Feasibility of Individual Carotenoid Quantification in Mixtures Using UV-Vis Spectrophotometry with Multivariate Curve Resolution Alternating Least Squares (MCR-ALS)

Nawel Achir,1 Adrien Servent,1,2 Marvin Soto,1,3 and Claudie Dhuique-Mayer1,2

1UMR Qualisud, Univ Montpellier, Avignon Université, CIRAD, Institut Agro, Université de La Réunion, Montpellier, France
2CIRAD UMR Qualisud, F-34398 Montpellier, France
3Centro Nacional de Ciencia y Tecnología de Alimentos (CITA), Universidad de Costa Rica (UCR) Ciudad Universitaria Rodrigo Facio, Código Postal 11501-2060, San José, Costa Rica

Correspondence should be addressed to Nawel Achir; nawel.achir@supagro.fr

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A fast and low-cost analytical method to determine the concentrations of carotenoids (β-carotene, lutein, and lycopene) from mixed standard solutions or from fruit extracts (kiwi fruits, tomato paste, pink grapefruit juice, kiwi-pineapple smoothie, and apricot nectar) was tested. The methodology was based on UV-Vis spectrophotometry and Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS). Results showed that relative concentrations of β-carotene, lutein, and lycopene in solvent were successfully determined by this technique with an error inferior to 6%. In real extracts, the procedure succeeded well in identifying the major carotenoid type of the fruit samples but also a more complex profile of a fruit mixture. The results also showed that accuracy of carotenoids determination by UV-Vis spectrophotometry-MCR-ALS in fruit extracts was conditioned by their spectral characteristics (III/II ratios and λmax), their relative proportion, and the extract purity.

1. Introduction

Carotenoids are a family of lipophilic light-absorbing chromophores responsible for the yellow, orange, or red color of many fruits and vegetables [1–3]. They can be found in very various amounts and profiles as a function of the crop [4–6]. These molecules can be classified as a function of their structure and biological role (Figure 1). Carotenes are nonoxygenated carotenoids and oxygenated carotenoids are xanthophylls [3]. The most common carotenes are β-carotene and lycopene. The bicyclic β-carotene is the most widespread in vegetables and fruits and can be found in carrot, orange-fleshed sweet potato, apricot, mango, palm fruits, etc. [7]. Of the acyclic carotenes, lycopene is the most prevalent and is the major carotenoid in tomato, pink grapefruit, and watermelon [2, 8]. Xanthophylls are widely distributed in fruits and vegetables. Lutein is present in high levels in leaves, green fruits and vegetables, and yellow flowers [3, 9]. Carotenoids are valuable natural colorants that are added to many processed foods, in the form of natural extracts or as pure chemically synthesized compounds, in order to give the desired coloring properties [10].

Carotenoids also exhibit numerous health promoting properties. Close to 90% of carotenoids in the human diet that are recovered in human plasma are represented by β-carotene, α-carotene, lycopene, lutein, and cryptoxanthin [11, 12]. β-carotene is a source of vitamin A with an equivalency ratio for β-carotene to vitamin A estimated as 12:1 [13]. Lutein is a macular pigment that plays an important role against cataracts and age-related macular degeneration [14]. Lastly, lycopene is the most potent antioxidant among carotenoids, and dietary intake has been shown to be associated with a decreased risk of chronic diseases, such as cancer and cardiovascular disease [15].

