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In vitro digestion for control and monitoring of food effects in relation to micellarization index of carotenoids

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i. Running Title: Carotenoid in vitro digestion

ii. Abstract

The multifactorial system that influences the availability of macro-, micro-nutrients, and phyto-constituents with relevant bioactivities has been assessed by *in vitro* digestion protocols, which have become an effective technique to support the health-through-food strategy. The measurement of the significance of food structure, food matrix, synergies and competitive effects, processing features and even some physiological issues has created valuable scientific and technological information, and the development of harmonized protocols. Now it is possible to make further advances by applying this knowledge to obtain data regarding the potential availability of target compound(s) in the food source within a *standard* meal context. This protocol describes the measurement of the micellarization index of carotenoids from dietary rich-sources with test meals accounting the effects of high *vs*. low fat and normo-, hypo- and hyper-caloric content.

iii. Key words *In vitro* digestion protocol, Micellarization index, Carotenoids, Food-effects, Bioaccessibility

1. Introduction

Bioaccessibility, defined as the amount of a compound that is released from its matrix in the gastrointestinal tract, becoming available for absorption, then absorbed by the enterocytes and finally turned into a bioactive form, is the ultimate concept applied to food science and nutrition to enhance the reliability in that the nutritional benefits are achieved. It is a critical issue in the design of food formulations with bioactive ingredients, because it considers the effects of the events taking place during digestion that are significant influencing factors for limiting or enhancing the *in vitro* bioaccessibility of target compound(s), and with the aim of predicting the outcome *in vivo*. Hence, straightforward procedures, *i.e. in vitro* digestion protocols have been designed using a decision-making approach related with the kind of compound(s) which bioaccessibility is determined, the question that needs to be answered, or the mechanism to be figured out [1]. However, the existence of almost tailored procedures for undertaking specific questions made difficult the comparison of data from different laboratories to draw a comprehensive scenario of the lineal combination of critical factors affecting digestibility and absorption of food components. A significant effort has been made by the INFOGEST project [2], which aim was to define a consensus protocol easy to set-up in any laboratory, with experimental conditions that should be applied to obtain a satisfactory grade of confidence in the results, to allow their comparison between different laboratories and to establish further improvements in the technique.

In vitro bioaccessibility of carotenoids has been a continuous topic of interest since the onset of this century. The findings obtained with *in vivo* approaches yielded a priceless legacy to conceive reliable *in vitro* digestion protocols, which measure the bioaccessibility of carotenoids under different experimental conditions. In the case of carotenoids as in other lipophilic compounds, bioaccessibility is often made equal to micellarization, that is, the technique determines the quantity of ingested carotenoids incorporated into mixed micelles and, consequently, ready for absorption. However, it should be noted that micellarization is not the single element required to measure bioaccessibility of carotenoids, but efficiency of the cellular absorption and first-pass metabolism are contributors to the complete bioaccessibility value. Hence, from the sequential steps involved in the digestion and absorption of carotenoids – release from food matrix, transfer to lipid droplets, emulsification and micellarization, and the subsequent routes of cellular absorption, trafficking of lipids and first-pass metabolism – the *in vitro* digestion protocols are designed to measure those factors affecting the four initial steps, while different protocols are required to estimate the efficiency of the others. Regarding the sort of factors that influence bioaccessibility of carotenoids, they are grouped within food structure,

food matrix and processing features, physiological issues, and genetic and host-related aspects, which meaning has been reviewed recently [3, 4]. Again, the design of *in vitro* digestion protocols does not allow ascertaining the influence of all those factors. Thus, the impact of genetic and host-related variations is not accessible through this technique, while the inclusion of experimental conditions according to the gastrointestinal capability of different population groups (new-born and infants, the elderly and patients with certain hepatobiliary disorders) has started to be included in the design of *in vitro* digestion protocols only recently [5, 6].

