Interlaboratory exercise for the analysis of carotenoids and related compounds in dried mango fruit (*Mangifera indica* L.)

HIGHLIGHTS

- State-of-the-art analytical techniques are applied on the analysis of carotenoids from mango peel.
- MS, UV-Vis and DAD Detection by absorbance (UV-Vis, DAD) and mass spectrometry allow a satisfactory identification and quantification of α and β-carotene, (9Z)-β-carotene, β-cryptoxanthin, and zeaxanthin
- Analytical quantification of lutein demands extra efforts, as no satisfactory agreement has been reached.
- The development of a Certified Reference Material is proposed based on the analytical results

ABSTRACT

An interlaboratory comparison was done for the analysis of carotenoids in freeze-dried mango. The study was performed from July 2018 to September 2018. Mango fruit was freeze-dried, homogenized, and packaged under vacuum conditions in portions of 6 g (test sample). Two test samples were sent to the participating laboratories for analysis. Laboratory results were rated using Z-scores in accordance with ISO 13528 and ISO 17043. The standard deviation for proficiency assessment (also called target standard deviation) was determined using a modified Horwitz function and varied between 10 and 25%, depending on the analyte. Out of 14 laboratories from 10 different countries, 9 laboratories (64%) obtained a satisfactory performance ($Z \leq 2$) for the analysis of β-carotene. While for 7 laboratories that analyzed α-carotene, (9*Z*)-β-carotene, β-cryptoxanthin, and zeaxanthin, 4 laboratories (57%) obtained a satisfactory performance. However, only 2 laboratories out of 7 (29%) obtained a satisfactory performance for lutein. Based on the comparability of the analytical results, this study concludes that freeze-dried mango pulp can be used as a reference material for the analysis of α and β-carotene, (9*Z*)-β-carotene, β-cryptoxanthin, and zeaxanthin by applying different analytical procedures for their extraction and quantification.

Keywords: Reference material, isomers, interlaboratory analysis, lipophilic compounds, liquid chromatography, quality assurance

Abbreviations:

BHT: butylated hydroxytoluene; IS: internal standard; CRM: certified reference material.

1. INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most traded and consumed tropical fruits, with a worldwide production of around 45 million metric tons in 2019. The increasing trends in mango consumption are associated with its flavor and its nutritional content, since it is a rich source of carbohydrates, organic acids, vitamins, phenolic compounds, and carotenoids (Khalid et al., 2020). Of these compounds, particular interest has been given to carotenoids, a versatile group of bioactives that actively participate in the risk reduction of various adverse conditions (*e.g.*, cancer, cardiovascular diseases, metabolic disorders, eye, skin and bone diseases). Additionally, it has been shown that cognition, early development, immunity, among other beneficial activities, are modulated by the intake of carotenoids.

Fruit and vegetables are the primary sources of nutrients and other compounds that, isolated or in combinations, positively affect human health. In this sense, fruits are the richest sources of carotenoids, and their daily intake provides adequate concentrations of these health-enhancing compounds. As recommended by the WHO, a daily intake of 400 g of fruits and vegetables to seize their beneficial effects. Besides fruits and vegetables consumption, dietary supplements intake accompanies a healthy lifestyle. Worldwide, around 4 million deaths were attributed to insufficient intake of fruit and vegetables in 2017 (FAO, 2020). With these premises, efforts have been put together to develop supplements, functional foods, nutraceuticals or nutricosmetics. The socioeconomic importance of these compounds is therefore undeniable (Meléndez-Martínez et al., 2021a, 2021b).

Carotenoids are classified as carotenes and xanthophylls. Carotenes are exclusively formed by carbon and hydrogen (hydrocarbon chains), while xanthophylls have oxygen in the molecule, which can form oxygenated functional groups (*e.g.*, epoxy, ketone, carbonyl) and be present in

45 the matrix as free carotenoids or esterified with fatty acids. Taking into account these characteristics, combinations of polar and non-polar
46 solvents are suitable for the extraction of carotenoids in low-moisture samples. Moreover, for analytical purposes, both polar and non-polar
47 organic solvents are synergistically used to extract different carotenoids from the same biomass in relatively short times. The selection of the
48 solvent and subsequent clean-up process is a key step for the correct characterization of the carotenoid composition of a plant matrix to avoid
49 underestimating their composition and concentration (Saini and Keum, 2018).

50 After extraction, the separation of the compounds is fundamental for their identification and characterization. In this context, high
51 performance liquid chromatography (HPLC) with C_{18} chromatography columns have been widely applied to separate carotenoids. However,
52 UHPLC systems with C_{18} columns with smaller particle sizes (sub 2-μm) offer a better separation and shorter run times. In addition, triacontyl-
53 bonded (C_{30}) columns, with higher hydrophobicity as compared with the $C_{18,}$ have improved the resolution for carotenoid separation. However,
54 longer run times are required (Giuffrida et al., 2020, 2018).

55 Coupling these devices to different types of detectors (absorbance by UV-Vis, mass spectrometry (MS)) allows both qualitative and
56 quantitative determination of carotenoids. UV-Vis has been widely applied for the identification of carotenoids; however, since molecules with
57 the same molecular structure (isomers), *i.e.*, α-cryptoxanthin and zeinoxanthin can co-elute, an overlapping signal might be obtained. In contrast,
58 MS instruments overcome spectral interferences and detect ions according to their mass-to-charge ratio (*m/z*). The information obtained on the
59 molecular structure depends upon the molecular mass of the analyte and the fragmentation pattern. Although the last is influenced by the mobile
60 phase and the ionization technique, studies have shown that different ionization techniques obtain specific carotenoids fragments. This approach

61 can be helpful to determine carotenoids with the same molecular mass but different fragmentation patterns, *e.g.*, geometrical and structural
62 isomers (Amorim-Carrilho et al., 2014; Hoffmann and Stroobant, 2007).

63 Even if different techniques have been applied to characterize plant matrices in terms of carotenoid content, several steps in the analytical
64 process, including sampling, sample pretreatment, extraction, choice of mobile phases, and the choice of the separation column, will influence
65 the outcomes. Moreover, variations during sample handling (*i.e.*, preparation and extraction or inconsistencies through analyte recovery and
66 injection variability) can be overcome by using an internal standard (IS), which is a compound with a similar structure to the analytes present in
67 the extract. Typically, it is carried out by dividing the analyte peak response (height or area) in the sample by internal standard peak response in
68 the sample corrected for theoretical internal standard peak response (Craft and Furr, 2018). Additionally, antioxidants (*e.g.*, BHT, butylated
69 hydroxytoluene (BHT), pyrogallol, or ascorbic acid) may be added to prevent oxidation or isomerization of the target components. Similarly,
70 sodium bicarbonate is added when the extract is performed in acidic fruits as a mild neutralizing agent to prevent epoxy to furanoid carotenoid
71 rearrangement (Saini and Keum, 2018).