Because these compounds are of nutritional and sensory importance, and because they cannot be synthesized by
humans so they have to be provided by food, many techniques were developed to analyze and quantify them. Their fine separation is for instance useful to gain insights into their biological role in nutritional studies [16]. Precise quantification is necessary to provide reliable data for food databases. However, many carotenoid-rich plants are found in developing countries where expertise and/or analytical resources are still insufficient [17]. In parallel, there is a growing demand for healthy and phytonutrient-rich food in developed countries so modern agriculture will gain in producing not only high-yield but also high-nutritional potential crops. This new strategy implies high throughput quantification of carotenoids. Therefore, phytonutrient analysis in general and carotenoids analysis in particular must meet both precision and material (time, technicity) constraints [18–20]. The two main protocols used for carotenoid quantification are UV-Vis spectrophotometry and the HPLC methods [21–24]. The first one uses the characteristic visible light absorption spectrum of carotenoids. Due to their system of conjugated double bonds, their spectrum consists of a three-peak shape (namely, I, II, III) between 400 and 500 nm. The three \( \lambda_{\text{max}} \) vary as a function of the carotenoid structure (Figures 1 and 2(a) and Table 1). In addition to \( \lambda_{\text{max}} \), the 3-peak-absorption shape of the carotenoid spectra enables the calculation of the \( \% \text{III/II} \) ratio which is another important parameter to identify them as it is different as a function of the molecule (Figure 2(a)). This parameter is the ratio of the height of the longest-wavelength absorption peak (III) and that of the middle absorption peak (II), taking the minimum between the two peaks as baseline, multiplied by 100 [3, 26, 27].

Carotenoids in solution obey the Beer–Lambert law. Their absorbance is directly proportional to the concentration which is usually determined using the molar extinction coefficient (cmol) in an appropriate solvent [28]. Therefore, absorbance measurements can be used to quantify the concentration of a pure carotenoid (standard) or to estimate the total carotenoid concentration in a mixture or extract of carotenoids from plants [29, 30]. This technique is rapid and low cost and has been optimized by much research. The main improvements consist in removing interference and particularly that of chlorophylls [21, 23] and optimizing the solvent extraction procedure [22] in order to increase accuracy and rapidity. Other spectrophotometric techniques were tested as near Infrared spectroscopy but proved to be less robust than UV-Vis spectrophotometry for quantification purposes [24]. However, despite its convenience, the main drawback of this method is the lack of quantification information compared to analyses by HPLC. Indeed, the UV-Vis method does not enable the distinction between carotenoids in a given mixture as the most common protocols use a mean absorption coefficient and a mean absorption wavelength [21, 31]. This problem limits its use for nutritional or adulteration control purposes. HPLC analysis is a more accurate and appropriate method to get individual concentrations of the different carotenoids in a fruit, a dish, or a formulated product [1, 10, 32]. This method is, however, time- and solvent-consuming and requires higher costs to establish and maintain this higher technicity. In addition, HPLC analyses sometimes require a long saponification step involving the use of a hazardous substance, potassium hydroxide solution in methanol, to remove the xanthophyll esters that can make quantification difficult [33]. Indeed, xanthophylls can be esterified by various fatty acids resulting in a hundred possible combinations of different molecular weights and polarities and unidentified and unresolved chromatographic peaks. The saponification step is not useful in the UV-Vis method since esterification does not significantly affect carotenoid spectra [3]. In addition, it was proven that saponification leads to significant carotenoid loss [21].

Since physical separation is a difficult task, it is interesting to explore mathematical separation on the basis of the
UV-Vis spectra. The main mathematical treatment on spectra used up till now for carotenoid analysis was the subtraction of absorbance to a certain wavelength to remove interference [21, 23]. For instance, Lichtenthaler developed formulas in different organic solvents to get a more reliable global carotenoid content in green tissue extract solutions by removing the contribution of chlorophyll. However, further and more specific information could be obtained from the spectrum by using chemometric tools. Among them, Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) is a powerful tool in solving mixture analysis problems. Indeed, MCR-ALS can provide a bilinear description of the observed data. As the total absorbance is the sum of the individual absorbance for each species according to the Beer–Lambert law, MCR-ALS can dissociate the spectral contribution of pure compounds involved in the mixture and also give their relative concentration. Therefore, MCR-ALS is not a statistical method like partial least square regressions which is usually used with near infrared spectroscopy, where footprint spectra (a hundred) are correlated to chemical features. MCR-ALS has, until then, mainly been used in the pharmaceutical or chemical engineering fields and proved to be efficient to monitor evolving reactions during processes [35–37]. However, this mathematical procedure is now used in the domain of food science to analyze complex natural

**Figure 2:** Spectra of (a) pure standards of β-carotene, lutein, and lycopene (solutions 1–3 of Table 2 and (b) mixtures of standards (solutions 4–13 of Table 2). Calculation of %III/II as indication of spectral fine structure is presented for lycopene spectra (%III/II = III/II × 100).

