JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 8753–8760 8753
DOI:10.1021/jf902113n

HPLC Assay of Tomato Carotenoids: Validation of a Rapid Microextraction Technique

Sylvie Sérino, Laurent Gomez,* Guy Costagliola, and Hélène Gautier

UR 1115 "Plantes et Systèmes de culture Horticoles", INRA, F-84000 Avignon, France

Carotenoids are studied for their role as pigments and as precursors of aromas, vitamin A, abscisic acid, and antioxidant compounds in different plant tissues. A novel, rapid, and inexpensive analytical protocol is proposed to enable the simultaneous analysis of four major tomato carotenoids: lutein, lycopene, β-carotene, and phytoene. Microextraction is performed in the presence of sodium chloride, *n*-hexane, dichloromethane, and ethyl acetate on fresh tomato powder that has been finely ground in liquid nitrogen. The carotenoids are extracted by agitation and centrifugation and then analyzed by HPLC using a diode array detector. The principal advantage of this extraction resides in the absence of an evaporation step, often necessary to assay tomato carotenoids other than lycopene. Whatever the carotenoid, tests for accuracy, reproducibility, and linearity were satisfactory and indicative of the method's reliability. The stability of extracts over time (several days at −20 °C) as the satisfactory sensitivity of the assay whatever the fruit ripeness had a part in the robustness of the method. Reliable, rapid, simple, and inexpensive, this extraction technique is appropriate for the routine analysis of carotenoids in small samples.

KEYWORDS: Carotenoids; lutein; lycopene; tomato; microextraction; HPLC

INTRODUCTION

Considerable research has been devoted in recent years to clarifying and better understanding the role of secondary metabolites in plants (*1*). These include carotenoids, a subject of growing interest. As pigments, they intervene in the visual quality of fruits and vegetables and their perception by consumers, and they are also precursors of aromas (*2*, *3*), abscisic acid (synthesized in response to water stress), and other derivatives involved in growth and development (*4*). Carotenoids and products arising from their metabolism are also endowed with antioxidant properties that may protect a plant against abiotic (*5*, *6*) and biotic (*7*, *8*) stress and contribute to the nutritional quality of fruits and vegetables (*9*) as well as their health value (*10*, *11*), although this may be modulated in the case of dietary supplements (*12*, *13*). Within only a few years, there has been exponential growth in research to localize them in plants (*14*), understand their metabolism (*15*), and try to increase their levels in fruits and vegetables by modifying environmental factors, cultivation techniques, or cropping times (*16*, *17*) or through genetic manipulation (*18*−*21*).

Different extraction techniques, and numerous colorimetric and chromatographic methods to assay carotenoids in plants, were the subject of a review in 2006 (*22*). Many are lengthy and complicated, usually because of the constraints linked to the preparation of extracts. This is common in the case of HPLC analyses, which are widely used for the separate quantification of carotenoids but require the evaporation (*23*−*26*) and sometimes the saponification of extracts (*27*, *28*). Faced with the need to routinely process several hundred tomato samples, we thus felt it would be appropriate to develop a simpler alternative method.

*Author to whom correspondence should be addressed (e-mail gomez@avignon.inra.fr).

Inspired by the principles underlying the QuEChERS method (quick, easy, cheap, effective, rugged, and safe) (*29*), used for the rapid extraction and purification of pesticides in plants, we have thus developed a novel HPLC analytical method for carotenoids in the tomato. Preliminary assays (data not published) showed that it was not necessary to evaporate the extraction solvents to achieve satisfactory HPLC quantification limits under these operating conditions. No purification step was deemed useful, whatever the degree of ripeness of the tomato. Both preparation time and quantity of extraction solvents necessary were thus significantly reduced, and these tests made it possible to define the most appropriate operating procedure.

To validate this novel extraction procedure, its precision, accuracy, linearity, and robustness were estimated. In the absence of any other reference method, we chose to use the extraction method described by Fish et al. (*30*), supplemented by the addition of an evaporation step for the *n*-hexane fraction, according to the technique described by Schofield et al. (*23*). This made it possible to achieve the assay of carotenoids using HPLC.

MATERIALS AND METHODS

Plant Materials. The study focused on three varieties of tomato at different degrees of ripeness (from green to red) to obtain a broad range of carotenoid concentrations and search for any matrix effect. Twenty-five samples (14 red, 5 green, 5 orange, and 1 green/orange) were harvested, plunged immediately in liquid nitrogen, and stored at −80 °C. These fresh frozen samples were then finely ground in liquid nitrogen and stored again at −80 °C.

To prevent their denaturation, it was essential to store these powders frozen at a low temperature, including during collection of the assay samples for analysis (working over liquid nitrogen).

Extraction Methods (Figure 1). To protect caroténoids from degradation and oxidation, the extraction was conducted under limited light.

