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Interlaboratory studies of HPLC procedures for the analysis of carotenoids in foods

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This paper describes the use of a potential reference material in interlaboratory studies for the analysis of carotenoids in a mixed vegetable material. Seventeen European laboratories have carried out collaborative studies to assess the accuracy of HPLC procedures for the measurement of lutein, zeaxanthin, lycopene, α-carotene and β-carotene in a vegetable mix. The studies investigated possible problem areas including chromatographic systems, standardization of carotenoid stock solutions, extraction procedures and data handling. The results suggested that the effect of the chromatographic system is probably not a major variable, although some systems achieved a more discrete separation of carotenoid isomers than others. In the more experienced laboratories, variation in the standardization of the carotenoid solution was not thought to be a significant problem. However, there were greater variations for lycopene calibration and measurement. Preliminary conclusions from these studies suggested that the preparation of the carotenoid extract may account for about 13% of the overall variance of around 23%. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Vegetables and fruits are complex foods containing a wide variety of substances and several specific compounds which are widely believed to confer protective properties for human health. Since the carotenoids are amongst the most abundant micronutrients in vegetables and fruits, the need for accurate qualitative and quantitative data on these compounds has become increasingly important. The availability of a certified reference material (CRM) is essential to the development and harmonization of methods and their application to nutritional and clinical studies. However, before reference values can be assigned to a CRM, the accuracy of the methodology used for measurement must be demonstrated. This paper describes the development and evaluation of a candidate reference material and its use in interlaboratory studies for the analysis of carotenoids.

DEVELOPMENT AND EVALUATION OF A REFERENCE MATERIAL

Following an initial feasibility study, a candidate reference material (RM) was prepared. The selected vegetables were size reduced, mixed and pureed. Individual vegetable materials were selected for their content of one or two predominant carotenoids: sweetcorn for lutein and zeaxanthin, tomatoes for lycopene and carrots for α- and β-carotene. The puree was lyophilized, milled and packaged under nitrogen in foil laminate sachets and stored at −18°C. Immediately after packing, 20 sachets were taken at regular intervals throughout the complete batch for homogeneity testing. This was performed by comparing the within-sachet variation (or method repeatability) with the between-sachet variation. In addition to homogeneity testing, another series of sachets were maintained at temperatures between +37 and −40°C for periods of up to 3 years to assess stability during both short- and long-term storage. The former was designed to test for effects of adverse shipment conditions on carotenoids.

DESIGN OF INTERLABORATORY STUDIES

Prior to carrying out the studies it was necessary to: (1) assess the likely problems; (2) define the methodology; and (3) establish suitable protocols. The possible areas of variation were identified as follows.

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- Chromatography: what is the effect of differences in column and solvent systems used in different laboratories on carotenoid values? To what extent did the different systems achieve discrete separation of the carotenoids of interest and did the systems separate the individual carotenoids and their isomers?
- Standardization: what steps should be taken to reduce possible errors in the standardization of the carotenoid stock and working solutions?
- Extraction: what are the effects of differences in extraction procedures used in different laboratories, and data handling/quantification on carotenoid values?

Approach

It was considered impractical to use a common chromatographic system. Therefore all laboratories were asked to use their own procedure, supplying details of their methods and specimen chromatograms.

To assess the possible variation in spectrometer readings between laboratories, a spectrometer calibration solution together with a standard carotenoid solution were circulated for measurement. Throughout the study all laboratories were asked to use the same extinction coefficients and absorption maxima, together with a 'purity' check by HPLC when calculating concentrations of carotenoid stock solutions.

To determine differences in analysis, which might be caused by differences in standards, a sample extract was circulated to all laboratories for analysis using both circulated and in-house standards. To assess the effect of extraction, laboratories were asked to use a common extraction method alongside their in-house method. An internal standard was used to estimate possible losses during the extraction. All laboratories used a common data handling approach, which included using peak area rather than peak height measurements.

SECOND INTERLABORATORY STUDY ON CAROTENOIDS (1993–1994)

Aims

There were three main aims of the study. First, to carry out a calibration check of spectrometers using a common Holmium reference solution. Secondly, to measure the absorption and concentration of a circulated β-carotene solution. Thirdly, to compare the concentration of carotenoids in the RM using both common and 'in-house' extraction methods.