72 The analytical performance of a laboratory can be evaluated by the execution of a ring trial in different laboratories analysing homogeneous
73 materials. These interlaboratory exercises form an integral part of the quality assurance and control programmes, in addition to initial and
74 ongoing in-house method validation (FAO and AGES, 2015). For accredited laboratories following ISO 17025, participation in proficiency tests
75 is a requirement of the quality management system.

Delivering reliable data is the basis for any laboratory, and this is achieved after performing strict quality control and quality assurance protocols to guarantee the production of traceable information with standard protocols for acquisition of data. To this end, several studies reported the interlaboratory analysis of carotenoids in food products. Luterotti et al. (2013) identified the key uncertainty sources (*e.g.*, protocols) and applied statistical analysis to indicate the conditions under which the biases between the results cannot be identified in an intra-and interlaboratory spectrophotometric and HPLC analysis of lycopene, β-carotene and total carotenoids in tomato products and yellow maize flours/grits. The authors concluded that ambiguous outcomes on carotenoids content could be obtained since high biases between carotenoids contents remain unidentified (significant *t*-test), accompanied by good R^2 correlation. Eriksen et al. (2017) developed an UHPLC-DAD-based method to analyze significant carotenoids in spinach, serum, chylomicrons, and faeces, which was further validated by an interlaboratory assessment. This external assessment showed no significant differences in the content of lutein or β-carotene on the samples. In a similar study, Dias et al. (2008) developed an in-house validated protocol for separation and determination of (all-*E*)-α-carotene, (all-*E*)-β-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin in tomato. Overall, good repeatability and low relative standard deviations were obtained for β-carotene, lycopene, and lutein.

The core of these studies was the quality assurance and control of the data delivered. Performing interlaboratory analysis, or in-house validations, could help identify weaknesses and specific critical points during the research. However, given the lack of reference materials with known concentrations, the quality control and assurance of the data obtained becomes fundamental for any laboratory. In this sense, it is clear that reference materials or available standards could be of substantial importance to control method performance and maintain the reliability of

the data produced (International atomic energy agency, 2003). However, the high cost for CRM production is a factor that influences the development of new reference materials (Lauwaars and Anklam, 2005; Zakaria and Rezali, 2014). Therefore, it is important to carry out research to produce new stable materials, characterized by their low cost, availability, and high concentration of easily extractable carotenoid components.

Consequently, this work describes an interlaboratory comparison exercise for carotenoids in freeze-dried mango pulp from Ecuador, a plant material chosen since it fulfils the requirements mentioned earlier (cheap material, immediate availability and rich in carotenoids). This exercise was organised by the Flemish Institute for Technological Research (VITO). The general objective was to compare the performance of the analytical techniques and the analytical standards for the correct identification and quantification of carotenoids in mango samples. Additionally, we aimed to provide a potential reference material that can be used in the future as quality control for the analytical measurement of carotenoids.

2. MATERIALS AND METHODS

2.1. Sample preparation

All the samples were prepared by the Department of Food Science and Biotechnology at Escuela Politécnica Nacional in Ecuador. For the analysis, mango (variety Tommy Atkins) was purchased on a local market in Quito, Ecuador. Samples were transported to the lab, hand washed and the mangoes with a maturity index of 5 (12 – 15 °Brix) were selected for the experiments. The fractions (peel, flesh and stone) were manually separated, and the pulp was freeze-dried until a final moisture content of 3.5%. The particle size was reduced with a coffee mill, sieved

to exclude particle size smaller than 0.425 mm, and the particles with a size greater than that were milled and sieved again. The process was repeated until a suitable amount of material was obtained. In total, around 400 g of mango powder was obtained. Then, samples of 6 grams each were transferred under vacuum to polyethylene-aluminum bags and sealed airtight for storage at -20 °C. In order to avoid degradation of compounds with light, the process was carried out under dim light.

2.2. Homogeneity study

Each bag of the whole batch was assigned with a specific number. Eight bags were selected using a random number generator, measured in duplicate under repeatability conditions. In first instance, a combination of acetone:methanol (70:30; $v.v^{-1}$) was applied, followed by an extraction with dichloromethane:methanol (50:50; $v.v^{-1}$), during 15 minutes in an ultrasound bath at 4 °C (solvent:material ratio of 1:10 $w.v^{-1}$) (Villacís-Chiriboga et al., 2021), and the analysis was performed via liquid chromatography with ultraviolet absorbance detection (LC-UV) at a wavelength of 450 nm. β-carotene was selected as a proxy for homogeneity assessment, as this analyte was present in significant quantity. The homogeneity was evaluated according to the procedure described by Fearn & Thompson (2001).

The Cochran test procedure for duplicate results was used to test for homogeneity of the data set. The use of average-normalised data in the homogeneity assessment was carried out according to ISO 13528 (2015), with a target acceptable study variation of 5%. The following equation was used [Eq. 1]:

$$\mathbf{[Eq.\ 1]} \qquad S_{sam}^2 = \frac{D_{max}^2}{\Sigma D_i^2}$$

Where:

123 $$S^2_{sam} = \text{Cochran's statistic test}$$

124 $$\mathbf{D_{Max}} = \text{the largest difference between duplicates}$$

125 $$\mathbf{D_i} = \text{difference of each pair of duplicates}$$

126 **2.3. Stability of the samples**

127 The sample bags were stored at -80 °C until dispatch. Stability data of reference material for these types of matrixes (*e.g.*, BCR-485) has
128 shown that samples stored at -20 °C are stable during 48 to 60 months. Measurements were executed within 3 weeks from dispatch. In view of
129 the nature of the samples (dry, no oxygen, airtight containers), and the stability of carotenoids when stored at -70°C (Dias et al., 2014), no
130 influence on sample stability was expected for the duration of this study. The stability of the materials was not assessed.

131 **2.4. Procedure for the interlaboratory trial**

132 This interlaboratory ring trial was organized by VITO and invitation letters were sent to 27 candidate laboratories. It was indicated that
133 participation would be free of charge (to reach as many participants as possible). The laboratories that subscribed, received the control materials
134 to be analysed. The condition for participation was that test results had to be submitted within the stipulated deadline. Fourteen laboratories from
135 10 countries, on a total of 27 laboratories on the candidate list (52%) indicated their interest to participate in this interlaboratory exercise,
136 including the organizer, with their agreement to abide by the conditions for participation. The majority of the participating institutions were from
137 Europe (10 laboratories), 3 institutions were from South America and one was situated in North America. These laboratories received an
138 individual laboratory code to report their measurement results.