Published on Web 09/21/2009

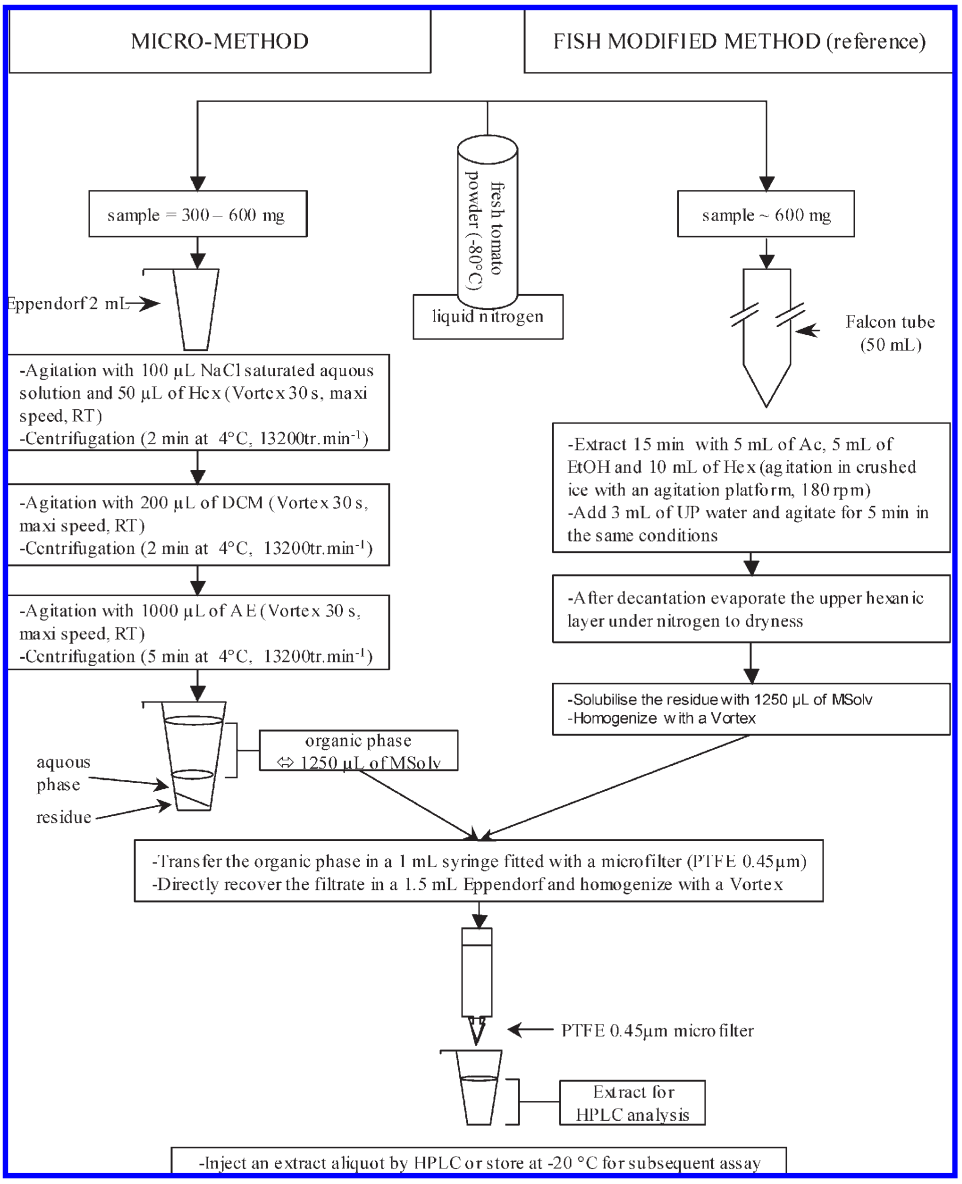

Figure 1. Procedure for the micromethod and the Fish modified method. Hex, *n*-hexane; DCM, dichloromethane; AE, ethyl acetate; Ac, acetone; EtOH, ethanol; UP water, ultrapure water; MSolv, mixture of AE:DCM:Hex (80:16:4, v/v/v); RT, room temperature.

Modified Fish Method. This method was used as the reference. The original technique developed by Fish et al. (*30*), a reduced volume version of that designed by Sadler et al. (*31*), produced extracts that were too dilute for the HPLC assay of carotenoids. To concentrate the extract, we thus added a step for evaporation of the *n*-hexane fraction, borrowed from the method described by Schofield et al. (*23*); this also enabled a reduction in the *n*-hexane concentration of extracts. Indeed, because *n*-hexane is not an injection solvent appropriate for optimum use with the C18 columns chosen for the HPLC assay, it was preferable to markedly reduce its presence in the extracts.

An initial extraction was performed on a assay sample of approximately 600 mg, in the presence of 5 mL of acetone, 5 mL of ethanol, and 10 mL of *n*-hexane, for 15 min, the tubes being held horizontally between two layers of crushed ice on an agitation platform (180 rpm). After the addition of 3 mL of water, a second extraction for 5 min was performed under the same agitation conditions. Following decantation, the upper *n*-hexane phase was evaporated under a nitrogen flux, protected from light. The dry residue was immediately solubilized with 1250 μL of MSolv [a mixture of EA:DCM:Hex (80:16:4, v/v/v)] to obtain a final extract with a volume and solvent composition identical to that of the micromethod. The extract thus obtained was homogenized with a vortex, filtered (PTFE 0.45 μm, Interchim, Montluçon, France), and then assayed immediately by HPLC or stored at −20 °C until assay.

