Intralaboratory assessment of analysts' proficiency for carotenoid analysis using a certified reference material

Avaliação intralaboratorial do desempenho de analistas na análise de carotenoides utilizando material de referência certificado

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ABSTRACT

Carotenoid analysis is inherently challenging, requiring the analysts' expertise and attention to many details. To guarantee the reliability of carotenoid data generated in our laboratory, aside from method development, optimization and validation, periodic evaluation of the analysts' performance is carried out. This paper reports the results obtained in one of our evaluations, using a certified reference material. Five analysts with varying experience in carotenoid analysis participated. The same liquid chromatograph and standard curves were used, restricting the evaluation to the analysts' performance. The HPLC method consisted of extraction with acetone, partition to petroleum ether, saponification with 10 % methanolic KOH, washing with water, concentrating in a rotary evaporator, drying with nitrogen, dissolving in acetone, separation, identification and quantification. The z-score for each carotenoid was calculated. There was very good agreement in terms of the carotenes and β-cryptoxanthin for the five analysts. For lutein and zeaxanthin, the analyst with little experience in carotenoid analysis obtained lower values, but the z-scores were still satisfactory. One analyst who had experience only with carotene analysis also got lower concentrations for the xanthophylls. This was due to the fact that ethyl ether was not used in partitioning the carotenoids from the extracting solvent to petroleum ether.

Keywords. analysts' proficiency, carotenoid analysis, certified reference material, intralaboratory evaluation, accuracy, precision.

RESUMO

A análise de carotenoides é um desafio inerente, pois requer experiência e atenção dos analistas para vários detalhes. Para garantir a confiabilidade dos resultados, nosso laboratório faz avaliações periódicas do desempenho do método analítico e dos analistas. Este trabalho apresenta os resultados de uma das avaliações utilizando-se material de referência certificado. Participaram do estudo cinco analistas com diferentes tempos de experiência em análise de carotenoides. Foi utilizado o mesmo cromatógrafo líquido de alta eficiência e a mesma curva analítica para restringir a avaliação apenas ao desempenho do analista. A metodologia consistiu na extração com acetona, partição para éter de petróleo, saponificação com 10 % KOH metanólico, lavagem com água, concentração em roto-evaporador, secagem com nitrogênio, dissolução em acetona, separação, identificação e quantificação. Os z-scores foram calculados para cada carotenoides. Houve boa concordância para carotenos e β-criptoxantina para todos os analistas. Para luteína e zeaxantina, o analista com pouca experiência obteve valores menores, mas os z-scores ainda foram satisfatórios. Um analista com experiência apenas em análise de carotenos também obteve concentrações menores para xantofilas; e esses resultados ocorreram pelo fato de não utilizar éter etílico na partição dos carotenoides do solvente de extração para o éter de petróleo.

Palavras-chave. proficiência de analistas, análise de carotenoides, material de referência certificado, avaliação intralaboratorial, precisão, exatidão.

INTRODUCTION

Carotenoids are among the food constituents of major interest in relation to human health. Aside from the well-known vitamin A activity, other biological activities have been attributed to these compounds such as reduction of the risk of developing certain types of cancer, cardiovascular diseases, macular degeneration and cataract[1-3]. These health-promoting actions are widely attributed to the carotenoid's antioxidant activity, by its ability to sequester singlet oxygen and react with free radicals[4-6]. However, other modes of action have been cited: modulation of carcinogen metabolism, regulation of cell growth, inhibition of cell proliferation, enhancement of cellular differentiation, stimulation of cell-to-cell communication, enhancement of the immune system and photoprotection[1,3,7].

Due to its role in human health and as natural pigments, the need for accurate qualitative and quantitative data on food carotenoids is widely recognized. Because the carotenoids differ in their health-promoting efficacy and coloring property, separation, conclusive identification and individual quantification are necessary. This analysis is inherently difficult, requiring the analyst's expertise, experience and attention to many details. Thus, aside from representative sampling and method validation, the analyst's proficiency should be verified.