2.5. Protocol for requesting the data

Test materials were dispatched to the participants under ambient conditions. Each participant received 2 bags containing around 6 g of freeze-dried mango pulp. Moreover, a letter was included with guidelines explaining that samples upon arrival in the laboratory, must be stored in a freezer. Once open, they must be kept away from light and oxygen to maintain stability. Participants were asked to perform 3 replicate analyses on 2 different days using the same procedure (which was free to choose). An Excel file was used for reporting, with detailed information on the protocol, equipment and reagents, besides the identification and quantification of the analyzed compounds.

2.6. Methods of analysis of the participants

The methods applied by the participants are described in Table 1. The amount of sample used for the extraction varied among the labs, ranging between 10 mg and 3000 mg. Regarding the solvents used, acetone, methanol (MeOH) and tetrahydrofuran (THF) were mostly applied for the extraction. HPLC was used for the separation of the compounds, except for one laboratory reporting the use of UHPLC. Eight laboratories used C_{30} columns, while five laboratories used C_{18} columns. For the identification and quantification of carotenoids, one laboratory used MS and the other labs used a diode-array detector (DAD) (one reported results as UV-Vis).

2.7. Statistical analysis and measurement of standard uncertainty

Intra-day precision was evaluated by analyzing data of three extracts measured on the same day, while the inter-day precision was evaluated taking all the values from the two days. Both results were expressed as the coefficient of variation (% CV).

The standard uncertainty μ_A [Eq. 2] was used to calculate the relative uncertainty of measurement results ($\mu_{A,rel}$) [Eq. 3] as follows (Chen et al., 2021):

[Eq. 2] $$\mu_{\mathbf{A}} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \overline{X}_m)^2}{n(n-1)}}$$

[Eq. 3] $$\mu_{\mathbf{A,rel}} = \frac{\mu_A}{\overline{X}_m}$$

Where

$\mathbf{x_i}$ = Average test results of each laboratory

$\overline{\mathbf{X}}_\mathbf{m}$ = Average test results of all laboratories

n = Number of laboratories.

The precision of the mean values was calculated as 2×SD (Nübler et al., 2021). The performance of the laboratories was evaluated using the Z-score [Eq. 4] (ISO 13528, 2015), determined by fit-for-purpose standard deviations (FFP), which at the same time were estimated by the Horwitz equation (Horwitz and Albert, 2006).

[Eq. 4] $$\mathbf{Z_i} = \frac{X_i - X_a}{\sqrt{\mu^2(X_i) + \mu^2(X_a)}}$$

Where:

$\mathbf{X_i}$ = participants' result

$\mathbf{X_a}$ = Reference value (average of averages for the same compound)

$\mu(\mathbf{X_i})$ = reported standard uncertainty of the result X_i

$\mu(\mathbf{X_a})$ = standard uncertainty for the assigned value

3. RESULTS AND DISCUSSION

In the last years, there has been significant progress in the research on carotenoids in food, feed and plant-based materials. However, the lack of interlaboratory exercises for the analysis of these compounds has hindered the compliance for quality requirements during quality control and quality assurance. This is even more evident if we consider that carotenoids are known as unstable bioactives and that certified reference materials (CRM) maintain a relatively high production cost.

3.1. Homogeneity study

β-Carotene concentration was measured to assess the homogeneity of the samples. The Cochran value (C_{CAL}) was obtained by calculating the variability between the replicates (data not shown) and compared against the critical value (C_{CRI}) of 0.68 (95% confidence level). The calculated value was lower than the critical limit (0.56 < 0.68). Hence the whole set was retained since adequate homogeneity was found. Moreover, a test statistic S_{sam}^2 < critical value (0.00125 < 0.0058) was obtained, so the test passed the criterium for homogeneity.

3.2. Reported results

All the participants reported concentrations above the limit of quantification (LOQ) for β-carotene in the sample provided. However, a large variability in reported results was found for the other carotenoid components, whereby some laboratories reported up to 16 different compounds while others only focused on 1 compound. Overall, 37 different analytes were reported by all participating laboratories. Based on all data received, performance was evaluated based on quantification of α-carotene, β-carotene, (9*Z*)-β-carotene, β-cryptoxanthin, lutein and zeaxanthin (Table 2). In agreement with other reports, β-carotene was the major carotenoid in mango, with measured concentrations ranging from 7.85 to 30.0 $\mu g.g^{-1}$ dry weight (DW), followed by lower concentrations of (9*Z*)-β-carotene (1.22 – 6.22 $\mu g.g^{-1}$ DW), zeaxanthin (0.39 – 2.95 $\mu g.g^{-1}$ DW), α-carotene (0.40 – 4.50 $\mu g.g^{-1}$ DW), β-cryptoxanthin (0.16 – 1.98 $\mu g.g^{-1}$ DW) and lutein (0.58 – 4.09 $\mu g.g^{-1}$ DW). Despite the differences in carotenoid concentrations, the compounds described in this study have also been previously described as the main carotenoids in the variety Tommy Atkins (Marcillo-Parra et al., 2021; Ruales et al., 2018).

The measured concentration of quantified analytes depends on the extraction and subsequent clean-up. Besides, other processing parameters during extraction also influence the outcome. Some laboratories saponified the extract, which hydrolyzes carotenol esters along with triglycerides and proteins resulting in a simplified chromatogram with only non-esterified carotenoids, while the use of BHT could have stabilized the carotenoids in the extracts due to its ability to avoid oxidation. However, the exposition of the extract to light, high temperature, acids, or the combination of such factors could have induced the *E*-*Z* isomerization of the carotenoids in the sample, which is not avoided by the presence of BHT (Arvayo-Enríquez et al., 2013). On the other hand, the addition of little proportions of ammonium acetate or triethylamine

(usually ≤ 0.1%) to solvents in the mobile phase are thought to improve the recovery of carotenoids from the column and the peak shapes (Melendez-Martinez et al., 2013; Rodriguez-Amaya, 2001).

As shown in Table 1, all the laboratories used reversed-phase columns, but of different types (C_{18} or C_{30}), which influence the resolution of the carotenoid molecules. These differences are very likely due to the interaction of carotenoids in the extracts with the stationary phase. C_{18} columns have been widely applied for the analysis of carotenoids in view of its hydrophobicity and the suitability for separation under a wide range of polarities and solvents. However, C_{30} columns are characterized by a higher hydrophobicity, and provide enough phase thickness to enhance interaction with carotenoids. C_{30} columns were specially developed for carotenoid analysis, since these columns can separate not only isomers (α-, β-), but also geometric isomers of carotenoids (*Z*/*E*) (Sander et al., 2002). This phase has also efficiently separated optical isomers of some carotenoids (Meléndez-Martínez et al., 2009). Moreover, the operation of the columns is influenced by the chromatographic packing, which is a function of the column wall, particle migration and arrangement (Dorn et al., 2017). In this sense, Zelenyánszki et al. (2019), showed that commercially available columns are axially heterogeneous. Greater homogeneity was observed in shorter columns.