Micromethod. Carotenoid extraction was performed directly in a 2 mL Eppendorf tube containing an assay sample of approximately 400 mg of tomato powder. It was achieved by means of alternating periods of agitation (vortex, at maximum speed) and centrifugation (13200 rpm, 4 °C, model 5415R, Eppendorf, Le Pecq, France), in the following order: addition of 100 μL of saturated aqueous NaCl solution and 50 μL of Hex, agitation for 30 s, and centrifugation for 2 min; addition of 200 μL of DCM, agitation for 30 s, and centrifugation for 2 min; addition of 1000 μL of EA, agitation for 30 s, and centrifugation for 5 min.

An aliquot of the organic fraction (upper phase) was filtered and assayed by HPLC or stored at −20 °C to await assay.

Assay Method. This was identical for the two methods in order to compare the extraction techniques. The solvents in the mobile phase were those used by Mendes-Pinto et al. (*32*), but because the columns differed, the proportions were adjusted.

The assay was performed using HPLC with a DAD UV−visible detector (UV6000LP, Thermo Separation Products, Riviera Beach, FL) under the following conditions: coupling of two columns, Chromolith Performance RP-18e column (100 × 4.6 mm, Merck, VWR International, Fontenay-sous-Bois, France); precolumn, Chromolith (Merck, VWR International); column oven temperature, 28 °C; mobile phase, ACN: UP water:EA (53:7:40, v/v/v); flow rate of mobile phase, 1 mL min^{-1}; injection volume, 10 μL; wavelength range, 200−750 nm; four working

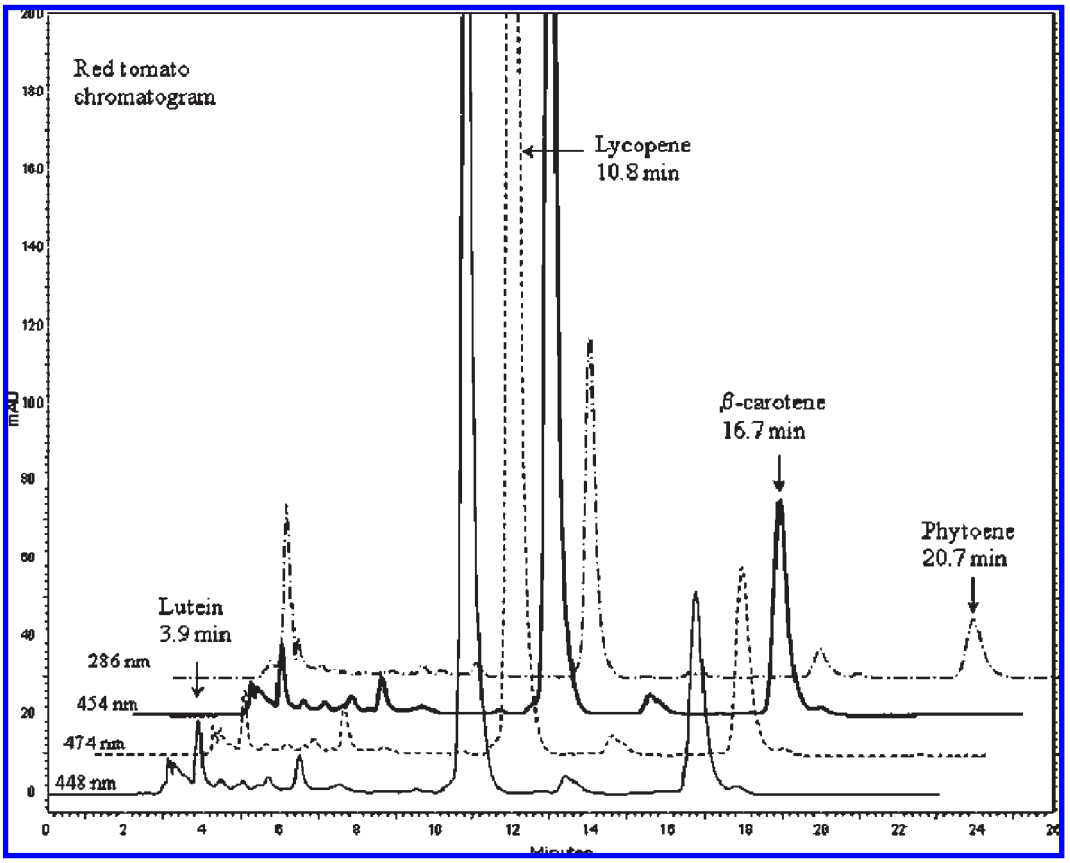

Figure 2. Red tomato extract chromatogram at λ_{max} for lutein (448 nm), lycopene (474 nm), β-carotene (454 nm), and phytoene (286 nm). See text for chromatographic conditions.

wavelengths, 474 nm for lycopene, 454 nm for β-carotene, 286 nm for phytoene, and 448 nm for lutein.