**Table 1:** Carotenoid absorption data from literature [3, 25].

|          | Molecular weight | \( \epsilon \) (mM\(^{-1}\). cm\(^{-1}\)) | \( \lambda_{\text{max}} \) (nm) | Solvent  | III/II (%) |
|----------|------------------|-----------------------------------------|---------------------------------|----------|------------|
| β-carotene | 537              | 139                                     | 450                             | Hexane   | 25         |
| Lutein    | 569              | 145                                     | 445                             | Hexane   | 60         |
| Lycopene  | 537              | 185                                     | 470                             | Hexane   | 65         |
samples. Indeed, MCR-ALS was successfully tested on voltammetric signals to quantify organic acids in fruit juices and on UV-Vis spectra to discriminate polyphenols in a hibiscus extract [38–41]. It was also employed for quantification and spatial distribution of salicylic acid in film-coated tablets using Raman spectrophotometry, or to get insight into conformational changes and protein folding [42, 43]. MCR-ALS was also used for β-carotene with transient absorption spectroscopy to monitor its excited state as a function of time and also to automatize isoprenoids analysis by HPLC [44, 45]. However, to our knowledge it has never been tested on mixtures of carotenoids for quantification purpose with a conventional UV-Vis spectroscopy protocol. It would, however, be very informative to test the potential of this technique on these interesting pigments from both a nutritional and organoleptic point of view because they exhibit quite remarkable spectral characteristics in the UV-Vis domain. These properties were interestingly exploited by Kupper et al. who showed that by fitting with Gauss-peak spectra (GPS) it was possible to quantify certain individual carotenoids in crude extracts of leaves of higher plants, brown algae, and cyanobacteria [31]. However, the GPS treatment required a mathematical treatment with complex equation and coding skills. In addition, GPS is based on modeling spectra by a set of Gaussian functions which is not as chemically meaningful as MCR-ALS. Indeed, MCR-ALS assumes that the observed spectra are a linear combination of the spectra of the pure components in the system which is the multiwavelength extension of Beer–Lambert law [46]. Another advantage of MCR-ALS is that a graphical user-friendly interface was developed in MATLAB and is freely available [47]. This tool enables constraints to be implemented easily, i.e., additional chemical knowledge, such as pure spectra to give even more sense to the resolution.

In this paper, we studied the use of a UV-Vis method for analyzing mixed carotenoids with the implementation of spectra treatment by MCR-ALS, to obtain the relative concentration of individual carotenoids from a mixture in solvents or a plant sample extract. To best analyze the possibilities, robustness, and limitations of this direct quantification method, we analyzed the spectra of mixed carotenoid standards (β-carotene, lutein and lycopene) in an appropriate solvent and afterwards on three different fruit or processed fruit product extracts. The accuracy and precision of this method were tested by comparison with HPLC results.

2. Materials and Methods

2.1. Chemical Products. β-Carotene, lutein, and lycopene standards were purchased from Extrasynthèse (Genay, France). Hexane, dichloromethane, ethanol, methyl-tert-butyl ether, methanol, potassium hydroxide, and pyrogallol were of analytical grade and obtained from Merk (Germany).

2.2. Raw Fruits. Fresh kiwi fruits (FK), pink grapefruit juice (PGJ), apricot nectar (AN), kiwi-pineapple smoothie (KPS), and tomato paste (TP) were purchased in local supermarkets (Montpellier, France). Contrary to PGJ and AN, KPS was flash pasteurized. KPS also contained apple juice (47%) and spirulina extract. These products were chosen to represent a diversity of matrices (species, raw, or processed) and of carotenoid profile.