In this milieu, and although a considerable number of *in vitro* digestion protocols is available, the procedures published by Garrett et al. [7] and Reboul et al. [8] contain the essential knowledge to consistently develop the measurement of the micellarization of carotenoids, or to be tuned with other experimental conditions, as it has been recently discussed [1]. However, these protocols have been applied to single carotenoid food sources (green vegetables and oils, carrots, tomatoes, peppers, oranges, grains, dairy products, food emulsions…) with the objective of gaining information of the factor(s) playing a role in the micellarization of carotenoids. This reductionist approach has been necessary to clearly correlate the observed effect(s) in the efficiency of the micellarization with the examined factor(s), or whether the latter has no influence in the former process. With this strategy a substantial amount of information has been obtained [9] to figure out several weighting factors in the micellarization of carotenoids, including food processing, technological treatments and cooking procedures, application of novel techniques, deposition in the food microstructures, texture, fibre networks and particle size, and amount and kind of lipids (preferably polar lipids). The next move forward could include a holistic assessment of the micellarization of carotenoids in the context of a standard meal or dietary pattern, according to the FDA guidelines for bioavailability and bioequivalence studies [10] and to the harmonized INFOGEST *in vitro* digestion protocol [2]. Hence, this protocol describes the measurement of the food effect in the micellarization of carotenoids considering normo-, hypo-, and hyper-caloric meals to tabulate predictive data of the potential bioaccessibility of carotenoids from dietary food sources, a protocol that could be extended to other lipophilic bioactives and vitamins.

2. Materials

2.1. Standard Meals

1. The standard meals are designed following the guidelines of the report for Human Energy Requirements, which are based on sex BMI and PAL [11] (*see* **Note 1**). For a single meal with a caloric intake of 650-750 kcal (*see* **Note 2**),

the macronutrient ratio is established according to the following reference intake ranges: >50% total carbohydrates, 15-30% total fat and 10-20% total protein (percentage of total energy intake per meal). In addition, the goal of 400 g of fruits and vegetables a day is followed [12]. The design of high or low caloric meals is made applying the same reference intake ranges for macronutrients, and the goal of fruits and vegetables daily intake is followed but increasing or decreasing *ca*. 30% the total caloric intake in the meal, respectively. Accordingly, the design of high or low-fat meals is made increasing or decreasing the mean reference intake of total fat a 60%, keeping the reference intake of protein in both cases, and decreasing or increasing the reference intake of total carbohydrates, respectively [10]. The food composition tables published by Mataix [13] are taken as reference to calculate the caloric content and macronutrient composition of the meals. To estimate the carotenoid intake in the designed meals, the European carotenoid database is used [14]. Table 1 includes example meals that we use routinely in our laboratory to measure the food effects in the micellarization index of carotenoids and lipophilic vitamins (*see* **Note 3**).

2.2. Preparing Simulated Gastric Fluids

1. Electrolyte stock solution: 0.625 M KCl, 0.625 M KH_2PO_4, 1.25 M $NaHCO_3$, 2.5 M NaCl, 0.187 M $MgCl_2x6H_2O$), 0.625 M $(NH_4)_2CO_3$ and 0.3 M $CaCl_2x2H_2O$ (*see* **Note 4**). Transfer each amount of weighted salt to 1 L graduate cylinders with 100 mL water. Make up to 1 L with water and mix. Store at 4 ºC.
2. Simulated salivary electrolyte stock solution: Transfer 15.1 mL of 0.625 M KCl, 3.7 mL of 0.625 M KH_2PO_4, 6.8 mL of 1.25 M $NaHCO_3$, 0.5 mL of 0.187 M $MgCl_2x6H_2O$ and 0.06 mL of 0.625 M $(NH_4)_2CO_3$ to a 500 mL graduate cylinder with 100 mL water. Make up to 500 mL and mix. Store at -20 ºC.
3. Simulated gastric electrolyte stock solution: Transfer 6.9 mL of 0.625 M KCl, 0.9 mL of 0.625 M KH_2PO_4, 12.5 mL of 1.25 M $NaHCO_3$, 11.8 mL 2.5 M NaCl, 0.4 mL of 0.187 M $MgCl_2x6H_2O$ and 0.5 mL of 0.625 M $(NH_4)_2CO_3$ to a 500 mL graduate cylinder with 100 mL water. Make up to 500 mL and mix. Store at -20 ºC.