These chromatographic conditions allow good separation of the different carotenoids present in tomato (**Figure 2**).

Reagents. *Pure and Analytical Standards*. Pure standards of lutein (Fluka, Sigma, Saint-Quentin Fallavier, France), lycopene (Sigma), and β-carotene (Fluka, Sigma) were purchased from Sigma, whereas phytoene was obtained from Carotenature (Lupsingen, Switzerland). To prepare each stock solution (SS), the standard was solubilized in pure DCM (80% of final volume). This volume was then adjusted to 100% with *n*-hexane. The SS concentrations of lutein (71 μg mL^{-1}), lycopene (45 μg mL^{-1}), β-carotene (100 μg mL^{-1}), and phytoene (40 μg mL^{-1}) were determined precisely by spectrophotometry, applying the Beer−Lambert law and using the specific absorption coefficients supplied by Britton (*33*,*34*).

To find the same solvent composition (MSolv) as the extracts to be injected for HPLC, each SS was diluted 5-fold in EA, and then daughter solutions were obtained by dilution in MSolv. As from SS/5, the standards were grouped to produce a standard mixture containing the four carotenoids.

In view of the high lycopene concentrations anticipated in red tomato extracts, two standards with a higher concentration (SS/2 and SS/3) were also prepared. The stock solutions and all dilutions were stored at −80 °C.

Solvents and Chemical Substances. The solvents employed were obtained from SDS (Peypin, France), and sodium chloride came from VWR (Fontenay-sous-Bois, France).

Search for a Matrix Effect. Internal standards were prepared using the standard addition technique on green, orange, and red tomatoes that had previously been analyzed. To achieve this, carotenoid solutions with known concentrations were first placed in Eppendorf tubes. After solvent evaporation under nitrogen, a fixed assay sample of tomato powder (400 ± 5 mg) was placed in each tube. Standard additions were performed at three levels of concentration for red tomatoes and two levels for orange and green tomatoes. At injection, the lowest level resulted in one addition equivalent to the limit of detection (LD) of phytoene (LD = 0.16 μg mL^{-1}), 4 times the LD of lutein (LD = 0.03 μg mL^{-1}) and β-carotene (LD = 0.05 μg mL^{-1}), and 16 times the LD of lycopene (LD = 0.02 μg mL^{-1}). The second and third levels of standard addition were respectively 8 and 40 times more concentrated than the first level, the highest level only being applied to red tomato. Extraction was then performed in accordance with the micromethod.

Stability of Extracts. Testing of the robustness of the micromethod consisted in estimating the stability of analytical extracts at −20 °C. For this, 22 extracts of samples from the first series of accuracy tests were reinjected after storage for 14 days at −20 °C.

Statistical Analysis of Data. The micromethod was validated by verifying its precision, accuracy, linearity, and robustness. For each test, the series were compared using the Wilcoxon signed rank test, and their correlation was verified with the Spearman rank test (*35*). According to Kennedy and Neville's definitions (*36*), the term "precision" refers to the closeness with which measurements agree with each other, whereas the term accuracy expresses the closeness of measurements to the true value.

To study the precision of the micromethod, an operative performed two series of analyses (separated by a 10 day interval) on the 25 tomato samples selected using separate assay samples. According to the ISO 5725 (*37*) standard regarding results obtained under these conditions, the reproducibility value R is the value below which there is 95% probability of finding the absolute difference between two results from a single assay.

The lower the R value, the better the reproducibility. This value was calculated using the equation

$$s_r = \left(\frac{1}{2q}\sum_{i=1}^{q} w_i^2\right)^{1/2}$$

$$R = 2.8 s_r$$

where q is the double-analyzed sample number and w_i is the absolute difference between pairs of results.

To take into account the order of magnitude of values obtained with the precision test, root mean square error (RMSE) values were also calculated.

Accuracy was verified by comparison with the modified Fish method. The 25 samples of tomato powder selected were analyzed using this method, and the results were compared with the mean of the results obtained by the micromethod during the precisision test.

The regression parameters obtained using the standard addition technique made it possible to evaluate linearity. The presence of a matrix effect was sought by comparing the carotenoid concentrations obtained using internal and external calibrations at different degrees of ripeness.

A test for robustness consisted of verifying the stability of analytical extracts stored at −20 °C.

All statistical analytical results are presented in **Table 1**.

