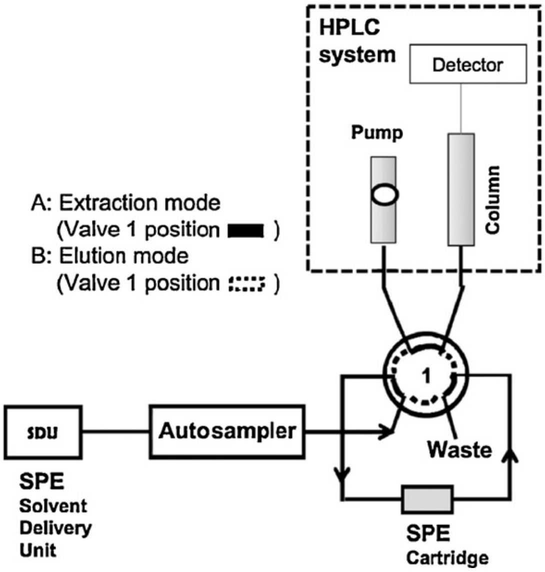

Fig. 1. Schematic diagram of the SPE–HPLC arrangement for automatic sample extraction – individual separation – determination. In the retention mode (valve 1 position ▬), an extraction cartridge is placed in the loop on the switching valve (1). The cartridge is washed and conditioned with liquid from the solvent delivery unit (SDU). An aliquot of the sample is led to the SPE cartridge by the loading solvent from the SDU. The analytes are retained and a washing solution is circulated through the cartridge to remove potential interferences. In the elution mode (valve 1 position –), the valve is switched to flush the analytes to the analytical column with the LC mobile phase. After the analytes have left the cartridge, the valve is switched again to the retention mode (A), and the extraction cycle repeated for a next sample, while the chromatographic step takes place.

step using ethanol for protein precipitation prior to extraction of the target analytes with an organic immiscible solvent (usually n-hexane) [17]. After centrifugation for phase separation, the hexane layer is evaporated to dryness under a nitrogen stream and the residue reconstituted with the appropriate organic solvent for injection into the HPLC system. This procedure is relatively tedious, complex, expensive, time-consuming, and with low precision and accuracy [4,17–20].

To know the impact of carotenoids in human health, a precise method that improves the repeatability and reproducibility of the already existing methods and provides better throughput is required for application in systematic epidemiologic studies, routine analysis and, in general, in cases in which the determination of these compounds in a high number of samples is needed. Thereby, the aim of this research was to fill this gap with a method to determine the target carotenoids in human serum with minimum user involvement.

2. Experimental

2.1. Instruments and apparatus

The experimental setup used for the automatic determination of the target analytes in serum samples is shown in Fig. 1.

An Agilent liquid chromatograph (Model 1100, Pittsburgh, PA, USA) consisting of a quaternary pump, vacuum degasser, and a diode array detector (DAD) with a tungsten lamp was used for the analysis of the target analytes. Hyphenated solid-phase extraction (SPE) was performed with a Prospekt2 system (Spark Holland, Emmen, The Netherlands) and an autosampler (Midas) with a 500 μL sample loop. The Prospekt2 system comprises a unit for SPE cartridge exchange – automatic cartridge exchange (ACE) – and a high pressure syringe dispenser (HPD) for SPE solvent delivery. Peek tube of 0.25 mm i.d. (VICI, Houston, TX, USA) was used to connect all valves of the Prospekt2 unit and this with the analytical column. A cartridge packed with Hysphere CN-SE (silica-based cyanopropyl phase second edition, from Spark Holland) as sorbent material 10 mm × 2 mm and 7 μm particle diameter was used for SPE. The analytical column was a 4 μm Novapak C18 column, 100 mm × 3.9 mm i.d. (Waters corporation, Milford, MA, USA).

A centrifuge (Selecta, Barcelona, Spain) and a vortex (Ika-Works, Wilmington, USA) were also used.