Materials supplied

Holmium wavelength calibration standard [Holmium perchlorate (15% w/v) in 10% perchloric acid)] was supplied by Sigma Chemical Co. (Poole, UK). β-Carotene was supplied as a N_2 dried solution, which when dissolved in 5 ml of hexane gave an absorbance of approximately 0.5 absorbance units at 450 nm. Two sachets of the RM were provided. All samples and calibrants were stored at −18°C on receipt.

Procedures

Wavelength and absorbance calibration
Spectrometers were zeroed at 450 nm using 10% perchloric acid solution (PCA). The absorption spectra of the Holmium calibration standard was scanned between 400 and 500 nm. The peak maxima (nm) at approximately 416, 451 and 485 nm were noted. Static absorbance measurements were carried out at 445, 446, 447, 448, 449, 450, 451, 452, 453, 454 and 455 nm.

Absorbance measurement on β-carotene solution
Hexane (5 ml) was added to the vial of dried β-carotene and was vortexed vigorously for 1 min to dissolve. The absorbance was measured at 450 nm in a 1-cm cell. A spectral scan (400–500 nm) was recorded and, if the indicated maximum absorbance was found to be different from 450 nm, the absorbance at this maximum was also recorded.

Purity test by HPLC
Hexane (5 ml) was added to the vial of β-carotene and the solution was prepared for chromatography using the normal 'in-house' procedure. The HPLC chromatogram was monitored at 450 nm, recording the total chromatographic area, the area of trans β-carotene, the area of total β-carotene (trans- + cis-isomers) or, where the isomers were not separated, the area of total β-carotene only. The purity of β-carotene was calculated as the area of the trans β-carotene (or total β-carotene) as a percentage of the total chromatographic area. The concentration of β-carotene solution (in a 5-ml volume) was calculated from the absorbance reading at 450 nm, using an extinction coefficient of 2592 ($E^{1\%}_{1cm}$) with correction for purity.

Measurement of concentration of circulated β-carotene solution using the 'in-house' β-carotene solution
In addition to the above, the chromatographic response of the 'in-house' β-carotene standard solution was measured and the concentration of the circulated 'test' β-carotene solution was calculated using 'in-house' standards. The same absorbance, extinction coefficient procedures and purity checks were made for the 'in-house' standard.

Calculation of concentration of standard solutions
The concentrations of individual 'in-house' carotenoid standard solutions were calculated using the absorbance and extinction coefficient data given in Table 1 and corrected for purities.

Measurement of the concentration of carotenoids in the RM
Duplicate analyses on one sachet of the RM were made using the 'in-house' procedure without saponification. The other sachet was analysed in duplicate using the common procedure. Some laboratories also carried out an additional analysis using their 'in-house' saponification procedure.

Analyses reported
Data were reported for the analysis of lutein, zeaxanthin and/or lutein + zeaxanthin (quantified against lutein), trans-lycopene, total lycopene (or total only), trans-α-carotene, total-α-carotene (or total only), trans-β-carotene, total β-carotene (or total only). All data were corrected for the recovery of an appropriate internal standard.

THIRD INTERLABORATORY STUDY ON CAROTENOIDS (1994–1995)

Aims

The aims of stage 1 were as follows. First, to carry out absorbance measurements on a solution of the RM extract. Secondly, to measure the concentration of carotenoids in the RM, using a circulated extract and carotenoid standards and compare with 'in-house' standards.

The aims of stage 2 were as follows. First, to compare the calculated concentration of 'in-house' carotenoid standard by analysis and quantification against a 'circulated' standard. Secondly, to measure the concentration of carotenoids in the RM using a circulated standard. Thirdly, to compare the common extraction and 'in-house' extraction procedures.

Materials supplied

The following materials were supplied for stage 1: a vial of a N_2 dried solution of the RM extract for absorbance reading; a vial of a N_2 dried solution of the RM extract for chromatographic analysis; a vial of a N_2 dried solution of a mixed carotenoid standard; and one spare vial of each.

The following materials were supplied for stage 2: a vial of N_2 dried solution of a mixed carotenoid standard; a vial of N_2 dried solution of an internal standard; two sachets of the RM; and spare vials of above.

Procedures

Stage 1

Absorbance reading The RM extract was dissolved and made to a 10 ml volume with hexane. The absorbance of this solution was measured spectrometrically at 450 nm in a 1-cm cell.