4. Simulated intestinal electrolyte stock solution: Transfer 6.8 mL of 0.625 M KCl, 0.8 mL of 0.625 M KH_2PO_4, 42.5 mL of 1.25 M $NaHCO_3$, 9.6 mL 2.5 M NaCl and 1.1 mL of 0.187 M $MgCl_2x6H_2O$ to a 500 mL graduate cylinder with 100 mL water. Make up to 500 mL and mix. Store at -20 ºC.
5. Stock solution of 1500 U of α-amylase per mL of simulated salivary electrolyte solution (*see* **Notes 5** and **6**).
6. Stock solution of 25000 U of porcine pepsin per mL of simulated gastric electrolyte stock solution (*see* **Notes 5** and **7**).
7. Stock solution of 800 U of pancreatin from porcine pancreas per mL of simulated intestinal electrolyte stock solution (*see* **Notes 5** and **8**).
8. Stock solution of 0.16 mmol of porcine bile extract per mL of simulated intestinal electrolyte stock solution (*see* **Notes 9** and **10**).
9. Laboratory blender with glass container (1-2 L capacity) with speed control and timer.
10. 100 mL GL45 clean glass laboratory bottles.
11. Magnetic stirrer with 4-6 stirring positions. Cross type PTFE magnetic stir bars 25 mm diameter.
12. Incubator chamber with adjustable temperature control to 0.1 ºC and internal power sockets for stirring unit(s).

2.3. Measurement and Adjustment of pH during In Vitro Digestion.

1. Adjustment of pH values at the initial and during the gastric and intestinal stages is made with 1 M HCl or 1 M NaOH.
2. The pH measurements should be performed with a pH electrode suitable for difficult samples containing colloids, emulsions, creams or very viscous samples (*see* **Note 11**).

2.4. Assessment of Lipolysis Efficiency

1. Folch solution, chloroform:methanol 2:1 (v/v), is made fresh each time.
2. 1 M HCl, 1 M NaCl and H_2O:H_2SO_4 (1:1; v/v).
3. Hexane:diethyl ether: glacial acetic acid 80:20:1 (v/v/v).
4. Silica gel coated TLC plates (10×20 cm, 0.25 mm, Si G60).
5. 200 μL syringe for application of sample on TLC plates.
6. TLC heater.
7. Scanner and software for image processing to calculate optical density.

2.5. Isolation of the Micellar Fraction

1. Benchtop centrifuge, temperature 4 ºC and equipped with rotors for low and high-speed centrifugation.
2. Centrifuge tubes of 15 mL and 50 mL capacity.
3. Nylon 25 mm (i.d.) × 0.22 μm pore size filters.

2.6. Extraction and analysis of the Carotenoid Fraction from the Meal Puree and the Micelles Fraction

1. Benchtop centrifuge, temperature 4 ºC.
2. Centrifuge tubes of 50 mL capacity.
3. Diethyl ether, hexane and NaCl (10%, w/v).
4. Nylon 13 mm (i.d.) × 0.22 μm pore size filters.
5. LC-MS/MS platform with UV-visible detector and APCI source coupled to UHR-TOF mass analyser.
6. C30 analytical column (3 μm particle size, 25 × 0.46 cm i.d.).
7. LC grade solvents for chromatographic separation: methyl-*tert*-butyl ether, methanol and water.
8. Standard stock solutions of astaxanthin, β-carotene, β-cryptoxanthin, canthaxanthin, lutein, lycopene, and violaxanthin, which are prepared at a concentration of 25 mg L^{-1} following the procedures described in [15]. Once the exact concentration is determined, working stock solutions for external calibration curves are prepared at 5 concentration levels ranging from 0.15 to 10.0 mg L^{-1}.