2.2. Chemicals and standard solutions

Deionised water (18 mΩ) from a Millipore Milli-Q water purification system was used to prepare all aqueous solutions. Violaxanthin (viola), β-criptoxanthin (cripto), neoxanthin (neo), anteraxanthin (antera), lycopene (lyco), zeaxanthin (zea), mutatoxanthin (mutato), canthaxanthin (cantha), lutein (lut), β-carotene (β-caro) and α-carotene (α-caro) from Carotenature (Im Budler 8 CH-4419, Lupsingen, Switzerland) were used as standards. Methanol, ethanol, acetone, acetonitrile, tetrahydrofuran (THF), sodium dodecyl sulfate (SDS) from Scharlab (Barcelona, Spain), heptane, di-tert-butyl-4-methylphenol (BHT) from Panreac (Barcelona, Spain), ammonium formate from Sigma (Sigma–Aldrich, St. Louis, MO, USA) were used. All chemicals were HPLC grade and used without further purification.Stock standard solutions were prepared by dissolving 0.1 g of each analyte in 50 mL methanol in the case of viola, cripto, antera, zea, mutato, and lut, and in 100 mL in the case of neo. 50 mL ethanol was used to dissolve cantha; 100 mL tetrahydrofuran (THF) for lyc, and 200 mL THF for β-caro and α-caro. All stock solutions contained 30 mM BHT to prevent oxidation [21]. The concentration of the stock solutions were checked using the extinction coefficient of each standard. Standard working solutions were prepared by dilution of the appropriate volumes of the stock solutions in methanol to a final concentration of the analytes 0.5 μg mL^{-1}, and used for optimisation of both the chromatographic step and the preconcentration effect achieved by the automatic SPE step. Deionised water was used as loading solvent in the preconcentration step and as washing solution in the cleanup step. The initial chromatographic mobile phase was used for elution of the analytes from the cartridge. Then, methanol and deionised water were used to purge the tubing system of Prospekt2 at the end of the SPE step.

2.3. Preparation of serum samples

A total of 80 serum samples were collected from blood donors (age range: 18–65 yr, 48 men, 32 women). Venous blood was collected into a plastic Vacutainer® tube from Becton Dickinson (Franklin Lakes, NJ, USA) without additives (red top). The tube, without opening it to ambient air, was placed in ice or kept refrigerated until processing. Blood samples were processed – centrifuged at 2375 × *g* for 15 min at 4 °C – within 1 h after collection. Serum was collected, placed in plastic tubes and stored at or below −80 °C until analysis. Appropriate volumes of standard solutions of the analytes were added in the validation step.

For protein precipitation, the serum samples were equilibrated at room temperature for at least 30 min, then vortexed for 30 s. 200 μL of serum was pipetted into glass tubes and 400 μL of THF was added to each, then vortexed for 1 min, and allowed to settle for 10 min. The mixture was centrifuged for 6 min at 855 × *g*, and the supernatant transferred to an autosampler vial.

2.4. Proposed method

The supernatant from the previous step, transferred to an injection vial, was placed in the autosampler tray, thus being ready for analysis. 500 μL of the content of the injection vial was injected

Table 1
Automatic solid-phase extraction step. Sequence of automatic operations.

Step	Time (min)	Solvent	Volume (mL)	Disp. flow (mL min^{-1})
Start autosampler	00:00	–	–	–
New cartridge	00:10	–	–	–
Solvation	00:46	Methanol	2	5
Equilibration	01:22	Water	2	5
Equilibration	02:34	Water	2	3
Sample application	03:19	Water	1	3
Rinse	03:41	Water	1	3
Elution	04:05	Mobile phase	–	–
Purge	04:36	Methanol	2	5
Purge	05:00	Water	4	5

into the system following the sequence of automatic operations described in Table 1.