Measurement of the concentrations of carotenoids in the RM Dichloromethane (DCM) or tetrahydrofuran (THF) (0.5 ml) was added to the other vial containing RM extract and this was sonicated for 2 min (or vortexed for 30 s). It was then diluted to 5 ml with the mobile phase and an aliquot was injected onto the chromatographic column. Analysis was performed in duplicate. The 'in-house' standards were analysed according to the normal practice.

Stage 2

Preparation of carotenoid standard and internal standard DCM or THF (0.5 ml) was added to the vial of carotenoid standard, sonicated for 2 min (or vortexed for 30 s) and diluted to 5 ml with the mobile phase. THF (2 ml) was added to the vial of internal standard (echirerone) and sonicated for 2 min (or vortexed for 30 s).

Measurement of the concentration of carotenoids in the RM Analyses of duplicate extractions were carried out by all laboratories using the common procedure (Hart & Scott, 1995). Some laboratories also analysed another sachet using their 'in-house' procedure. All data were corrected for the recovery of the internal standard.

Table 1. Extinction coefficients of carotenoid standards

Standard	Solvent	Wavelength (nm)	Extinction coefficient ($E^{1\%}_{1cm}$)
Lutein	Ethanol	445	2550
Zeaxanthin	Ethanol	452	2480
Lycopene	Hexane	472	3450
α-Carotene	Hexane	444	2800
β-Carotene	Hexane	450	2592

Table 2. Stability of carotenoids in the mixed vegetable reference material stored at −18°C (μg/g)

Carotenoid	0–12 months[1,2]	18 months[1]	36 months[1]
Lutein	12.6	13.5	12.5
Zeaxanthin	9.5	11.5	10.7
Lycopene	11.7	11.6	11.4
α-Carotene	10.4	10.7	9.7
β-Carotene	22.1	24.6	22.0

[1]Mean of duplicate determinations.
[2]Mean of duplicate determinations after 0, 3, 6 and 12 months.

Analyses reported

Stage 1

Data were reported for lutein, zeaxanthin [and/or lutein + zeaxanthin (quantified against lutein)], trans-lycopene, total-lycopene (or total only), trans-α-carotene, total-α-carotene (or total only), trans-β-carotene, total β-carotene (or total only).

Stage 2

Data were reported as for stage 1. In addition, the concentrations of the individual 'in-house' stock carotenoid solutions were calculated using the absorbance and extinction coefficients as before. The calculated concentrations of the 'in-house' standards were compared by analysis and quantification against the circulated standard mixture.

Note: The second study and the first stage of study 3 were carried out using a vegetable mix prepared for IFR, stage 2 of study 3 was carried out using a mix prepared for the EU.

RESULTS AND DISCUSSION

Homogeneity

The within-sachet and between-sachet CVs (%) were 4.0, 4.8 (lutein), 3.9, 5.9 (zeaxanthin), 3.8, 8.9 (lycopene), 4.1, 7.9 (α-carotene) and 3.9, 7.4 (β-carotene). It was concluded that within the accepted level of analytical variation, no significant inhomogeneity was detectable.

Short-term stability

There was no significant losses for a period of up to 28 days over the temperature range +25 to −40°C. However there was a trend, particularly for lycopene, α- and β-carotene, to decline after 7 days at 37°C. It was concluded that during a period of transport of up to 7 days, all carotenoids would probably remain stable at temperatures of up to 37°C. As a precaution, the RM would be transported under cooled conditions.

Long-term storage

Sachets were stored at −18, −30 and −40°C for a period of up to 3 years. Results are shown in Table 2 for samples at −18°C only. No significant instability was detected for any of the carotenoids analysed. The carotenoids will be further monitored at regular intervals in the future.

Interlaboratory study

Spectrophotometer calibration with Holmium reference solution (stage 1)

The manufacturer's data indicated peak maxima at 416.0, 450.5 and 484.5 nm and an absorbance reading of 0.832 at 451 nm. Mean values of all participating laboratories ($n = 10$) showed peak maxima at 416.6, 451.3 and 485.2 nm. For readings between 445 and 455 nm ($n = 13$) seven laboratories recorded peak maxima at 451 nm of between 0.793 and 0.838, and a mean of 0.820. This agreed favourably with the manufacturer's value of 0.832. Of the other five laboratories, three also recorded maximum values at 451 nm, but figures ranged from 0.533 to 0.727; one laboratory recorded a peak maximum (0.791) at 452 nm, and one laboratory did not record a peak maximum over this range.