3. Methods/Study Design

3.1. Standard meals

1. Once the meal is ready to eat, grind it finely in an electric food mixer until a puree is obtained.
2. Keep the puree at 40 ºC until the application of the *in vitro* procedure. Take three portions (5 g) to determine the carotenoid content in the puree.

3.2. Three-stages *In vitro* Digestion Procedure and Isolation of the Micellar Fraction

1. The procedure is based on Minekus et al. [2]. Mix 30 g of the puree with 21 mL of simulated salivary electrolyte stock solution, and place 3 aliquots (17 mL) of the puree in 100 mL GL45 clear glass laboratory bottles (*see* **Note 12**) with a cross type magnetic bar. Mix each aliquot with 1 mL of salivary α-amylase

stock solution (1500 U/mL), 50 μL of 0.3 M $CaCl_2$ and 1.95 mL of water. Incubate the mixture at 37 ºC (20 mL) for 10 min or 2 min with magnetic stirring at 1 000 rpm.

2. Mix each aliquot (oral bolus) of the preceding step with 15 mL of simulated gastric electrolyte stock solution, 3.2 mL of porcine pepsin solution and 10 μL of 0.3 M $CaCl_2$. Adjust the pH of the mixture to 3.0 with 1 M HCl (*see* **Note 13**) and make the final volume equal to 40 mL. Place the bottle in the incubator chamber at 37 ºC for 2 h with magnetic stirring at 1000 rpm.
3. The gastric chyme is mixed with 22 mL of simulated intestinal electrolyte stock solution, 10 mL of pancreatin solution, 5 mL of fresh bile and 80 μL of 0.3 M $CaCl_2$. Adjust the pH of the mixture to 7.0 with 1 M NaOH (*see* **Note 13**) and make the final volume equal to 80 mL. Place the bottle in the incubator chamber at 37 ºC for 2 h, with magnetic stirring at 1000 rpm.
4. The intestinal chyme of each bottle is divided in 40 mL aliquots and transferred to 50 mL centrifuge tubes. Each bottle is washed with two portions of 5 mL of water and added to the centrifuge tubes to make the final volume equal to 50 mL. Isolate the aqueous suspension from the undigested and solid residues by low speed centrifugation at 4 000×g for 20 min. Transfer 10 mL aliquots of the aqueous suspension to 15 mL centrifuge tubes and apply the high-speed centrifugation step at 15 000×g for 8 min. Isolate the micellar fraction by filtering the clarified suspension through a 0.22 μm Nylon filter.

3.3. Extraction of carotenoids from the puree and micelles

1. The extraction method is based on Ríos et al. [16]. Mix the puree (5 g) or the micellar fraction (5 mL) with diethyl ether (5 mL) and hexane (2 mL) and mix in a vortex for 2 min. Add 5 mL of NaCl (10%, w/v) and mix in a vortex for 2 min. Centrifuge the sample at 4 000×g for 5 min.
2. Isolate the organic layer in a rotatory flask and repeat the extraction procedure (avoiding the addition of NaCl solution). After centrifugation, isolate the organic layer again and evaporate the combined organic extracts to dryness in a rotatory evaporator at 25 ºC.
3. Dissolve the extract in 0.5 mL of hexane and filter it through a nylon 13 mm (i.d.)×0.22 μm pore size filter. Store the sample at -20 ºC until HPLC-MS/MS analysis (within 1 week).

3.4. LC-MS/MS Analysis

A great variety of LC systems, MS hardware configurations and software tools is available for LC-MS/MS analysis of carotenoids, including column, solvent composition, isocratic or gradient elution, flow rate and post-column operation, APCI ionization protocol, parameters and arrangement of the MS analyser, and conditions for MS/MS ionization and scan of product ions. The following description is the practice routinely used in our laboratory [17].