2.3. Standard Solution Preparation. Four individual 100 mL standard solutions of β-carotene, lutein, and lycopene were made at concentrations of 1.98, 5.45, and 4.18 mg L$^{-1}$, respectively, in hexane. To properly dissolve lutein and lycopene, 1 mL of ethanol and dichloromethane was added, respectively, before hexane and the mixture was put in an ultrasound bath for 30 s. The concentrations of each solution were determined using molar extinction coefficient (εmol) in the appropriate solvent checked by spectrophotometry (Table 1).

Standard carotenoid solutions of β-carotene, lutein, and lycopene were mixed with different volume proportions leading to a total concentration of ~5 mg L$^{-1}$. The different relative concentrations are presented in Table 2 before UV-Vis analysis. Solutions 1 to 3 correspond to pure compounds. Solutions 4 to 13 were prepared with β-carotene lutein and lycopene at different concentrations to estimate the sensitivity of the MCR-ALS procedure.

2.4. Preparation of Fruit Carotenoid Extracts. Extraction procedures and conditions for analysis were described previously [48, 49]. Briefly, samples were ground if necessary and then weighed (200 mg for tomato paste, and 1000 mg for pink grapefruit juice, apricot nectar, kiwi-pineapple smoothie, and kiwi fruit) in 20 mL glass tubes. 2 mL of an ethanol solution containing 1% pyrogallol was added in each tube. For each product, 3 samples were prepared in tubes for UV-Vis and 3 for HPLC.

2.4.1. UV-Vis Sample Preparation. 2 mL of distilled water and 5 mL of hexane were added in the tubes. After mixing in a tube rotator (Boekel Scientific, USA) during 15 min and decantation, the organic phase was recovered with a Pasteur pipette, and the aqueous phase was extracted once more with 5 mL of hexane. The organic phase was pooled and protected from light prior to UV-Vis spectrophotometry analysis. Dilution (1/10) in hexane was made only for tomato paste extract to reach an absorbance intensity of between 0.7 and 0.9 in the 450–500 nm range.

2.4.2. HPLC Sample Preparation. Only tubes containing kiwi were subjected to saponification to remove esters from xanthophylls for 30 min in a water bath at 70°C by adding 1.5 mL of saturated potassium hydroxide (12 N). After incubation, the tubes were cooled in an ice bath. For all tubes (kiwi and the others) 2 mL of distilled water and 5 mL of hexane were added to samples. Then, after mixing and decantation, the organic phase was recovered, and the aqueous phase was extracted two more times with 5 mL of hexane.

2.4.3. Spectrophotometry Analysis. Analysis were performed using a UV-Vis spectrophotometer (Jasco V-630 spectrophotometer, Japan) by recording the absorbance spectra of all the samples in the 300–600 nm range.
For all tubes, the organic phases were combined and evaporated to dryness using a vacuum evaporation system (GeneVac EZ-2, SP Scientific, UK). Finally, the residue from samples for HPLC analysis was redissolved in 500 μL of methyl tert-butyl ether/methanol (80/20) and placed in an amber vial prior to HPLC analysis.

2.5. Determination of Carotenoid Concentrations

2.5.1. UV-Vis Spectrophotometry Measurements and MCR-ALS Analysis. The spectra of pure standard and standard mixtures as well as fruit extracts were acquired in the same conditions. For each solution, 3 spectra were acquired from 250 to 600 nm (every 0.5 nm) with a diode array spectrophotometer (Specord S600 Analytik Jena, Germany).

Total carotenoids were calculated using the Beer–Lambert law from the absorbance at 450 nm using a mean absorption coefficient of 135310 L.mol$^{-1}$.cm$^{-1}$ and converted into mg.kg$^{-1}$ using a mean molecular weight of 548 g.mol$^{-1}$ [21].