Table 1. Summary of Statistical Analysis Results[a]

						RMSE (mg kg^{-1})	
Figure	carotenoid	*n*	Wilcoxon test	Spearman test	reproducibility test	series 1	series 2
3	lutein	25	0.0422	0.9954	0.1138	0.0342	0.0345
3	lycopene	25	0.3038	0.9907	2.2013	0.0493	0.0497
3	β-carotene	25	0.0255	0.9831	0.4694	0.0458	0.0463
3	phytoene	25	0.0362	0.9527	0.5698	0.0545	0.0554
4	lutein	25	0.0000*	0.9546			
4	lycopene	25	0.0391	0.9865			
4	β-carotene	25	0.1014	0.9615			
4	phytoene	25	0.2455	0.9736			
6		12	0.9774	1			

[a] See text for justification. *, significantly different at $P < 0.01$

RESULTS AND DISCUSSION

Preliminary assays (data not shown) enabled optimization of the extraction and assay steps for the micromethod analysis of tomato carotenoids prior to their actual validation. Using NaCl-saturated aqueous solution instead of UP water improved the separation between organic and aqueous phases. The present extraction method (sequential additions of the different solvents followed by centrifugation) was preferable to a single extraction with the solvent mixture because the latter did not offer sufficient recovery rates for carotenoids, notably lycopene (~50%). An exhaustive extraction test showed that the conditions retained enabled the recovery of 98% of lutein, 100% of lycopene and β-carotene, and 97% of phytoene present in the tomato extract.

Tests also showed that an assay sample ranging from 300 to 600 mg, with a constant extraction volume, exerted no significant effect on the carotenoid concentrations measured in the tomato samples.

The proven stability of sample extracts for 15 h at ambient temperature and protected from light made it possible to perform HPLC analyses routinely and without interruption by using an autosampler. Use of a refrigerated autosampler (6 °C) extended this period of stability to at least 24 h.

Because a mobile phase gradient was not necessary to separate lutein, lycopene, β-carotene, and phytoene in 23 min, working in an isocratic mode enabled time savings because no stabilization period was required between injections.

Various injection volumes of between 5 and 50 μL were tested. A volume of 10 μL guaranteed a satisfactory chromatographic

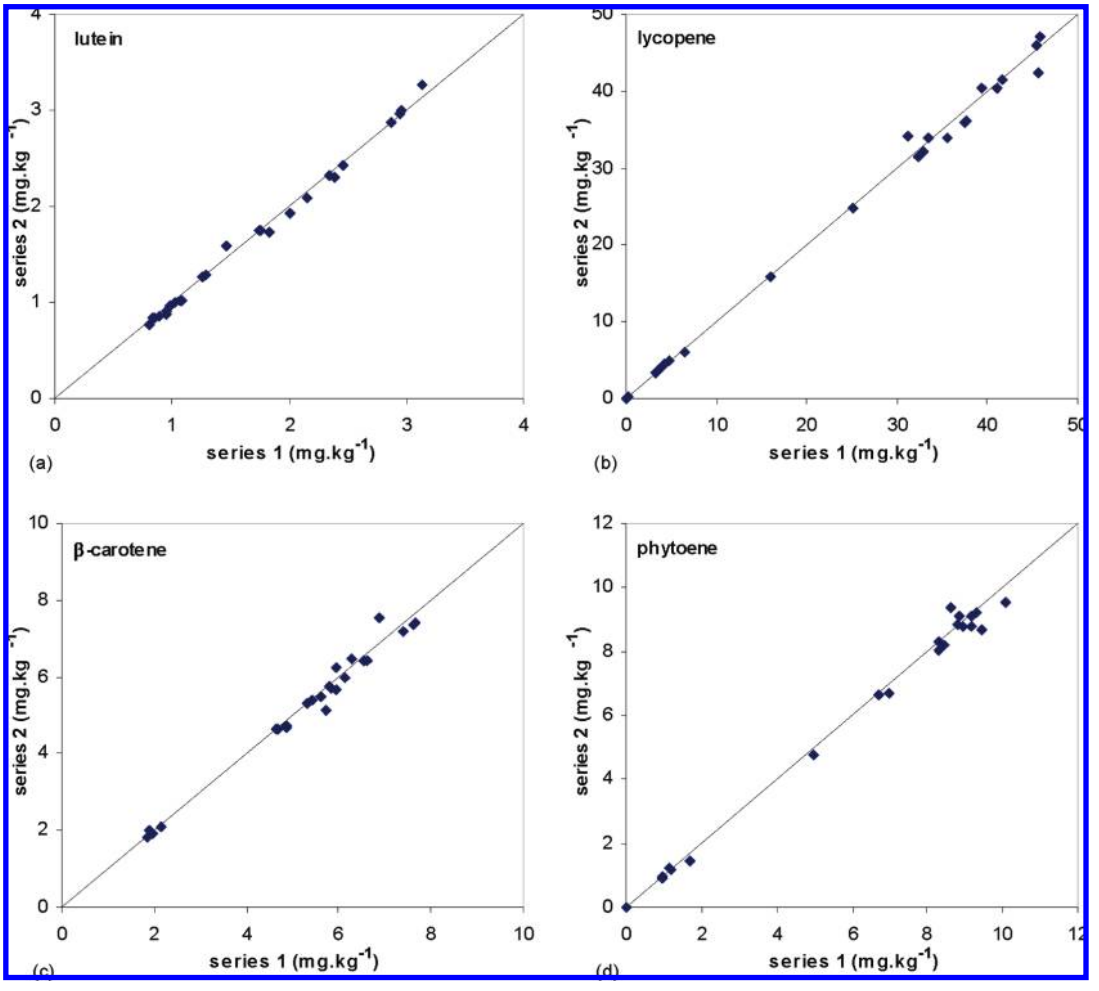

Figure 3. Precision of the micromethod. Double micromethod analyses of carotenoid concentrations in 25 tomato samples were made.