Absorbance and concentration of a circulated solution of β-carotene (stage 2)

Reported absorbancies ($n = 14$) of the β-carotene solution varied between 0.455 and 0.505 with a mean of 0.487 (CV = 3.4%). The two lowest values (0.455 and 0.457) were from laboratories which carried out this measurement a few weeks after the others, and may be a result of degradation of the sample. The average 'theoretical value' for total β-carotene concentration (calculated from the extinction coefficient and corrected for analytical purity) was 1.84 μg/ml. All values reported were ±6% of the mean. The mean value for total β-carotene measured against the 'in-house' standard was 1.85 μg/ml. Only one laboratory fell just outside ±10% of the mean.

Comparing the two stages, it is difficult to interpret some of the low data reported in stage 1. However, β-carotene has a relative broad spectrum around 451 nm, whereas the calibration solution peak was very sharp and this may in part account for some of the error. In general, the mean peak maxima (nm) values for the calibrating solution were well within acceptable limits of variation, and eight of the 11 laboratories recorded absorbance values within the range 0.791–0.820.

The agreement between laboratories for the comparison of the calculated and analysed concentration against 'in-house' β-carotene solutions was considered very acceptable. It would suggest, for β-carotene at least, errors due to spectrometer differences are likely to be small.

Measurement of the concentration of carotenoids in RM using common and 'in-house' methods (stage 3)

Results are summarized in Table 3. Twelve laboratories produced data using the common method, six also provided data using various 'in-house' methods. The mean data for the individual carotenoids were similar for both the common and the 'in-house' methods. The coefficient of variation was 24% for the common, and 17% for 'in-house', methods. Using the common method the largest variation was for total lycopene (40%), others ranged from 11% for total α-carotene to 30% for trans-lycopene. Four of the laboratories did not use, or correct for, the recovery of an internal standard. The mean recovery value for those laboratories using an internal standard was around 93%. There was an indication that for four of the six laboratories using their own methods the values were generally lower than the values from the same laboratories using the common method.

Third interlaboratory study

Absorbance measurement of solution of RM extract (stage 1)

In order to check that any variability in the analysis of

Table 3. Comparison of the concentration of carotenoids (μg/g) in mixed vegetable reference material analysed using the common extraction method and 'in-house' methods with 'in-house' carotenoid standards (second interlaboratory study)

Carotenoid	Common procedure			In-house procedure		
	n^1	Mean	CV (%)	n^1	Mean	CV (%)
Lutein	9	10.6	27	4	11.2	31
Zeaxanthin	9	9.0	22	4	8.2	23
Lutein + zeaxanthin	9	17.7	19	4	18.4	21
Trans-lycopene	8	8.6	30	2	9.9	11
Total lycopene	11	10.2	40	5	9.2	13
Trans-α-carotene	6	8.1	23	2	9.4	9
Total α-carotene	8	9.3	11	5	9.3	30
Trans-β-carotene	8	17.9	23	2	21.2	3
Total β-carotene	11	20.9	19	5	19.7	12
Mean CV (%)			24			17

[1] n = number of laboratories.

Table 4. Concentration of carotenoids (μg/g) in a circulated extract of the mixed vegetable reference material quantified using circulated and 'in-house' standards (third interlaboratory study)

Carotenoid	Circulated standards			In-house standards		
	n^1	Mean	CV (%)	n^1	Mean	CV (%)
Lutein	9	10.0	7	9	9.7	11
Zeaxanthin	9	8.1	14	9	7.7	19
Lutein + zeaxanthin	14	18.2	6	13	18.7	19
Trans-lycopene	10	8.8	13	10	8.6	18
Total lycopene	13	10.9	24	13	11.0	32
Trans-α-carotene	7	9.0	6	6	8.7	8
Total α-carotene	12	9.2	7	11	9.0	11
Trans-α-carotene	10	19.7	5	10	19.6	7
Total β-carotene	12	21.2	6	12	20.9	10
Mean CV (%)			10			15

[1] n = number of laboratories.

the RM was not due to variation in the extracted sample, the laboratories were asked to measure the absorbance of a solution of the extract. The range of results ($n = 14$) was 0.525–0.600 (mean 0.573) with a CV of 3.6%. Excluding two lower values of 0.525 and 0.536, the range was 0.560–0.600 (mean 0.580) and a CV of 1.8%. This would suggest that any variation in the analysis of carotenoid concentrations would not be due to variation in the circulated extract.