To obtain the relative proportion of the different carotenoids, MCR-ALS was done on the whole spectra. Preprocessing steps consisted in removing the short-wavelength region (250–350 nm) and in normalization. MCR-ALS analysis was done with Matlab (The MathWorks, Inc., Natick, MA, USA) using the toolbox built by Jaumot et al. [41, 50]. Briefly, Multivariate Curve Resolution aims to achieve a bilinear decomposition in 2 matrices of an experimental absorbance matrix $D(m,n)$ which corresponds to a combination of spectrophotometric measurements. The two matrices correspond to the concentrations $C(m,k)$ of the individual compounds and their normalized spectra $S^T(k,n)$. The underlying law that links the two-way data matrix $D(m,n)$ to $C(m,k)$ and $S^T(k,n)$ is a generalization of the Beer–Lambert law applied to all wavelengths:

$$D(m,n) = C(m,k)S^T(k,n) + E(m,n),$$

with $k$, the number of chemical species in the unknown mixture, and $E(m,n)$, the error matrix.

Estimation of the number of components $k$ is done with singular values of the data matrix $D$. Initial estimations of spectra are obtained from pure variable detection methods. Resolution of (1) is carried out by an iterative process using the ALS algorithm that stops when the error ($E$) is minimum. Results were improved by the addition of optimization constraints with chemical sense: the nonnegativity constraint algorithm “fast nonnegative least squares” and spectra were put at the same height (no closure constraint).

The goodness of fit of the results to the experimental data is estimated with the residues, or the residual sum of squares calculated as follows:

$$\text{residues} = \sum (\text{SRC} - \text{ERC})^2,$$

with SRC the simulated relative concentration obtained with the MCR procedure from the $C$ matrix of (1) and ERC the experimental relative concentration of individual carotenoids (provided by the known concentration of standards incorporated in the mixture or using the HPLC results for fruit extracts).

Mean error of the model expressed in percentage, providing the idea of accuracy of concentration prediction, was calculated as follows:

$$E = \frac{\sqrt{\sum (\text{SRC} - \text{ERC})^2/n}}{\sum \text{ERC}/n} \times 100.$$
10–60 min, 4% A-11% B- 85% C; 60–70 min, isocratic 4% A-11% B-85% C; 70–71 min, 100% B; 71–72 min, with a return to the initial conditions for rebalancing. All-E-β-carotene and their isomers and all-E-lutein were detected at 450 nm, and all-E-lycopene and their isomers were detected at 470 nm. Z-lycopene content was expressed as the sum of all Z-lycopene isomers. Isomers were identified according to their relative retention times, i.e. elution order and the combined use of their spectral data. The identifications were based on previously published data obtained with the same mobile phase (water/methanol/methyl tert-butyl ether) and the same detection wavelength range [51, 52].

2.6. Statistical Analysis. Spectrophotometric and HPLC analyses were performed in triplicate for each sample. One-way ANOVA was used to analyze the difference in carotenoid compositions between the different products (difference considered significant when \( p < 0.05 \)). If significantly different, means were further compared using Tukey’s test. Statistical analyses were performed with Statistica® (StatSoft Inc., USA).

3. Results and Discussion

3.1. Concentration of Individual Carotenoids of Mixed Standard Solutions. Spectra of pure standards and their mixture are presented in Figures 2(a) and 2(b), respectively, according to their composition shown in Table 2. The spectra of pure compounds, (solution 1 to 3) and their typical three-peak shape, are illustrated in Figure 2(a). Lutein and lycopene spectra exhibited significant differences with a maximal absorption intensity at 440 and 470 nm, respectively. They differed from β-carotene with their high III/II ratio of 60%.

Figure 2(b) shows the variability of spectra shapes obtained from the standard mixture with relative concentrations for individual carotenoids ranging from 5 to 55%. Two MCR-ALS procedures were applied to these spectra to obtain simulated concentrations of individual carotenoids. Firstly, we applied the mathematical procedure directly on spectra of Figure 2(b) and secondly on spectra of Figure 2(b) augmented with pure standard spectra of Figure 2(a). In both cases, the number of components \( k \) (equation (1)) was logically chosen equal to three, since it is the number of the different carotenoids put in the mixed standard solutions. Results of the first simulation showed that the error was very high and discrepancy between simulated and real values too great (error above 20% for β-carotene and lutein). These results were explained by the fact that the pure spectra obtained (not shown here) exhibited confusions, particularly between β-carotene and lutein. Addition of the spectra of standards added constraints that improved markedly the resolution of MCR-ALS. Results of this simulation are presented in Figure 3 that shows real values plotted against simulated ones. Plain lines represent the first bisector of the plan and stand for a perfect accuracy of the model, that is to say, simulated results equal to the real values of relative concentrations of Table 2.