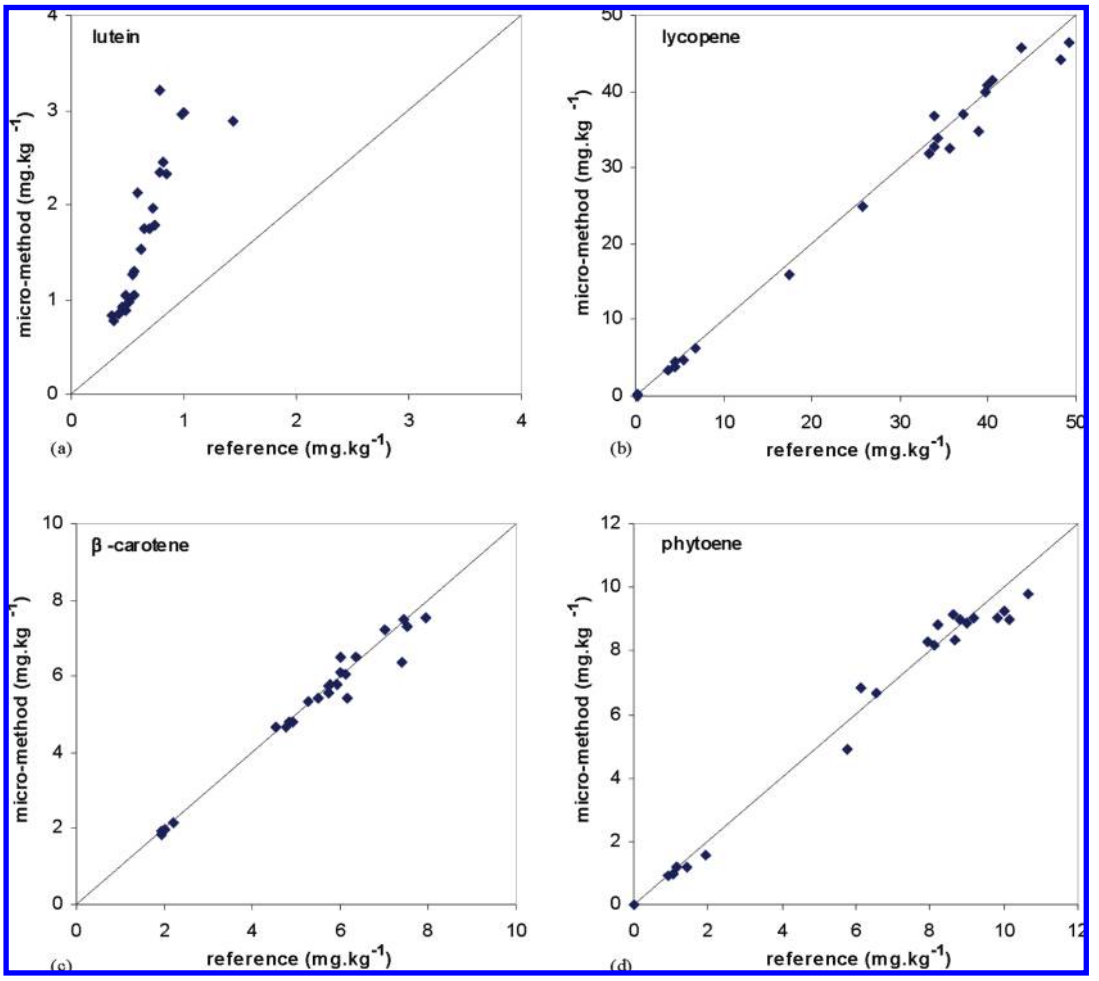

Figure 4. Accuracy of the micromethod: comparison between measurements of carotenoid concentrations in 25 tomato samples analyzed with the Fish modified method (reference) and the micromethod.

profile and quantification limit with our tomato extracts. By contrast, larger injection volumes affected peak separation because of the difference in chemical composition between the HPLC mobile phase and the analytical extract.

Precision. The results are shown in **Figure 3**. Both analytical series were very strongly correlated ($\rho > 0.95$), and no bias was detected ($P > 0.01$) whatever the carotenoid assayed (**Table 1**). Reproducibility values also demonstrated the precision of the micromethod, including for lycopene, its high R value (2.2013) being explained by the range of concentrations employed (0−50 mg kg^{-1}). To free ourselves of this variable, we thus calculated the RMSE of each of the series of measurements. Whatever the series, these RMSE values were between 0.03 and 0.06 mg kg^{-1} (or CVs of 2, 0.2, 1, and 1% for lutein, lycopene, β-carotene, and phytoene, respectively). This statistical test confirmed our view concerning the excellent precision of this micromethod, including for lycopene.