After separation, different detectors were reported for the identification and quantification of carotenoids. From the 14 laboratories, 12 used DAD, 1 UV/Vis, while MS was used by one laboratory. Since all the labs reported β-carotene, results obtained with DAD and MS detection are very similar (see Tables 1 and 2). However, the laboratory that used a UV-Vis detector reported a concentration significantly lower as compared to the other laboratories. As explained by Crupi et al. (2012), if calibrated correctly, the detection limits and reproducibility on the analysis of

carotenoids are similar for both MS and DAD. Thus, it could be possible that the variations in the result of the laboratory that used a UV-Vis detector are attributed to the sample handling, preparation, and the prevention of degradation or oxidation of carotenoids.

3.3. Validation of mango sample as reference material for carotenoid analysis

The quantification of the different carotenoid compounds in the mango samples was evaluated by means of both inter- and intra-day precision, expressed as the coefficient of variation (%CV) (see Table 3). The variation for the intra-day analysis was, on average, 6.28%, although in specific cases outliers were found (variations up to 69%). In the same line, the inter-day precision was 12.71%, average value. Similarly, variations up to 97% were found. Overall, it can be seen that the participants were characterized by a good precision (CV lower than 10% and values above 30% are considered outliners). These errors could be attributable to sample handling or presence of impurities (Farias Couto et al., 2013; Kimura and Rodríguez-Amaya, 1999). In a study performed by Stinco et al. (2014), a rapid resolution liquid chromatography (RRLC) method for analysis of carotenoids was developed and in-house validated on 12 commercial fruits and vegetables. The repeatability (%CV on the intra-day assay) ranged between 0.58% to 6.81%, and reproducibility (%CV for the inter-day assay) ranged from 4.66% to 11.87% for the analysis of unsaponified samples. When the samples were saponified, %CV values up to 26.38% and 27.61% were obtained for the repeatability and reproducibility tests, respectively. These results show the negative effect that saponification (which is mainly used to remove unwanted lipids, chlorophylls and/or to simplify the chromatograms when carotenoids are esterified) can have in the quantification of carotenoids, since this step could lead to isomerization or destruction of alkaline labile carotenoids. Although the extent of such effects depends on the saponification conditions, *e.g.*, temperature, reaction time and alkali concentration. In a similar study, an analytical method for

226 determination of carotenoids via RRLC in baby fecal samples was in-house validated. In line with previous results, unsaponified samples were
227 evaluated with repeatability values ranging from 0.86% to 6.94% and reproducibility values ranging from 2.36% to 9.92% (Stinco et al., 2019).
228 On the other hand, the relative uncertainty of the data set (See table 2) was between 9.13 and 31.67% for β-carotene and α-carotene,
229 respectively. These values follow the same trend as the %CV. In a similar way, a lower precision, expressed as a higher deviation from the
230 average value, was obtained after a large SD for β-carotene (± 12.24 $\mu g.g^{-1}$ DW), and a lower SD for β-cryptoxanthin (± 1.21 $\mu g.g^{-1}$ DW).
231 As explained above, Z-scores were calculated for all parameters for which more than 6 compounds were reported. The criteria of
232 classification was as follows: if the Z-score is $Z \leq 2$, the performance of the laboratory is satisfactory, while with a value of $2 < Z < 3$ the
233 classification of the laboratory is questionable and at a Z-score ≥ 3 the result was regarded as unsatisfactory. The graphical representation of the
234 Z-score for the analyzed carotenoids is given in Supplementary material SS.1.
235 The results on the performance of labs are displayed in Table 4. It can be seen that for the majority of the compounds, a Z-score lower than 2
236 was found, meaning a satisfactory result for the laboratories. However, the results for lutein were found to be unsatisfactory for 42% of the
237 laboratories. This variation could have been due to the extraction method used, destruction of this xanthophyll during saponification step and
238 deficient separation (Scott et al., 1996). In addition, the separation of geometric and structural isomers demands longer run times and the specific
239 use of a C_{30} column, since the C_{18} does not resolve geometrical isomers and inefficiently resolves positional isomers (*i.e.*, lutein and zeaxanthin)
240 (Simonovska et al., 2013).

241 Moreover, regarding β-carotene, there is no correlation between the amount of sample used for extraction and the method performance (data
242 not shown), even though the sample amount ranged between 10 mg and 3 grams. For the other carotenoids, for which the contents in the sample
243 were lower, applying 10 mg of sample resulted in the largest Z-scores. This indicates that a higher sample amount for carotenoid analysis is
244 advisable. Additionally, a smaller particle size (≈ 50 µm) could be positively related to a better extraction performance in analytical processes
245 (Saini and Keum, 2018). Moreover, as explained above, the solvent used for the extraction can also influence the extraction yield. From the
246 results displayed in Table 1, it can be seen that among all the solvents used (*e.g.*, H_2O, THF, MeOH, pentane, hexane, petroleum ether), most of
247 the laboratories included acetone within the solvents mixture.
248 The quality of the data generated from each lab was also evaluated on the basis of prevention of degradation or isomerization of the
249 carotenoids in the presence of antioxidants and the use of an IS. The 4 laboratories that didn't report any conservation method or addition of IS
250 were evaluated with Z-scores lower than 2, meaning that their performance was satisfactory for the evaluation of β-carotene. Of these
251 laboratories, four reported light protection during the procedure, which could provide a good explanation for the satisfactory result obtained. On
252 the other hand, since no addition of IS nor conservation was reported for the other laboratories, it is difficult to establish a reason for the
253 acceptable outcome. It could be due to a relative stability of carotenoids when solubilized in extractants, as has been previously shown (Patel et
254 al., 2019). Hence, results that were the least in agreement were all traceable to labs that did not use or report any form of conservation.
255 Carotenoids are unstable molecules that can undergo isomerization after extraction or severe purification steps (Martins and de Rosso, 2016),
256 which can affect the quantification. Moreover, it could be seen that specific laboratories were able to identify different configurations of the

same compounds, *i.e.*, (13*Z*)- or (15*Z*)-violaxanthin, (13*Z*)- or (15*Z*)-β-carotene (data not shown). Although some of these isomers might be separated on some C_{18} columns, C_{30} column offers a better separation of geometrical isomers, as already explained. With this information in mind, it is clear that the analysis depends both on the process and on the sample handling. Yet, as mentioned, given the instability of carotenoids, the use of BHT, pyrogallol or ascorbic acid, together with an IS could be used for analytical purposes. Moreover, a mild alkali (sodium bicarbonate) should be added for extraction to avoid epoxy to furanoid rearrangement (due to presence of violaxanthin in mango) (Rodriguez-Amaya, 2001).