As can be seen, the relative proportions of β-carotene, lutein, and lycopene obtained by MCR-ALS were very close to the experimental ones. Final error for individual carotenoid concentration determination was 6, 3, and 4% for β-carotene, lutein, and lycopene, respectively. Therefore, in the case of a pure carotenoid mixture, discrimination of β-carotene, lutein, and lycopene was good providing addition of pure spectra.

3.2. Concentration of Individual Carotenoids in Fruit Extracts

3.2.1. HPLC Results. The chromatograms of the 5 fruit extracts tested are presented in Figure 4: pink grapefruit juice (PGJ), tomato paste (TP), fresh kiwi (FK), apricot nectar (AN), and kiwi and pineapple smoothie (KPS). Lycopene (all-E- and Z-forms) was the major type in PGJ and TP (~70–80%) which was in accordance with literature [8, 48, 53]. Lutein was the major xanthophyll in FK [54] and KPS, and for apricot it was β-carotene [55]. β-Carotene was also present in KPS, because both pineapple and spirulina contain it, as well as in low concentrations in TP, KF, and PGJ of 5, 9, and 18%, respectively.

Nonidentified carotenoids represented a nonnegligible proportion. Assuming a spectrophotometric response coefficient close to that of common carotenoids, nonidentified species could represent about 2 and 6% in AN and PGJ, but 19% in TP and about 26% in KP and till 50% in KFS certainly due to its more complex composition (kiwi, pineapple, and spirulina as additive). These nonidentified carotenoids may consist of residual esterified xanthophylls. In addition, in processed food (PGJ, TP, and AN), these peaks can correspond to degradation products of carotenoids. The first indicator of carotenoid deterioration by thermal treatment is the percentage of isomerization as can be seen in the HPLC chromatogram of grapefruit juice, apricot nectar, or tomato paste. Total carotenoids obtained from HPLC are presented in Table 3. β-Carotene and lycopene were calculated as the sum of all isomers. Logically as it is a concentrated product, total carotenoids accounted for 218 mg.kg\(^{-1}\) in tomato paste and were about 20, 40, 80, and 1000-fold lower in PGJ, AN, KF, and KPS, respectively.

3.2.2. Spectrophotometric Results. Figure 5 shows the spectra of fruit extracts obtained without saponification. This kind of extraction was much more rapid by comparison with the saponification-HPLC procedure (cf Sections 2.5.2 vs. 2.5.3.). The saponification step is not mandatory since esterification does not change the spectra of esterified carotenoids [3]. As can be seen in Figure 5, spectra of extracts were much noisier than those of the standards particularly in the short-wavelength range 300–420 nm. This phenomenon may be due to several reasons. Firstly, the fruit matrix is complex and variable which can be seen on the spectra of kiwi fruit. The other fruit samples had similar spectra since they were homogenized during the process of juice or paste making. Fruit extracts were also more complex because in addition to the main carotenoids, small quantities of other nonidentified carotenoids were extracted together as can be seen in
chromatograms of Figure 4. In addition, we can assume a possible coextraction of small quantities of compounds that may absorb light at the same wavelength as carotenoids, like chlorophylls. This is obviously visible on the spectra of KF and KPS in the region 650–700 nm of the spectra. Finally, in real matrices, carotenoids undergo chemical changes such as isomerization as can be also seen in Figure 4. These changes affect the spectrum of the original carotenoid with a $\lambda_{\text{max}}$ shift and apparition of a cis peak [3]. These artifacts were even more visible in the UV region of the spectra from 300 to 350 nm but a slight background noise may remain at the highest wavelength.