Accuracy. Whatever the carotenoid, the Spearman test (**Table 1** for **Figure 4**) demonstrated that the micromethod and the modified Fish method (reference) were significantly correlated ($\rho > 0.95$). As shown in **Figure 4**, no bias was detected with respect to lycopene, β-carotene, or phytoene ($P > 0.01$). The fact that these two very different extraction methods produced analogous results (the regression line could be assimilated to the bisector) was indicative of the accuracy of the micromethod when these three secondary metabolites were assayed. However, in the case of lutein, a very marked bias ($P < 0.0001$) was observed between the two methods. The much lower values obtained with the reference method could be explained by the presence of hydroxyl groups, which endowed lutein with greater polarity than the other three carotenoids. Thus, during extraction using the reference method, a proportion of lutein remained entrapped in the "water/acetone/ethanol" phase and was not extracted in the weakly polar n-hexane fraction, which was retained and then evaporated for the assay. This problem did not arise with the micromethod, because none of the organic solvents used (Hex/DCM/EA), with markedly different levels of polarity, was removed during extraction. The lack of any reference method did not allow us to assess the accuracy of lutein assays using the micromethod. However, the recovery rates close to 100% estimated by the addition of an internal standard (see paragraph below) suggested that the micromethod was accurate for all of the carotenoids, including lutein.

Linearity of the Response and Matrix Effect. Whatever the carotenoid and degree of ripeness, the linear regression coefficients close to 1 were indicative of the good linearity of the method. The signal measured was clearly proportional to the carotenoid concentration (**Figure 5**).

Efforts were made to determine the presence of a matrix effect by comparing the values obtained by internal and external calibration (**Figure 6**). There was an excellent correlation between the two series ($\rho = 1$), whatever the carotenoid and degree of tomato ripeness. The similarity of the results ($P > 0.9$) testified to the absence of any matrix interference affecting the extraction or assay of added carotenoids.

Another way to perform this test was to use an external calibration to measure the concentrations of each extract and

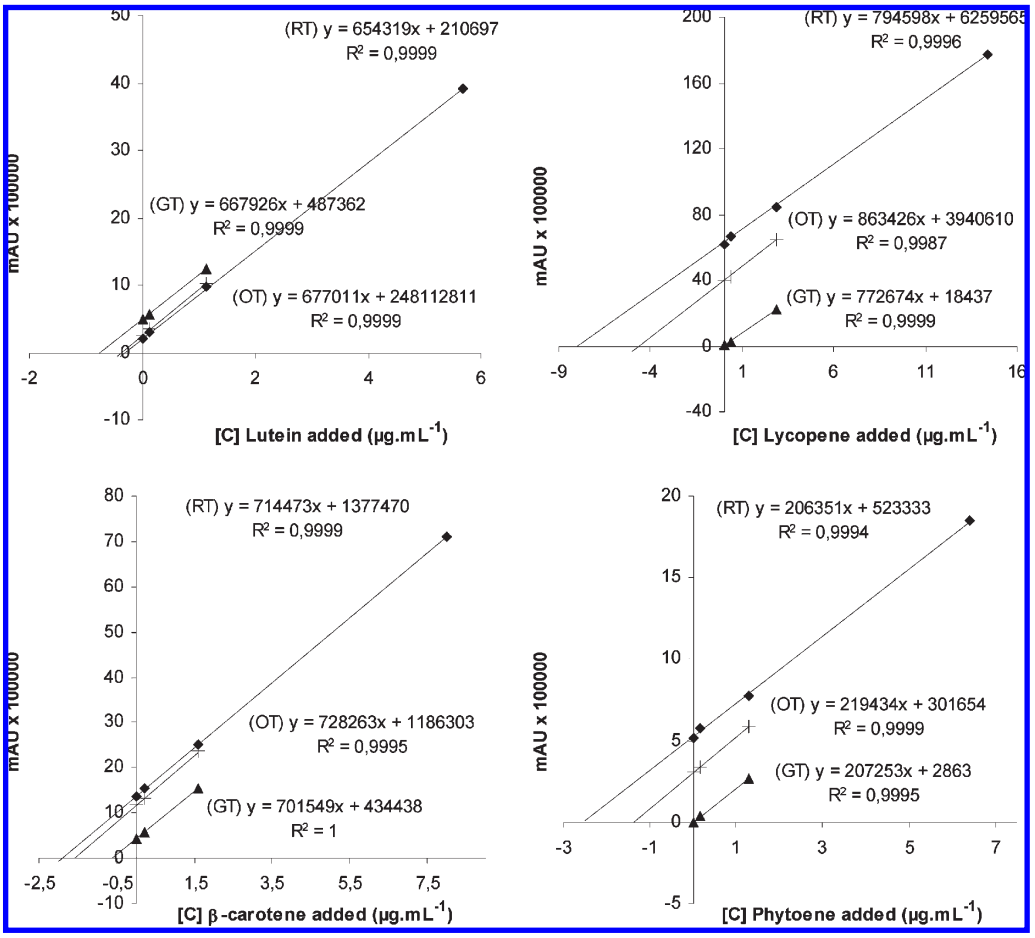

Figure 5. Internal calibration standard determination of carotenoids with micromethod in extracts at three tomato ripeness degrees. GT, ▲, green tomato; +, OT, orange tomato; ◆, RT, red tomato.