1. A binary solvent system consisting of methyl-*tert*-butyl ether:methanol:water 85:15:4 (solvent A) and methyl-*tert*-butyl methyl:methanol:water 7:90:3 (solvent B) is used at a flow rate of 1 mL min^{-1}.
2. Carotenoid separation is performed on a C30 analytical column (3 μm particle size, 25 × 0.46 cm i.d.). Injection volume is 20 μL per sample.
3. LC separation is performed using the following gradient setting: hold at 100% A for 10 min, then gradient from 100 to 50% A in 30 min, 50 to 100% B in 10 min, 100% A in 5 min, and finally isocratic 100% A for 5 min for re-equilibrating column.
4. A split post-column of 0.4 mL min^{-1} is directly introduced on the APCI source, which is operated in positive mode. The *m/z* scan in the UHR-TOF is in the 50-1200 D range. MS data are acquired in broad band Collision Induced Dissociation mode to obtain MS and MS/MS spectra simultaneously.
5. Data evaluation is carried out to identify the carotenoids by using an *in-house* mass database created *ex professo* that contains the monoisotopic masses, elemental composition and, optionally, the retention time and characteristic product ions for 360 carotenes, xanthophylls and xanthophyll esters. The process is represented in Figure 1. High-resolution mass spectrometry measurements are completed based on mass accuracy and in combination with the isotopic pattern. The characteristics of experimental mass spectrum and the MS^2 data are compared with the data available in the literature for carotenoid identification in food samples, as well as with authentic carotenoid standards isolated from their natural sources. For carotenoid quantification, external calibration curves are applied for individual free carotenoids, while xanthophyll esters are quantified with the calibration curves of their corresponding free xanthophyll counterparts.

As the carotenoids belong to the lipid profile of foods, they should incorporate into mixed micelles to be accessible to intestinal cells for absorption. Micellarization is the outcome of a complex process where several parameters are key players, but particularly the action of digestive secretions that include enzymes and bile salts impacts the efficiency of this stage. Therefore, to obtain reliable results is critical to measure the efficiency of the lipolysis in the chyme obtained after the *in vitro* digestion protocol.

1. Add 1 g of chyme to a 15 mL centrifuge tube and mix with 5 mL of the Folch solution, cap and mix for 10 min. Mixture could be stored at -20 ºC at this point if required.
2. Add 1 mL of 1 M NaCl to the tube and mix for 10 min.
3. Centrifuge for 10 min at 1 000 g at 4 ºC.
4. Discard the aqueous upper layer and transfer the solvent phase (*see* **Note 14**) to a rotatory flask and evaporate to dryness. Add 0.5 mL of chloroform to the dry lipid extract and vortex to dissolve.
5. Spot 200 μL of the lipid extract onto a TLC plate and develop the TLC plate with the elution solvent in the TLC chamber until solvent reaches 4/5 up plate.
6. Remove the TLC plate from the chamber and allow solvents to evaporate at room temperature.
7. Spray the TLC plate with H_2O:H_2SO_4 (1:1, v/v) solution and place it in a plate heater at 180 ºC for 15 min. Subsequently, let the TLC plate to reach ambient temperature and scan it. Use an image processing software to analyse one-dimensional images and obtain the grey scale intensity values (*see* **Note 15**).
    1. The micellarization index is expressed as percentage value regarding the total initial carotenoid content in the meal and the total carotenoid content in the micellar fraction.
    2. A minimum of three replicates per *in vitro* digestion process is required to determine whether the observed effect (if it is so) is statistically significant.
    3. The efficiency of the lipolysis of triacylglycerides is determined by measuring the grey intensity of bands corresponding to the intact triacylglycerides, mono- and di-acylglycerides and free fatty acids at the beginning and end of the digestion protocol (*see* **Note 15**).