Basically, the sample preparation process started with preparation of the cartridge by activation of its stationary phase with methanol, conditioning and equilibration with water; then, the sample was loaded into the cartridge with water (flow pumped by a high-pressure syringe dispenser – HPD in Fig. 1). Under these conditions, the compounds of interest were retained in the sorbent. The chromatographic step started by switching valve 1, thus eluting the analytes from the sorbent with the initial mobile phase.

Mobile phase A was 90:10 acetonitrile–water, and mobile phase B was THF. The initial mobile phase was held at 100% A for the first 6 min; then, a linear gradient up to 13 min to obtain 25:75 A–B, and then other linear gradient for 1 min led to a 50:50 A–B as final mobile phase. The flow rate was 1.5 mL min^{-1}. The column temperature was optimised and the optimum value for the best separation of the analytes was 20 °C. Absorbance was monitored at 450 and 460 nm. The total analysis time was 18 min, being required 7 min for re-establishing and equilibrating the initial conditions.

To reuse the cartridge, a last step for cleaning–regeneration was developed thus allowing the cartridges to be reused at least three times without notable performance changes (i.e. identical extraction recovery and peak shape, no appearance of ghost peaks, etc.); thus allowing a drastic reduction of the analysis costs. A last step to purge the Prospekt2 tubing system was developed. The chromatographic and detection steps of one sample and the SPE step of the next other overlapped, thus increasing the throughput.

The eluate was monitored simultaneously at 450 nm (for violaxanthin, anteraxanthin, mutatoxanthin, lutein and zeaxanthin) and 460 nm (for neoxanthin, canthaxanthin, β-criptoxanthin, lycopene, β-carotene and α-carotene).

An internal standard was not used since recovery and analyte retention was validated by cartridge dual test. In this way, sample spiking was not required, which lowered significantly the cost of the analysis.

3. Results and discussion

The sequence of the study to evaluate the influential variables consisted of two steps: the first, focused on the best separation–determination of the compounds using HPLC and, the second, focused on the most efficient cleanup–preconcentration of the target compounds by the automatic SPE system.

3.1. Study of the chromatographic separation step

The main variables with influence on the chromatographic separation (*viz.* composition of the mobile phase, flow rate and temperature of the analytical column) were studied to obtain the best separation of the analytes in the shortest time. Various binary and ternary solvents (*viz.* methanol, acetonitrile, THF, 2-propanol, and water in different proportions) in isocratic or gradient mode were used to obtain the best chromatographic separation of serum carotenoids. The most appropriate mobile phase was found to be 90:10 (v/v) acetonitrile–water as phase A, and 100% THF as phase B. The optimum gradient program is given in Section 2.4. The flow rate was 1.5 mL min^{-1}. The temperature of the column oven was studied from 15 to 50 °C and the best separation of the analytes was achieved at 20 °C.

3.2. Study of the automatic solid-phase extraction step

This study was aimed at obtaining maximum extraction efficiency and cleanup.

The SPE sorbent, sample volume, loading solvent (type, volume and flow rate) and elution flow rate were studied by a univariate approach because the high number of non-continuous variables (i.e. SPE sorbent, loading solvent, washing solvent) made the use of a typical multivariate design difficult. Spiked serum was used for the study of the SPE sorbent and other variables of this step.

The stationary phase of the cartridges was activated with 2 mL methanol, conditioned and equilibrated with the loading solvent, and then, the sample was applied.

3.2.1. Selection of the SPE sorbent

Eight types of SPE sorbents – *viz.* CN-SE (silica-based cyanopropyl phase second edition), C8-EC (end-capped silica-based octyl phase), C18-EC (end-capped silica-based octadecyl phase), C18-HD (spherical shape, end-capped, silica-based phase with a high loading of octadecyl chains), resin-GP (polymeric polydivinylbenzene phase), and resin-SH (strong-hydrophobic modified polystyrene-divinylbenzene) – were tested in order to select the best one in terms of preconcentration and cleanup. The best results were obtained with Hysphere CN-SE cartridges.