The second part of stage 1 was to measure the concentration of carotenoids in a circulated extract of vegetable mix quantified using both a circulated standard and 'in-house' standards (Table 4). This was used to assess the degree of variation due to 'in-house' standards. The mean values for the individual carotenoids were similar, however, the CV with the circulated standard was 10% compared to 15% with 'in-house' standards. In comparison to the earlier study, the CV for extracts prepared by the common procedure and quantified using 'in-house' standards was 24%. Again the largest variation was found for total lycopene. Bearing in mind that possible statistical outliers have not been excluded, a CV of around 10% for this type of exercise is considered very acceptable. Whilst the variation using the 'in-house' standards was higher at 15%, it is again considered acceptable. As might have been expected the variation is reduced for a circulated extract compared to extracts prepared in each laboratory using a common method.

Comparison of 'in-house' and circulated carotenoid standards (stage 2)

In order to assess the degree of variation due to possible errors in the measurement of 'in house standards', a comparison was made between the calculated concentration of 'in-house' standards with that by analysis against a circulated standard (Table 5). If all 'in-house' standards were comparable then it would be expected that the concentration of the 'in-house' value expressed as a percentage of the analysed value would be similar. It can be seen that whilst much of the data were within acceptable limits, that for lycopene was very variable.

The second part of stage 2 was to compare common and 'in-house' extraction using a common standard (Table 6). Previous analyses have demonstrated a similar CV between common and 'in-house' procedures using 'in-house' standards (about 20%) This variation was reduced to 10% using a circulated extract and standard. With a circulated extract and 'in-house' standards the variation was 15%. It has also been shown that the accuracy of the 'in-house' standard concentration may in some laboratories be a significant source of variation, particularly for lycopene. The variation of 21% using the common extraction method was similar to the 24% using the common method and 'in-house' standards (second study). However, the 34% variation with the 'in-house' extraction method was higher than

Table 5. Concentrations of 'in-house' carotenoid standards (calculated from absorbance readings and purity) were compared to the concentrations after analysis against a circulated standard (third interlaboratory study)[1]

Laboratory code No.	Lutein	Lycopene	α-Carotene	β-Carotene
3	98	93	90	95
4	95	95	91	89
5	93	90	94	99
6	93	100	108	104
7	87	72	96	87
11	98	76	88	86
14	—	88	98	100
15	83	217	100	117
16	88	69	77	76
17	—	—	98	95

[1]Concentration expressed as a percentage of the calculated value.

Table 6. Concentration (μg/g) of carotenoids in the mixed vegetable reference material analysed using common and 'in-house' methods, and common circulated carotenoid standards (third interlaboratory study)

	Common procedure			In-house procedure		
	n[1]	Mean	CV (%)	n[1]	Mean	CV (%)
Lutein	9	12.5	11	5	10.9	40
Zeaxanthin	9	11.0	15	5	8.7	41
Lutein + zeaxanthin	11	22.9	13	7	20.4	34
Trans-lycopene	9	13.9	33	5	13.8	47
Total lycopene	10	15.6	29	6	14.4	46
Trans-α-carotene	7	9.4	22	5	9.6	23
Total α-carotene	9	10.3	19	5	9.5	25
Trans-α-carotene	9	22.3	24	5	22.3	28
Total β-carotene	10	25.6	20	6	25.2	26
Mean CV (%)			21			34

[1]n = number of laboratories.

the 17% variation of the same analyses with the 'in-house' standards (second study).

CONCLUSIONS

First, the fact that with a circulated extract and standard a CV of 10% or less is obtained, the effect of the chromatographic system is probably not a major variable in measuring the carotenoid concentrations.

Secondly, the standardization of the carotenoid stock solution would not appear to be a significant problem in the more experienced laboratories with a CV of > 10%. However, there were indications of a higher variation particularly for lycopene which may, in part, account for the larger variability of lycopene results in this material.

Thirdly, the variation using the common method of extraction with either circulated or 'in-house' standards is 23%.

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