3.4. Development of CRM from mango powder for the analysis of carotenoids

The importance of CRM for analytical laboratories could be traced back in time to the 1970's, where the growing concern about the quality of data generated in food analysis led to the formation of specialized organizations (*i.e.*, National Institute of Standards and Technology, Bureau Communautaire de Référence, Institute for Reference Materials and Measurements, among others) and the production of many homogeneous stabilized biological materials both from animal and plant origin (Wise and Phillips, 2019). Over the years, with the progress of analytical technology and laboratory analysis, along with the increasing demand of analytical measurements of good quality, there has been an increase in the production of CRM for such purposes.

However, most analytical laboratories perceive CRM as expensive and prohibitive, depending on the type and the state of the matrix (*e.g.*, solid, powder, liquid or even needs for transport under special conditions, and many other considerations). Yet, the acquisition of a CRM and the performance of a quality control assessment should be viewed as an investment rather than as a expense, since the analysis repetition will be

reduced, and consequently the reagents will be used to a lesser extent, while the reliability of the lab will be enhanced (Venelinov and Quevauviller, 2003).

Besides price, the stability of the compounds is a factor that is also considered before acquiring and using a specific CRM. In the specific case of carotenoids, which are regarded as unstable compounds, the exact measurement of their concentrations might be influenced by sample handling, specific extraction procedures and other variables that are inherent to each laboratory.

The development of a CRM with mango demands the examination of several points. Although quality control for the analysis of lipophilic compounds can be performed with already available CRM suited for this purpose (BCR-485 mixed vegetables and SRM 968f- Fat-Soluble Vitamins in Frozen Human Serum), their availability and high cost (between 250 and 800 €) hinders their large scale use in carotenoid analysis. It is essential to limit the production and cost of CRM through cooperation between academia and industry. As Venelinov and Quevauviller (2003) explained, international networks covering the demands and promoting interchanges between users and consumers can be substantial to optimize the overall cost of CRM. However, to prepare a CRM from mango requires extra technical steps (*e.g.*, freeze-drying, milling). Even though these factors can be considered expensive, studies performed in freeze-drying optimization have shown that the initial investment represents the major part of the overall cost. In contrast, the operational cost represents between 5 and 9% (Stratta et al., 2020). Keeping the material in an airtight environment is essential to avoid moisture absorption. Additionally, freeze-drying avoids overheating the sample, provides

stability, minimizes chemical decomposition, avoids contamination during storage, maintains long-term viability, and facilitates product distribution. Under these conditions, it is predictable that carotenoids in the mango matrix will remain stable.

It should be stated that the participation of highly qualified laboratories, with expertise in carotenoid analysis by applying different in-house validated methods, makes us conclude that the use of freeze-dried mango for quality control in analytical laboratories is appropriate for use, not only because of its low cost but also because of the presence of extractable bioactive compounds suitable for analytical purposes. As such, freeze-dried milled mango could be considered a potential vegetal matrix for developing readily available, low-cost CRM for the analysis of carotenoids.

4. CONCLUSION

The results of this study provide insights on a worldwide interlaboratory comparability for the accurate determination of carotenoids in mango. The results have proven that β-carotene, α-carotene, (9*Z*)-β-carotene, β-cryptoxanthin and zeaxanthin were satisfactorily determined, independently from the analytical protocols of each laboratory. Moreover, this research provided valuable information for the development of reference materials based on mango to be used for the quality assessment in analytical laboratories. The economic perspective for the development of these materials seems to be promising, in view of the concentration of carotenoids in mango and their easy extractability. Overall, research involving analysis of carotenoids could be improved if new CRM material, produced from waste and scientifically tested, is used.

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DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Methods reported by the participants

Lab code	Sample intake (mg)	Sample pretreatment	Extraction	Clean-up	IS	LC	Column phase	Column dimensions	Detector/ Wavelength	Injection solvent	Conservation	Reference
1	800	Saponification with pyrogallol	THF:hexane	NR	None	HPLC	C_{18}	25 cm x 4.6 mm; 3 µm	DAD/450 nm	20 EtAc/80 (90 ACN/10 IPA)	Light protection with UV filters	[44]
2	500 – 1500	Saponification	THF:MeOH (1:1; v:v)	PVDF 0.45 µm filters	(All-*E*-)β-apo-carotenal	HPLC	C_{18}	10 cm x 4.6 mm; 3 µm in series with 25 cm x 4.6 mm; 5 µm	DAD/450 nm	ACN:MeOH:DCM (7:2:1; v:v:v)	BHT during extraction and light protection	[45]
3	1000	Enzymatic digestion + saponification	Acetone:Pentane (4:6)	Clean-up with 10% NaCl-solution and H_2O	(All-*E*-)β-apo-carotenal	HPLC	C_{30}	25 cm x 4.6 mm; 5 µm	DAD/450 – 470 nm	MeOH:ACN (9:1; v:v) + EtAC + 0.25% triethylamine	NR	[46]
4	500	Saponification of extract	MeOH:THF (1:1; v:v)	None	Echinenone	HPLC	C_{30}	25 cm x 4.6 mm; 5 µm	DAD/450 nm	MeOH:MTBE (1:1; v:v)	BHT during extraction and light protection	[47]
5	250	Saponification	Acetone	None	None	HPLC	C_{30}	25 cm x 4.6 mm; 5 µm	DAD /450 nm	MeOH:MTBE (1:1; v:v)	Light protection, nitrogen and low temperature	[48]
6	10 – 15	NR	H_2O:Acetone:ACN (2:4:4; v:v:v)	None	(All-*E*-)β-apo-carotenal	HPLC	C_{18}	15 cm x 4.6 mm; 5 µm	DAD /NR	H_2O:acetone:ACN (1:2:2; v:v:v)	Light protection	[49]
7	3000	Saponification	Acetone + Hexane:Petroleum ether (1:1; v:v)	PTFE 0.45 µm filters	(All-*E*-)β-apo-carotenal	HPLC	C_{30}	25 cm x 4.6 mm; 5 µm	UV-Vis/450	MeOH:MTBE:H_2O (v:v:v)	BHT for standard solutions	[50]
8	500	Saponification	Hexane:EtOH:Acetone (5:2.5:2.5; v:v:v) + hexane	None	(All-*E*-)β-apo-carotenal	HPLC	C_{18}	25 cm x 2.1 mm; 5 µm	DAD /450 nm	ACN:MeOH:EtAc (6:2:2; v:v:v)	NR	[51]
9	1000	NR	Acetone:MeOH (50:50; 1:1) + DCM:MeOH (50:50; 1:1)	None	NR	UPLC	C_{18}	10 cm x 2.1 mm; 1.8 µm	MS/NR	DCM	BHT during extraction	[16]
10	500 – 1000	NR	Light petroleum/ EtAc/MeOH (1:1:1; v:v:v)	PTFE 0.45 µm filters	NR	HPLC	C_{30}	25 cm x 4.6 mm; 5 µm	DAD/NR	EtAc	NR	[52]
11	1000	Saponification	Ethanol:hexane (4:3; v:v)	Clean-up with 10% NaCl-solution and H_2O	(All-*E*-)β-apo-carotenal	HPLC	C_{30}	25 cm x 4.6 mm; 5 µm	DAD/450 nm	EtAc + 0.25% (v/v) triethylamine/MeOH/ACN (50:45:5; v:v:v)	BHT during extraction	[53]
12	1800 – 2000	None	MeOH:THF (1:1; v:v)	None	Retinyl acetate	HPLC	C_{18}	25 cm x 3 mm; 5 µm	DAD/450	MeOH:Butanol (6:4, v:v)	BHT during extraction	[54]
13	120 – 130	NR	Hexane:Acetone (1:1; v:v)	NR	NR	HPLC	C_{30}	15 cm x 4.6 mm; 3 µm	DAD/285 – 450 nm	EtAc	NR	[35]
14	110 – 115	NR	H_2O + Acetone + Diethyl ether:Petroleum ehter (1:1 v:v)	NR	(All-*E*-)β-apo-carotenal	HPLC	C_{30}	25 cm x 4.6 mm; 5 µm	DAD/450 nm	MeOH:MTBE (1:1; v:v)	BHT during extraction	[55]