3.2.2. Loading solvent

Several aqueous mixtures of methanol and acetonitrile were tested as solvents for loading the sample into the SPE cartridge. In both cases, concentrations of organic solvents from 0 up to 50% were studied to release the carotenoids from protein transporters. In all cases, 15 mM SDS was added to avoid precipitation of serum proteins in the tubes. These experiments resulted in low recoveries, which could be ascribed to two possible mechanisms: inefficient protein precipitation at these concentrations of organic solvent and reduced solubility of carotenoids in the solvent mixtures due to their lipophilic character. Therefore, a prior step for protein precipitation was necessary. Several solvents such as ACN, MEOH, ETOH, and THF were assayed as protein precipitants and THF provided the best results, as its physical and chemical characteristics allow simultaneous precipitation of protein and extraction of carotenoids, thus avoiding losses of analytes as evaporation of the precipitating agent to dryness under a nitrogen stream and dissolution of the residue in a highly nonpolar solvent was avoided. The proportion THF–serum was also studied by assaying 1:1, 2:1, 3:1

Table 2
Characteristics of the method.

Analyte	Equation[a]	Linear range	Coefficient of regression (R^2)	Limit of detection (ng mL^{-1})	Limit of quantification (ng mL^{-1})
Neoxanthin	$y = 10.6 + 155.9C$	99–1500	0.984	30	99
Violaxanthin	$y = 27.9 + 801.1C$	49–900	0.993	15	49
Anteraxanthin+Mutatoxanthin	$y = 11.1 + 384.3C$	49–900	0.992	15	49
Lutein	$y = 45.4 + 1142.5C$	23–900	0.993	7	23
Zeaxanthin	$y = 14.6 + 692.2C$	23–1500	0.994	7	23
Cantaxanthin	$y = 16.5 + 373.2C$	23–900	0.990	7	23
β-Criptoxanthin	$y = 32.3 + 1176.9C$	10–1500	0.996	3	10
Lycopene	$y = 11.7 + 297.7C$	10–1500	0.992	3	10
β-Carotene	$y = 9.3 + 724.0C$	10–1500	0.998	3	10

[a] C is concentration expressed as ng mL^{-1}.

(v/v) ratios, the intermediate 2:1 ratio was selected as it provided a good precipitation of proteins and ensured total extraction of the analytes. The higher 3:1 ratio was rejected as the use of higher proportion of organic solvent did not improved the step.

Different methanol–water and acetonitrile–water mixtures were tested as loading solvent; nevertheless, the optimum loading solvent was pure water since small concentrations of organic solvents promoted partial elution of the target metabolites.

The volume and flow rate of the loading solvent were also studied. As a guideline, the initial load volume should be at least twice the sum of the loop, transport tubing and cartridge volume (*ca.* 1 mL). The load volume was studied between 1 and 8 mL and the flow rate between 0.5 and 4 mL min^{-1}. The selected load volume was 2 mL – from which approximately 1 mL was for loading the sample and the rest for rinsing the system – as higher volumes did not improve retention of the analytes. The selected load flow-rate was 3 mL min^{-1} – the highest flow rate which did not promote analytes loss.

3.2.3. Sample volume and elution time

Serum volumes ranging from 50 to 200 μL and 2:1 (v/v) diluted with THF were studied and the highest value was selected because it provided the highest sensitivity.

The study of the elution time, aimed at avoiding elution from the cartridge of matrix compounds that could interfere at the detector, provided an optimum value of 30 s. In this way, the elution of non-polar compounds retained in the cartridge was avoided. The appropriate value of the elution time is of particular importance to preserve the capability of the analytical column.

3.3. Calibration curves and linearity

Calibration plots were run for the target analytes using the peak area at the monitoring wavelengths as a function of the standard concentration of each compound. Slope, intercept and regression coefficients for all analytes are listed in Table 2.

3.4. Analytical sensitivity and detection limits

The limits of detection (LOD) and quantitation (LOQ) of each analyte were calculated as the concentration yielding signals three- and ten-times, respectively, higher than the noise. The LODs and LOQs are between 3–30 ng mL^{-1} and 10–90 ng mL^{-1}, respectively, for all analytes, as shown in Table 2.