4. Notes

1. The meals are designed for a 40-49 years old male, 1.70 m high and 65 kg weight, 18.5-25 kg/m^2 (body mass index, BMI) and a moderately activity level (Physical Activity Level, PAL = 1.70-1.99). With this data the caloric requirement is 2450 kcal.
2. The total daily caloric intake (2450 kcal) is distributed 20-25% at breakfast, 15-20% at mid-day/snack, 30% at lunch, 25-30% at dinner.
3. Pasta is cooked in boiling water (1 L per 100 g of pasta) for 12 min. Tuna fish is fried in iron at 120ºC for 3 min. Bacon strips are fried in iron at 120ºC for 3 min.
4. This salt is not added to the simulated electrolyte stock solutions as precipitation may occur.
5. Enzymes solutions need to be used on the same day. Weigh the enzyme carefully because it tends to disperse. The amount of enzyme to be used for the digestion is re-calculated according to the activity.
6. We measure the α-amylase enzyme activity with an Alpha-amylase microplate assay kit.
7. We measure the pepsin enzyme activity with a Pepsin microplate assay kit.
8. Pancreatin is a mixture of several digestive enzymes produced by the exocrine cells of the porcine pancreas. However, we measure the lipase enzyme activity to re-calculate the amount of pancreatin required.
9. We measure the total bile acid content in the bile extract with an assay kit .
10. Particle size distribution of the bile extract may considerably change among lots as it is depicted in Figure 2. This may affect the efficiency of the micellarization. Batch 1 is a fine powder while batch 2 should be grinded in a mortar to obtain the same particle size distribution.
11. The pH probe we use in our lab is equipped with temperature sensor and designed for low conductivity samples. Use cleaning solution or mild soap or detergent, and a toothbrush to remove oil/fat and chyme residues from the pH bulb after pH measurement.
12. The use of glass bottles is mandatory because intestinal enzymes bind to the plastic materials, so that efficiency of the enzymatic activity is reduced.
13. Determine the amount of 1 M HCl or 1 M NaOH required adjusting the pH value at the gastric and intestinal phase, respectively, in a test experiment so that their addition can be subsequently made quickly. Monitor the pH values at the beginning

and intermediate periods of both digestion phases in one of the replicates and adjust the pH value if necessary.

14. Be careful not to disturb the pellet or the solvent phase.
15. We use the free software ImageJ that performs density or grey scale measurement/calibration [18]. Optical density step tablets to calibrate an image are commercially available from Tiffen (Kodak) and Stouffer. The densitometry on bands corresponding to the triacylglycerides, mono- and di-acylglycerides and free fatty acids at the beginning of the digestion phase is the reference value for obtaining the lipolysis efficiency.

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Figure Captures

Figure 1. Scheme of the process for data evaluation after acquisition of HPLC-APCI-qTOF spectra.

Figure 2. Different particle size distribution of bile extracts purchased to the same dealer.

Tables

Table 1. Description of the meals to account the food effects in the micellarization index of carotenoids.

	Caloric content			**Fat content**	
	Normo	*Hypo*	*Hyper*	*Low*	*High*
	Pasta (80 g)	Salad (250 g)[3,4]	Salad (320 g)[2,3]	Pasta salad[3,5] (305 g)	Salad (320 g)[2,3]
	Tuna fish (50 g)[1]	Zucchini omelette (egg 55 g, zucchini 200 g, olive oil 5 g)	Bacon (40 g)	York ham (30 g)	Bacon (80)
	Fried tomato (60 g)	Bread (30 g)	Blue cheese (25 g)	Olive oil (8 g)	Egg (88 g)
	Salad (320 g)[2,3]	Apple (150 g)[3]	Chips potatoes (50 g)	Bread (60 g)	
	Olive oil (5 g)		Bread (50 g)	Watermelon (250 g)[3]	
	Bread (50 g)		Orange juice (100 mL)[3]	Pear (150 g)[3]	
	Orange (200 g)[3]				
Caloric content (kcal)	700	470	900	650	688
Carbohydrates (%)	60	68	52	78	30
Fats (%)	20	19	30	8	50
Proteins (%)	20	13	18	14	20