NR = Not reported; **MeOH** = methanol; **ACN** = acetonitrile; **EtOH** = ethanol; **THF** = tetrahydrofuran; **DCM** = dichloromethane; **EtAc** = Ethyl acetate; **IPA** = isopropyl alcohol; **MTBE** = Methyl-tert-butyl ether

Table 2. Concentration of the reported carotenoids, all expressed in µg.g^{-1} DW as average ± SD (n = 6)

	Compounds [µg.g^{-1} DW]*					
Labcode	**α-carotene**	**β-carotene**	**(9Z)-β-carotene**	**β-cryptoxanthin**	**Lutein**	**Zeaxanthin**
1	0.40 ± 0.03^{a}	18.69 ± 0.30^{cd}		0.89 ± 0.07^{b}	1.94 ± 0.09^{c}	1.77 ± 0.05^{b}
2	1.04 ± 0.43^{b}	19.67 ± 1.11^{cd}	3.20 ± 0.37^{e}		3.95 ± 0.39^{e}	<LOD (0.019)
3		18.00 ± 1.96^{c}				
4		19.55 ± 0.44^{cd}	2.53 ± 0.10^{d}			
5	0.96 ± 0.13^{b}	18.96 ± 1.28^{cd}	1.94 ± 0.18^{b}	0.91 ± 0.11^{bc}	3.11 ± 0.12^{d}	1.82 ± 0.13^{b}
6	4.54 ± 0.79^{e}	19.93 ± 1.89^{cd}	1.22 ± 0.16^{a}	0.16 ± 0.04^{a}		1.73 ± 0.16^{b}
7		12.09 ± 1.54^{ab}				
8		28.44 ± 1.36^{f}				
9		23.23 ± 6.44^{e}		1.98 ± 0.14^{e}	0.78 ± 0.56^{a}	0.39 ± 0.18^{a}
10		13.27 ± 1.73^{b}	1.86 ± 0.12^{b}			
11	2.43 ± 0.15^{d}	9.65 ± 0.93^{a}		1.63 ± 0.12^{d}	1.36 ± 0.47^{b}	
12	0.71 ± 0.11^{ab}	20.99 ± 0.45^{de}	6.22 ± 0.17^{f}	1.00 ± 0.03^{c}	3.97 ± 0.12^{e}	1.44 ± 0.10^{b}
13	1.87 ± 0.06^{c}	29.98 ± 0.86^{f}	2.22 ± 0.09^{c}	1.89 ± 0.08^{e}	4.09 ± 0.12^{e}	2.95 ± 1.17^{c}
14		7.85 ± 0.70^{a}				
Average	1.71	18.59	2.74	1.21	2.71	1.45
SD	1.32	6.12	1.53	0.61	1.32	0.97
Relative uncertainty (%)	31.67	9.13	22.77	20.47	18.84	19.98
Precision of mean values	2.65	12.24	3.06	1.21	2.52	1.46
CV (%)	77.59	32.91	55.77	50.14	46.14	61.84

LOD = Limit of detection.

DW = Dry weight

Different letters in the same column indicate statistically significant differences among the concentration detected for each compounds in each laboratory ($p<0.05$).

425 **Table 3.** Intra and inter-day precision measurement, all expressed as the average (n = 3) of CV (%)
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	α-carotene			β-carotene			(9Z)-β-carotene			β-cryptoxanthin			Lutein			Zeaxanthin		
	Day 1	Day 2	Interday	Day 1	Day 2	Interday	Day 1	Day 2	Interday	Day 1	Day 2	Interday	Day 1	Day 2	Interday	Day 1	Day 2	Interday
Labcode																		
1	5.7	4.1	6.7	0.7	1.8	1.6				7.5	3.7	7.7	2.9	3.1	4.8	0.7	2.5	3.0
2	6.0	3.2	40.8	6.5	4.1	5.6	2.6	12.0	10.7				1.1	3.5	10.0			
3				3.9	13.5	10.9												
4				2.4	0.8	2.3	2.4	1.5	3.5									
5	3.5	2.6	13.6	1.1	1.1	6.8	2.1	0.7	8.6	1.2	2.6	11.4	1.5	1.2	3.7	0.6	2.5	2.2
6	6.3	16.2	17.5	9.9	2.1	9.5	1.1	1.9	11.9	15.5	6.9	21.1				9.6	2.5	7.7
7				7.4	12.2	12.7												
8				5.2	4.1	4.8												
9				19.6	17.8	27.7				0.1	9.2	6.6	14.5	68.7	96.9	13.8	2.5	56.9
10				13.8	8.7	13.0	2.1	6.8	5.8									
11	5.6	5.1	5.5	13.2	2.9	9.6							19.0	3.5	34.5			
12	14.1	7.7	14.7	0.9	2.9	2.1	6.3	0.6	2.5	0.3	3.6	2.9	1.8	3.8	3.0	2.6	2.5	12.8
13	2.2	2.7	2.9	3.3	2.3	2.9	2.1	3.3	3.5	3.8	3.2	3.9	3.4	2.6	3.1	60.4	2.5	51.7
14				2.6	3.6	8.9												

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Table 4. Study performance based on Z-score obtained for each analyte.