3.5. Evaluation of the precision of the method

Within-laboratory reproducibility and repeatability were evaluated in a single experimental set-up with duplicates by experiments carried out with a serum pool. Two measurements per day were carried out on 7 days. The results obtained are listed in Table 3. The repeatability, expressed as relative standard deviation (RSD), ranged between 2.83 and 5.06% for the target analytes; and the within laboratory reproducibility, also expressed as RSD, ranged between 3.80 and 7.40%.

3.6. Evaluation of the accuracy of the method

The accuracy of the method and potential matrix effects were studied by analysis of non-spiked and spiked human serum samples. First, a two-cartridge configuration of the Prospekt2 system [22] was employed to estimate the recovery factor (defined as the proportion of analytes retained in the first cartridge) where non-spiked human serum samples were used. Two cartridges were put in serial; hence the amount of analyte not retained in the first cartridge could be retained in the second. Then, the compounds retained in both cartridges were sequentially eluted to the chromatographic column for independent analysis. Recovery was calculated as amount in cartridge 1/[amount in cartridge 1 + amount in cartridge 2], where the first and second cartridges were Hysphere CN. The results obtained are listed in Table 3.

To evaluate the recovery a pool of spiked serum with high multistandard concentration (1 μg mL^{-1}, the highest level assayed in calibration) was analysed with the single configuration. This high concentration was used to ensure that the SPE cartridges were not

Table 3
Repeatability (Sr), within-laboratory reproducibility (Swr) and recovery for each analyte.

Analyte	Repeatability (%)	Reproducibility (%)	Recovery (%)
Neoxanthin	5.06	7.40	62.5
Violaxanthin	2.83	3.80	100
Anteraxanthin+Mutatoxanthin	3.04	5.86	73.5
Lutein	3.90	5.97	74.1
Zeaxanthin	3.85	5.96	73.1
Cantaxanthin	3.31	6.51	82.2
β-Criptoxanthin	3.60	4.62	85.4
Lycopene	3.93	4.98	88.1
β-Carotene	4.08	6.92	100

Table 4
Profile of the target analytes in the population under study (expressed as mean ng mL^{-1} ± standard deviation).

Parameter	Values found	Values Ref. [1]	Values Ref. [4]	Values Ref. [6]	Values Ref. [23]
Age (n = 80)	41 ± 8				
Neoxanthin	N.Q.	–	–	–	–
Violaxanthin	N.D.	–	–	–	–
Anteraxanthin+Mutatoxanthin	N.Q.	–	–	–	–
Lutein	30 ± 30	120	250 ± 280	110 ± 40	400 ± 170
Zeaxanthin	30 ± 20	30	70 ± 40	30 ± 20	50 ± 30
Canthaxanthin	60 ± 40	–	–	–	20 ± 20
Criptoxanthin	110 ± 820	130	220 ± 140	70 ± 40	190 ± 150
Lycopene	590 ± 380	320	–	130 ± 70	300 ± 230
Carotene	180 ± 140	310	500 ± 660	400 ± 260	630 ± 380

N.Q.: non quantified; N.D: non detected. These values correspond to average concentrations of the target metabolites in the selected cohort. Concentrations below LLOQ were considered zero. Data in reference [1] are the mean of women and men. Data in reference [23] are control data.

saturated when the samples are used. In this case, the recovery was calculated as [final concentration – original concentration]/spiked concentration, evaluated by analysis of five replicates in the same day.

3.7. Comparison of the proposed method with other methods

The method developed is automated, fast, uses minimal sample handling and photometry detection and has high precision an accuracy, although an internal standard is not used. These characteristics yield a low-cost method. This aspect, together with its throughput, makes the method suitable for routine analysis. Various HPLC methods have been described in the past for determination of carotenoids, and all of them include a liquid–liquid extraction step for deproteination, centrifugation, evaporation to dryness and reconstitution prior to injection into an HPLC system, as described in the introduction [4,17–20]. These steps are relatively tedious, complex, expensive, time-consuming, and with low precision and accuracy [4,17–20]; shortcomings which were drastically reduced by the on-line automatic SPE step here proposed.