From the absorbance at 450 nm, total carotenoids were calculated and reached 226 mg kg$^{-1}$ in tomato paste and were about 20-, 25-, 35-, and 300-fold lower in PGJ, AN, KF, and KPS, respectively. Therefore, carotenoid content in HPLC results was equal or 1.5 to 3 times below the spectrophotometric results. These differences were also observed in literature. For instance, Biehler et al. also reported a concentration of total carotenoids in tomato paste by spectrophotometric higher than that of the HPLC results. The underestimation of HPLC results was attributed to the extraction step where about 20% of carotenoids is lost. This loss can be even higher when a saponification is required in case of xanthophyll-rich fruits. In addition, Biehler et al. also reported the possible overestimation in spectrophotometric results due to other compound interference and particularly chlorophylls.

To get the individual proportion of carotenoid from the UV-Vis data but also of unknown compounds, MCR was applied to the fruit extract spectra of Figure 5 augmented with standard solution spectra of Figures 2(a) and 2(b) to improve the resolution. As said before, pretreatments consisted in removing the UV region 250–350 nm and to normalize spectra. Both treatments were essential to reduce spectra noise for the fruit extracts and to perform properly MCR-ALS. Two options of initialization were tested: 3 and 4 compounds (named $k$ in equation (1)). This number corresponds to the supposed number of pure compounds present in the mixture and was used for MCR-ALS initialization. Indeed, conversely to standard mixture, the number of compounds in the fruit extract was unknown. The first test of 3 was chosen because it corresponded to the number of the major carotenoids. We also tested 4 components to create an unknown compound that artificially gathered all the noise of the other constituents of the extract. This last solution helped to lower the error of the relative quantification of carotenoids. The results after MCR-ALS resolution in terms of spectra of pure compounds are presented in Figure 6. Pure spectra obtained after resolution based on the experimental spectra from 350 to 750 nm are presented in Figure 6(a) and from 400 to 600 nm in Figure 6(b).

From the comparison with spectra of standards, we could easily identify lutein (S1), lycopene (S2), $\beta$-carotene (S4) and the unknown (S3). Figure 6(a) shows that it was possible to differentiate an unknown compound exhibiting absorbance in the 350–425 and 650–700 nm region which is typical of chlorophylls. From the result of MCR-ALS relative proportions, unknown contribution could be estimated at 5% for TP and PGJ, 15% for AN, 30% for FK, and nearly 80% for KPS which is consistent with the expected content of other extractible compounds, particularly chlorophylls, in the different samples. Contribution of chlorophylls was very high in the commercial KPS, probably because of the addition of spirulina (percentage of this ingredient not given). The content of non-carotenoid compounds that were removed from the total carotenoids are given in Table 3 in the line “TC by spectrophotometry + MCR.” By doing this, the overestimation of spectrophotometric estimation was significantly reduced and was at most 1.3-fold and 1.5-fold for AN and KF, respectively.

Relative proportions of lutein, lycopene, and $\beta$-carotene were obtained after resolution in the rage 400–600 nm (with pure spectra obtained in Figure 6(b)) for TP, FK, KPS, AN, PGF, and 4 different mixtures of KPS, AN, and PGF. Results are given from HPLC and spectrophotometry-MCR-ALS results in Figures 7(a) and 7(b), respectively.

As can be seen, for each extract, the major expected form could be elucidated: lutein for KF, $\beta$-carotene for AN and lycopene for PGF and TP (Figure 7(a)). Regarding the carotenoid species, lycopene and $\beta$-carotene were the best
elucidated form as on every matrix, the error was about 10% vs 35% for lutein. This effective discrimination can be linked to the highest total carotenoid concentration with 200 mg kg$^{-1}$ for tomato paste and 12 mg kg$^{-1}$ for pink grapefruit juice, respectively. Because of this concentration, even if the extract was diluted for spectral measurements, the noise due to other coeluted compounds became minor. We can observe some aberrant concentrations of lutein in TP