Labcode	α-carotene	β-carotene	(9Z)-β-carotene	β-cryptoxanthin	Lutein	Zeaxanthin
1	-3.1	-0.4		-1.3	-1.9	1.5
2	-1.6	0.1	1.1		3.0	-6.7
3		-0.7				
4		0.1	-0.5			
5	-1.8	-0.2	-1.9	-1.3	0.9	1.7
6	6.6	0.3	-3.7	-4.3		1.3
7		-3.8				
8		4.6				
9		2.0		3.2	-4.8	-4.6
10		-3.2	-2.1			
11	1.7	-5.0		1.7	-3.4	
12	-2.3	0.8	8.5	-0.8	2.8	-0.1
13	0.4	5.4	-1.3	2.8	3.3	6.9
14		-5.8				
Number of participants	7 (54%)*	14 (100%)	7 (54%)	7 (54%)	7 (54%)	7 (54%)
Satisfactory	4 (57%)	9 (64%)	4 (57%)	4 (57%)	2 (29%)	4 (57%)
Unsatisfactory	2 (29%)	5 (36%)	2 (29%)	2 (29%)	3 (43%)	3 (43%)
Questionable	1 (14%)	0 (0%)	1 (14%)	1 (14%)	2 (29%)	0 (0%)

* = number of participants (% with respect to the number of laboratories)

$Z \leq 2$ = satisfactory; $2 < Z < 3$ = questionable; $Z \geq 3$ = unsatisfactory

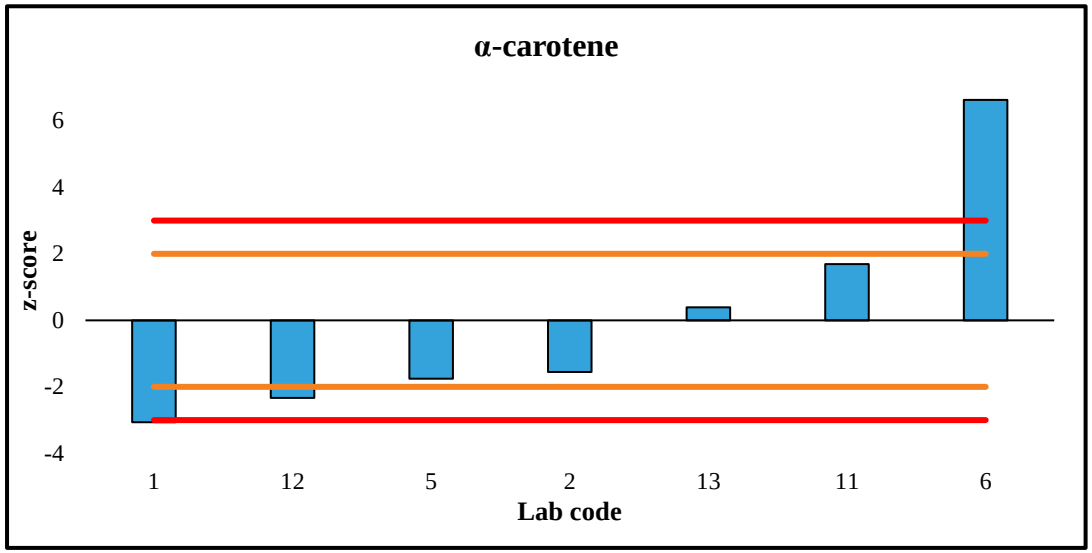
Interlaboratory exercise for the analysis of carotenoids and related compounds in dried mango fruit (*Mangifera indica* L.)

José Villacís-Chiriboga, Griet Jacobs, John Van Camp, Kathy Elst, Jenny Ruales, Verónica Marcillo-Parra, Volker Böhm, Andrea Bunea, Martina Cirlini, Neal Craft, Bruno De Meulenaer, M. Graça Dias, Giacomo Lazzarino, Antonio J. Meléndez-Martínez, Pieter Versloot, Adriana Z. Mercadante, Begoña Olmedilla-Alonso, Johana Ortiz-Ulloa, Carla M Stinco, Stefan Voorspoels

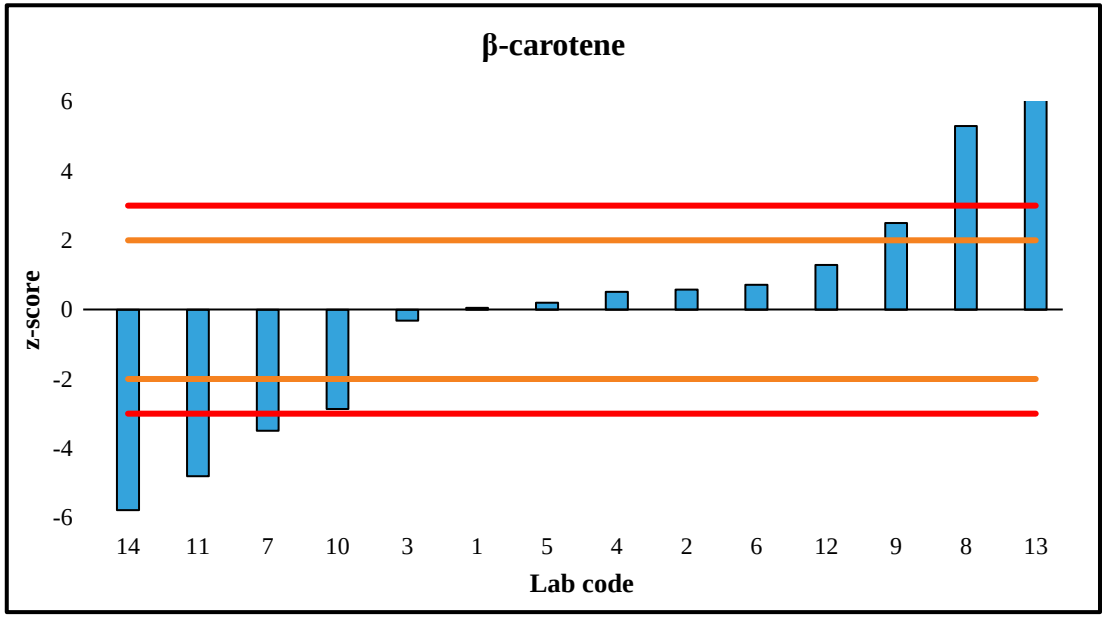
Supplementary Material SS1.