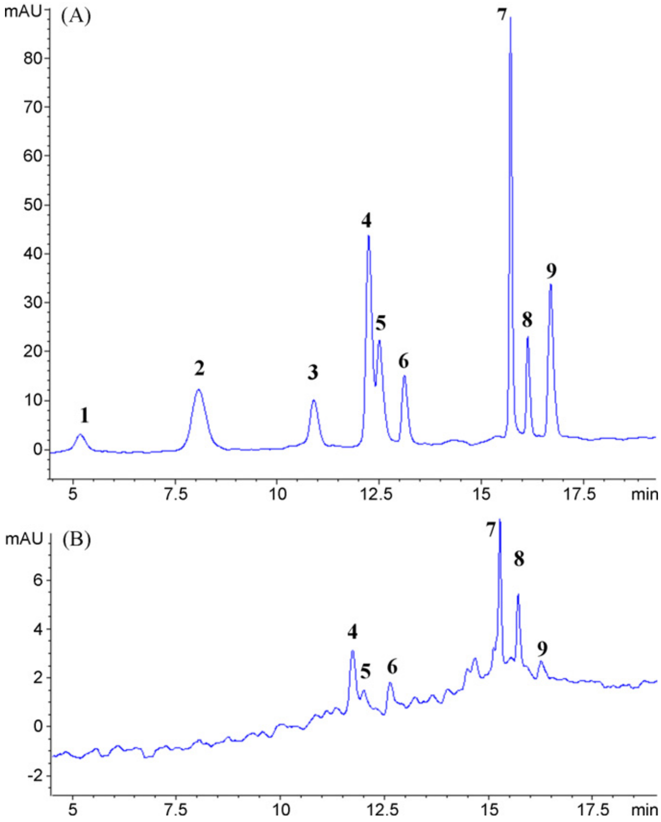

Fig. 2. Representative chromatogram of a carotenoid standard mixture (A) and a serum sample (B). Analytes: (1) neoxanthin; (2) violaxanthin; (3) anteraxanthin+mutatoxanthin; (4) lutein; (5) zeaxanthin; (6) canthaxanthin; (7) criptoxanthin; (8) lycopene; (9) carotene.

The use of a photometric detector provides enough sensitivity and selectivity for determination of the analytes in serum samples and makes the method simple, cheap (both in equipment acquisition and maintenance costs), and thus available for routine analysis.

3.8. Application of the method

In order to demonstrate the applicability of the proposed method, it was used to determine the target analytes in serum from eighty apparently healthy volunteers (age range: 18–65 yr; 48 men, 32 women). A typical chromatogram of the samples is shown in Fig. 2.

Demographic profile and mean standard deviation of the concentration of each analyte in the samples are shown in Table 4. The results obtained are similar to those from other authors in healthy people [1,4,6,23].

4. Conclusions

Automation of sample preparation by the use of on-line SPE with automatic valve switching and cartridge exchange constitutes an excellent approach for unattended analysis of the target compounds in human serum. In our case, only a prior simple protein precipitation is developed manually. Minimising human intervention makes the method easy to apply and improves the precision and accuracy of the results. In addition to automation, the proposed approach provides a closed system for maximum protection against atmospheric-induced degradation and high precision because the number of sample transfer steps is significantly diminished.

The proposed method is cheap, fast, and showed a good sensitivity, precision and accuracy thanks to the use of an automatic closed system. Furthermore, a sample can be automatically prepared (by solid-phase extraction) while running the chromatographic step of the previous one, so that continuous analysis can be done, providing a throughput assessment of analytes in human serum of 3.3 samples h^{-1}.

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