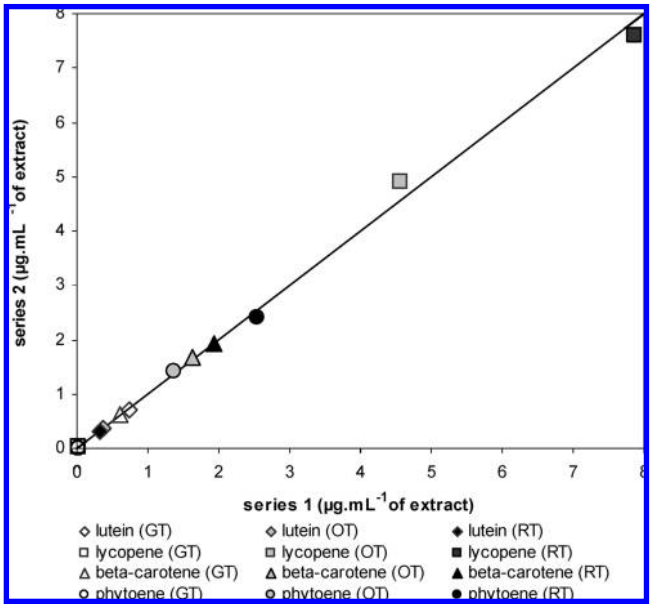

Figure 6. Comparison between measurements of carotenoid concentrations with internal (series 1) and external standards (series 2) in extracts. GT, green tomato; OT, orange tomato; RT, red tomato.

then deduct the quantities of standard additions. The same initial carotenoid concentrations were still obtained (data not shown), thus proving that 100% of the carotenoids added were extracted and analyzed.

These results were very important from a practical point of view because they validated the assay with an external calibration for the micromethod.

Robustness. To optimize a method, it is generally necessary to dissociate the different steps of the procedure over time. The stability of extracts thus ensures robustness, for example, by allowing for delays or the repetition of chromatographic assays and the automated processing of a large series of samples. The storage of extracts at −20 °C for 2 weeks did not significantly affect lycopene and phytoene concentrations ($P > 0.01$, data not shown), but slightly reduced the concentrations of β-carotene and lutein (mean of CV < 2%). Thus, this new method allows the storing of the extracts, which is relevant to optimize the planning of high numbers of samples.

We have succeeded in developing a miniaturized and rapid extraction method for the HPLC assay of four major carotenoids in tomato (lutein, lycopene, β-carotene, and phytoene). Because extraction is performed directly in a 2 mL Eppendorf tube, no glassware is necessary. For this reason, and particularly thanks to a considerably reduced volume of organic solvents when compared with numerous other methods used for carotenoid extraction (*28*, *38*), this rapid method (approximately 70 assays per day) is inexpensive and more environmentally friendly. In particular, it consumes 16 times less extraction solvents than the modified Fish method (*30*) (reference method). However, the most important aspect of this very small extraction volume (1250 μL) is that it is possible to remove the evaporation step (*9*, *23*, *38*), which is restrictive and quite lengthy and is normally necessary to concentrate the extract and achieve sufficient HPLC sensitivity.

Removal of the solvent evaporation step also means that potential degradation phenomena can be prevented, as carotenoids are photosensitive and easily oxidizable compounds (*39*, *40*). Some authors used BHT as an antioxidant to limit these phenomena (*30*), others did not (*23*, *28*). We did not feel this was necessary, insofar as the extraction of carotenoids was very rapid and did not include an evaporation step, thus limiting the risks of oxidation.

In terms of statistical analyses, the reliability of the micromethod was established. It appeared to be precise and accurate for the four carotenoids, the recovery rates of which were > 97%. The micromethod was linear for a very broad range of concentrations, whatever the carotenoid and degree of tomato ripeness. It would now be useful to determine whether this method for carotenoid analysis can also be applied to freeze-dried plant powders or to processed products.

The analytical extracts were satisfactorily conserved at −20 °C for a period of 15 days. In the absence of any proven matrix effect, it is possible to use external calibration. These observations endow this micromethod with considerable flexibility of use.

ABBREVIATIONS USED

NaCl, sodium chloride; Hex, *n*-hexane; DCM, dichloromethane; EA, ethyl acetate; Ac, acetone; EtOH, ethanol; UP water, ultrapure water; MSolv, mixture of EA:DCM:Hex (80:16:4, v/v/v); SS, stock solution; HPLC, high-performance liquid chromatography; DAD, diode array detector; LD, limit of detection; RMSE, root-mean-square error.

ACKNOWLEDGMENT

We thank Dr. F. Lescourret for assistance in the reprocessing of statistical data and V. Vhawken for revising the English of the manuscript.

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Received June 19, 2009. Revised manuscript received July 21, 2009. Accepted August 31, 2009.