Access to interlaboratory evaluation of method and analyst performance, although the preferred procedure, is very limited. Intralaboratory evaluation is needed and standardized protocols have been established[8]. Method accuracy can be verified in the laboratory by recovery tests, method comparison and analysis of a certified reference material. Spiked analytes do not behave in the same way as the endogenous compound, thus the validity of recovery studies of analytes like carotenoids, which are naturally well protected by membranes and cell walls and can be linked to other components in food samples, is questionable. Obtaining comparable results with methods of differing principles/procedure indicate good reliability of the methods. Analysis of a certified reference material is the preferred procedure for verifying method and analyst capability for obtaining accurate results. The analytical process from extraction to instrumental measurement can be assessed. For carotenoids, two certified reference materials have been developed: Community Bureau of Reference BCR 485 (freeze-dried mixed vegetables) and NIST SRM 2383 (baby food composite)[9,10].

To ensure the reliability of carotenoid data generated in our laboratory, aside from method development, optimization and validation, periodic evaluation of the analysts' performance is carried out. This paper reports the results of one of their evaluations, using a certified reference material.

MATERIAL AND METHODS

Experimental

Five analysts, with experience on carotenoid analysis varying from one month to 4 years and one analyst with experience only with carotenes, participated. NIST standard reference material 2383 baby food composite was used. The same HPLC equipment and standard curves were employed so that the evaluation was restricted to the analyst's performance in preparing the extract for HPLC analysis. Analysis was done in triplicate by each analyst.

Carotenoid analysis

The carotenoids were determined using a method developed and evaluated for leafy vegetables by Kimura and Rodriguez-Amaya[11] and validated using a lyophilized vegetable mix certified reference material by Kimura et al[12].

About 3 g of the homogeneous SRM was weighed and the sample was ground with acetone and Hyflosupercel with a mortar and pestle. The extract was filtered through a sintered glass funnel. Extraction and filtration were repeated until the residue turned colorless (usually 3 times). The carotenoids were transferred to about 50 mL petroleum ether:ethyl ether (2:1) by partition, in a separatory funnel with the addition of water. Saponification of the extract after partition to petroleum ether:ethyl ether was carried out by adding equal volume of 10 % methanolic KOH and 0.1 % butylated hydroxytoluene to the extract and, after flushing with nitrogen, leaving the stoppered flask in the dark at room temperature overnight (about 16 h)[13]. The saponified extract was then washed five times with water, dried with anhydrous sodium sulfate, concentrated in a rotary evaporator, and brought to dryness under nitrogen. Immediately before injection, the carotenoids were dissolved in 2 mL HPLC grade acetone and filtered through a 0.22 μm PTFE syringe filter; a 10 μL aliquot was injected into the liquid chromatograph. All the necessary

precautions were taken to avoid alterations or losses of the carotenoids (e.g. exclusion of oxygen, protection from light, avoiding high temperature and contact with acids, use of high-purity, peroxide-free solvents, completion of the analysis within the shortest possible time) and other errors during analysis[14].

The HPLC system consisted of a Waters separation module, model 2690 (Waters Corp., Milford, Mass., U.S.A.), equipped with quaternary pump, autosampler injector, degasser and a photodiode array detector (model 996), controlled by a Millenium workstation (version 2010). Detection was at the wavelengths of maximum absorption (max plot).

The column was monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 x 150 mm. The mobile phase consisted of acetonitrile (containing 0.05 % of triethylamine), methanol and ethyl acetate, used at a flow rate of 0.5 mL/min. A concave gradient (curve 10) was applied from 95:5:0 to 60:20:20 in 20 min, maintaining this proportion until the end of the run. Reequilibration took 15 min.

Identification of the carotenoids was done according to Rodriguez-Amaya[14], with the combined use of retention time, co-chromatography with standards, and the visible absorption spectra. Quantification was by external standardization. Standards were isolated from roquette leaves (lutein), maize (zeaxanthin), papaya (β-cryptoxanthin), watermelon (lycopene) and carrot (α-carotene and β-carotene) by open column chromatography on MgO:Hyflosupercel (1:1, activated for 4 h at 110 ºC) packed to a height of 20 cm in 2.5 cm i.d. x 30 cm glass column[12]. The columns were developed with increasing amounts of ethyl ether and acetone in petroleum ether; the purity of the carotenoid isolates was monitored by HPLC. The mean purity of the standards was 97 % for lutein, 97 % for zeaxanthin, 93 % for β-cryptoxanthin, 96 % for lycopene, 93 % for α-carotene and 96 % for β-carotene. The concentrations of the standard solutions were corrected accordingly.