[1]Fish could be replaced by other meat products that provides the same carbohydrates, fat and protein content. [2]This salad contains iceberg lettuce (100 g), tomato (100 g), onion (30 g), carrot (50 g), pepper (30 g), olive oil (10 g). [3]This item(s) could be substituted/composed by another rich-source of carotenoids. [4]This salad contains iceberg lettuce (150 g), tomato (50 g) onion (30 g), cucumber (15 g), olive oil (5 g). [5]This salad contains pasta (125 g), tomato (100 g), corn (30 g), carrot (50 g).

Figure 1

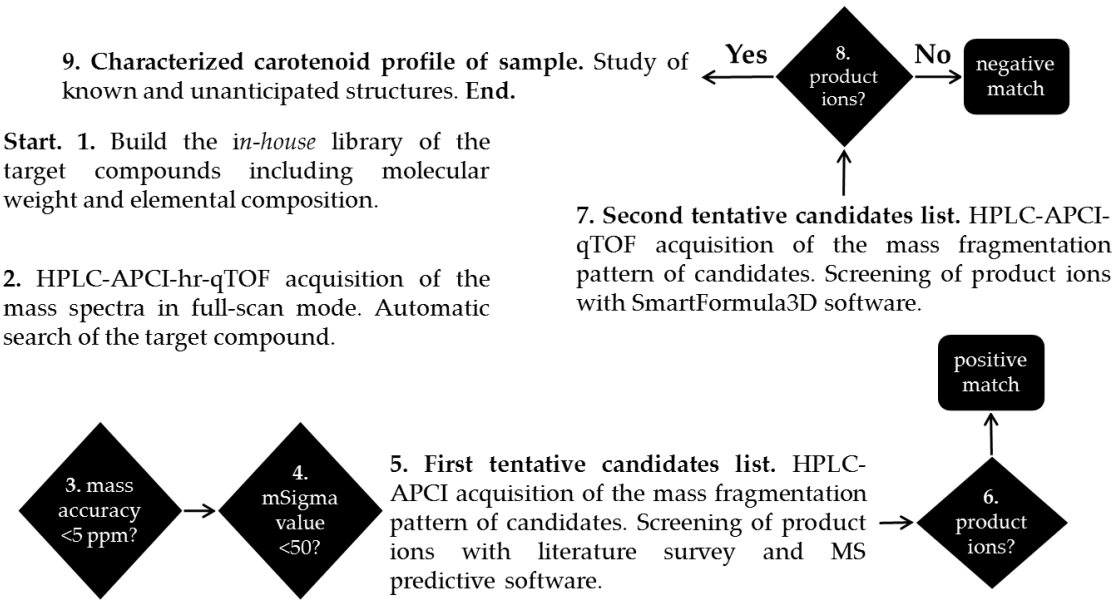
9. Characterized carotenoid profile of sample. Study of known and unanticipated structures. End.
Yes
8. product ions?
No
negative match
Start. 1. Build the in-house library of the target compounds including molecular weight and elemental composition.
7. Second tentative candidates list. HPLC-APCI-qTOF acquisition of the mass fragmentation pattern of candidates. Screening of product ions with SmartFormula3D software.
2. HPLC-APCI-hr-qTOF acquisition of the mass spectra in full-scan mode. Automatic search of the target compound.
positive match
3. mass accuracy <5 ppm?
4. mSigma value <50?
5. First tentative candidates list. HPLC-APCI acquisition of the mass fragmentation pattern of candidates. Screening of product ions with literature survey and MS predictive software.
6. product ions?

Figure 2