Figure 4: Chromatograms of the 5 fruit extracts measured at 450 nm. BC: all-E-β-carotene; LYC: all-E-lycopene; LUT: lutein; Z-BC: Z-β-carotene isomers; Z-LYC: Z-lycopene isomers.
and lycopene in AN which were inferior to 5%. Conversely, the higher error for lutein could be due to a more complex extract and spectral proximity with other xanthophylls, pigments, or degradation compounds as can be seen in the chromatograms of Figure 4. This fact may affect the efficiency of MCR-ALS resolutions and create spectrums that differ from the spectra of standards. This phenomenon is illustrated in Figure 6(a), where S1 and S4, whose main contributors are lutein and \( \beta \)-carotene, respectively, move away from the pure spectra of Figure 2(a). Even if the MCR-ALS spectral resolution is better by taking a shorter range of wavelength from 400 to 600 nm (Figure 6(b)), this fact may explain the overestimation of lutein, contrary to \( \beta \)-carotene, in KPS as well as in all the mixtures that contain it: KPS-AN-PGJ, KPS-AN, KPS-PGF, and AN-PGJ.

Therefore, simple UV-Vis spectrophotometry associated to MCR-ALS treatment can enable good relative quantification in a mixture of pure carotenoid standards with a good sensitivity but also be used to get an estimation of the relative proportion of each specie in a real extract. Indeed, this technique was efficient to discriminate \( \beta \)-carotene and lycopene from other carotenoids with an error inferior to 10%. Therefore, with a simple UV-Vis spectrophotometer and a standard extraction protocol used to determine total carotenoids, addition of the MCR-ALS procedure can give more insights into the pigment composition of the food extract. In comparison to the usual and widespread “total carotenoid” experiments and with the same material, this methodology can give semiquantitative information about the carotenoid profile in a very reduced time (a few min) in comparison to 30–60 min with HPLC. Therefore, this methodology could be applied for high throughput analysis such as for food process controls where the carotenoid profile could be affected by thermal treatment, varietal screening, or ripening monitoring where hundreds of fruits/vegetables must be analyzed. However, this paper also showed the limitations of such techniques that is mainly linked to the types of carotenoids that need to be separated. MCR-ALS worked very differently as a function of the spectra features of the compounds in mixture and its composition. The more the spectra are different, the more the resolution can be possible and relevant. This fact is not constant like the molecules we wanted to separate: spectra may be very different (like \( \beta \)-carotene and lycopene) or the same (like \( \beta \)-carotene and \( \beta \)-cryptoxanthin). Therefore, the performance of the method can be high for some plants but not at all for others. As a consequence, this methodology worked very well for fruits that contained lycopene and \( \beta \)-carotene like tomato and pink grapefruit and could be relevant for watermelon, guava, etc. More generally, our
observations showed that separation could be possible with a difference of $\lambda_{\text{max}}$ of at least 10 nm and/or III/II ratio difference of 30%. Therefore, violaxanthin whose maximal absorption wavelength is around 440 nm could be separated from $\beta$-carotene and lycopene [43, 56]. Lastly, for different sectors of the agrifood industry, MCR-ALS could be useful in estimating other pigments such as chlorophylls.

However, in the case of a mixture of pigments that have very similar features in the UV-Vis such as $\beta$-carotene and lutein, use of MCR-ALS can lead to important ambiguities on the composition and lead to a much lower accuracy. Therefore, this technique cannot replace HPLC analysis especially when specific nutritional calculation has to be carried out. HPLC remains much more robust and reliable for a fine and accurate carotenoid profile. Also, substances to be analyzed have a low absorption coefficient in the relevant spectral region, or which are only minor components of the mixture cannot be determined with high accuracy. Therefore, it is recommended that substances should have different spectral features and be of similar concentrations to achieve a proper discrimination. Finally, the removal of the UV region < 350 nm can be useful to limit interferences, and the signal/noise ratio must be high, so a good spectrophotometer with a low spectral bandwidth is required.

Figure 6: 4 pure spectra obtained after MCR-ALS procedure on standards and fruit extract spectra (a) from 350–750 nm and (b) from 350–600 nm.
Future improvements can be made, by adding a physical purification step, or mathematically by adding spectra of other compounds in the mixture if they are known.

Data Availability
The data that support the findings of this study are available from the corresponding author, Nawel Achir, upon reasonable request.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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