The standard curves were constructed by the injection in triplicate of standard solutions at five different concentrations. The curves passed through the origin and were linear at the concentration range expected of the samples, the coefficients of correlation obtained being higher than 0.99.

Calculation of the z-score

The z-score was calculated for each carotenoid for each analyst, as follows: z-score = $(x - \mu)/\sigma$ (with x being the individual laboratory result, μ the NIST mean value, and σ the NIST standard deviation). This calculation of the z-score is widely used in laboratory proficiency testing programs. A z-score may be either positive or negative, reflecting either a higher or lower result compared to the assigned value. Generally a z-score less than or equal to 2.0 is considered satisfactory, between 2.0 and 3.0 questionable, and greater than 3.0 unsatisfactory[8].

RESULTS AND DISCUSSION

The chromatogram shows that the HPLC column provided baseline separation of the six carotenoids in the SRM baby food composite. Table 1 presents the carotenoid concentrations obtained by the analysts and the certified or reference values (means and standard deviations) furnished by NIST. It can be observed that all the analysts obtained good values for the carotenes and β-cryptoxanthin. For the xanthophylls, the analyst with little experience and the one who had experience only with carotenes, obtained low values for lutein and zeaxanthin.

Although the concentrations obtained by the analyst with little experience in carotenoid analysis were low for lutein and zeaxanthin, the z-scores were satisfactory (z-score<2) (Figure 1). The analyst who had experience only with carotenes obtained results which was questionable for lutein (2<z-score<3) and was not satisfactory for zeaxanthin (z-score>3). The other analysts (more than one year of experience in carotenoid analysis) obtained good results for all carotenoids (z-scores less than 1).

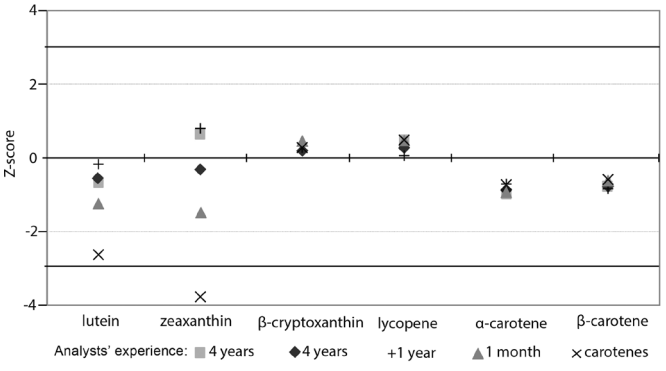

Figure 1. Z-scores for lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene. Solid lines indicate ±3.0 z-score and dashed lines indicate ±2.0 z-score

Reevaluating the analytical results of the analyst who had a z-score greater than 3.0, it was discovered

Table 1. Means and standard deviations of carotenoid concentrations obtained by the analysts, means and standard deviations of certified or reference values furnished by NIST

Experience of analyst	Carotenoid concentration (mg/g)[a]					
	Lutein	Zeaxanthin	β-Cryptoxanthin	Lycopene	α-Carotene	β-Carotene
4 years	0.94 ± 0.07	0.95 ± 0.02	1.53 ± 0.08	7.70 ± 0.14	0.62 ± 0.02	2.64 ± 0.06
4 years	0.97 ± 0.03	0.83 ± 0.04	1.44 ± 0.01	7.56 ± 0.20	0.64 ± 0.01	2.63 ± 0.06
1 year	1.10 ± 0.06	0.97 ± 0.05	1.48 ± 0.02	7.07 ± 0.27	0.67 ± 0.01	2.59 ± 0.08
1 month	0.72 ± 0.09	0.65 ± 0.06	1.52 ± 0.03	7.70 ± 0.29	0.64 ± 0.00	2.72 ± 0.09
Carotenes only	0.30 ± 0.02	0.33 ± 0.01	1.46 ± 0.03	7.73 ± 0.18	0.67 ± 0.03	2.74 ± 0.12
NIST values	1.16 ± 0.33	0.86 ± 0.14	1.38 ± 0.31	7.00 ± 1.5	0.85 ± 0.24	3.12 ± 0.63

[a] mean ± standard deviation of triplicate analyses

that the low levels were due to the fact that she did not utilize ethyl ether (together with petroleum ether) in the partition step. Petroleum ether is commonly used pure for analysis of carotenes. Ethyl ether makes the ether layer more polar, avoiding the loss of xanthophylls (more polar than the carotenes because of the presence of hydroxyl groups) to the subsequently discarded water phase. The saponification step might have also influenced the results. Necessary to hydrolyze carotenol esters, saponification is error prone and this analyst did not have experience in this step either. Repeating the analysis with the addition of ethyl ether, the results obtained were satisfactory for both lutein and zeaxanthin, the z-scores being less than 2.0, 1.58 for lutein and 1.86 for zeaxanthin.

The importance of experience can also be perceived in terms of precision. The analyst with only one month experience obtained greater standard deviations, particularly in terms of lutein and zeaxanthin, greater than those of the analyst with experience only with carotenes.

Scott et al[15] carried out an interlaboratory study with the participation of 17 European laboratories, using a candidate reference material (lyophilized mixed vegetables). The results indicated that the HPLC systems were not responsible for variations in the analytical data obtained nor was the preparation of the standard solutions a significant problem in the more experienced laboratories. They concluded that the preparation of the extract was the principal factor responsible for the variation of results. This conclusion is reaffirmed in the present study, in which the same HPLC system and standard curves were used by all participating analysts.

Phillips et al[10] reported the results of the USDA's National Food and Nutrient Analysis Program for a total of 2554 values obtained by nine laboratories for 259 certified or reference concentrations of 26 certified reference materials. For carotenoids, more than 20 % of z-scores were outside ± 3.0, demonstrating the difficulty in measuring these analytes in foods.

CONCLUSION

The intralaboratory evaluation of the analyst's performance in determining carotenoids, using a certified reference material, reinforced the importance of the preparation of the carotenoid extract for HPLC analysis and demonstrated once again the inherent difficulty of carotenoid analysis. Even with a previously validated method, the experience of the analyst was a decisive factor in obtaining good results. Evaluation of the analyts' performance is fundamental to ensuring the reliability of analytical results.

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REFERENCES

1. Olson JA. Carotenoids and human health. Arch Latinoam Nutr.1999;49:7-11.
2. Tapiero H, Townsend DM, Tew KD. The role of carotenoids in the prevention of human pathologies. Biomed Pharmacother. 2004;58:100-10.
3. Krinsky NI, Johnson EJ. Carotenoid actions and their relation to health and disease. Mol Aspects Med. 2005;26:459-516.
4. Young AJ, Lowe GM. Antioxidant and prooxidant properties of carotenoids. Arch Biochem Biophys. 2001;385:20-7.
5. Stahl W, Sies H. Antioxidant activity of carotenoids. Mol Aspects Med. 2003;24:345-51.
6. Kiokias S, Gordon MH. Antioxidant properties of carotenoids in vitro and in vivo. Food Rev Int. 2004;20:99-121.
7. Stahl W, Ale-Agha N, Polidori MC. Non-antioxidant properties of carotenoids. Biol Chem. 2002;383:553-8.

8. Thompson M, Ellison SLR, Wood R. The international Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories. Pure Appl Chem. 2006;78:145-96.

9. Sharpless KE, Margolis S, Thomas JB. Determination of vitamins in food-matrix Standard Reference Materials. J Chromatogr. 2000; 881:171-81.

10. Phillips KM, Wolf WR, Patterson KY, Sharpless KE, Holden JM. Reference materials to evaluate measurement systems for the nutrient composition of foods: results from USDA's National Food and Nutrient Analysis Program (NFNAP). Anal Biochem. 2007;389:219–29.

11. Kimura M, Rodriguez-Amaya DB. A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. Food Chem. 2002;78:389-98.

12. Kimura M, Kobori CN, Rodriguez-Amaya DB, Nestel P. Screening and HPLC methods for carotenoids in sweetpotato, cassava and maize for plant breeding trials. Food Chem. 2007;100:1734-46.

13. Kimura M, Rodriguez-Amaya DB, Godoy HT. Assessment of the saponification step in the quantitative determination of carotenoids and provitamin A. Food Chem. 1990;35:187-95.

14. Rodriguez-Amaya DB. A guide to carotenoid analysis in foods. Washington, D. C.: International Life Sciences Institute Press; 1999.

15. Scott KJ, Finglas PM, Seale R, Hart DJ, de Froidmont-Görtz I. Interlaboratory studies of HPLC procedures for the analysis of carotenoids in foods. Food Chem. 1996;57:85-90.