Graphical representation of the Z-score for the analyzed carotenoids

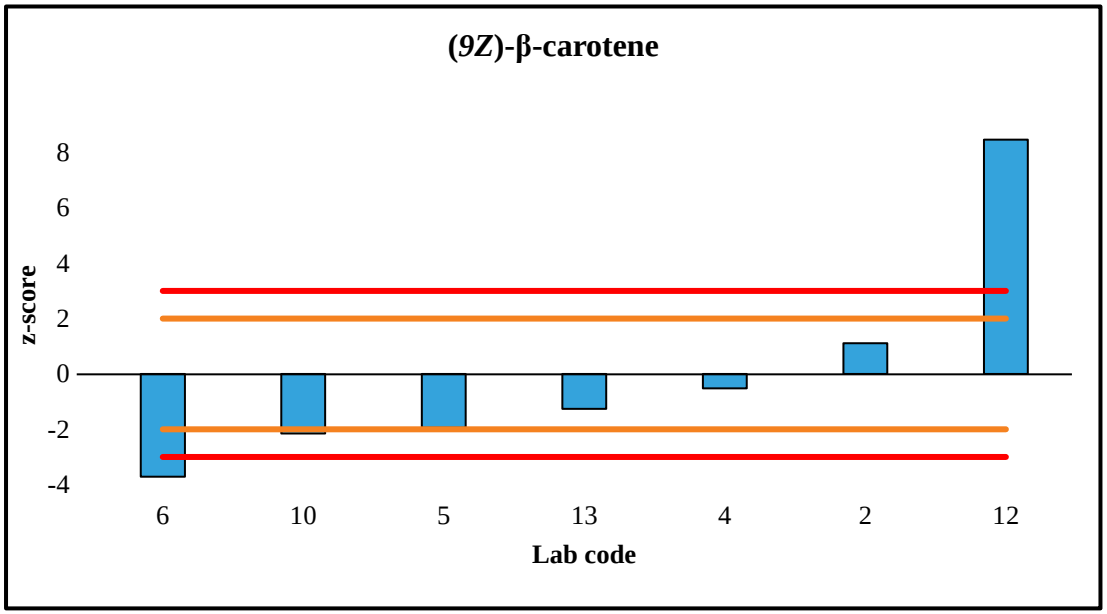
a)



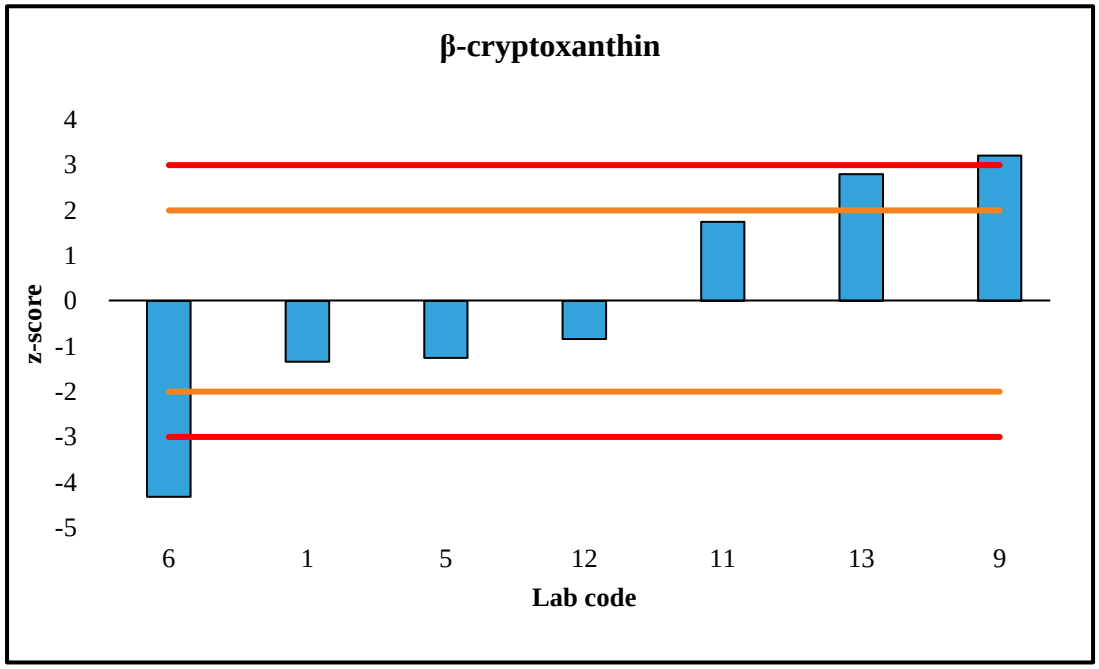
b)



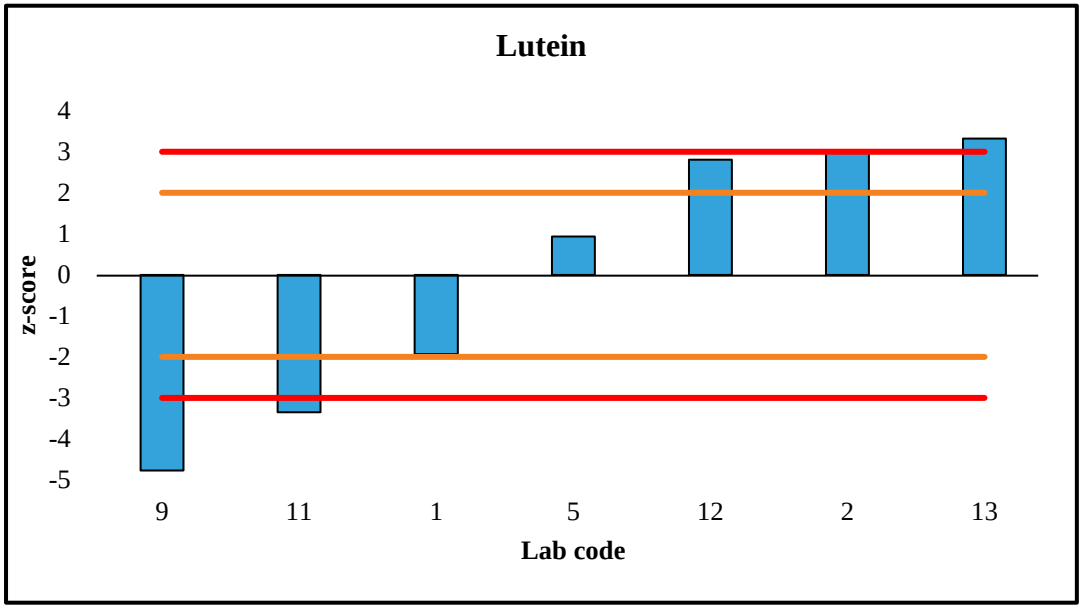
c)



d)



e)



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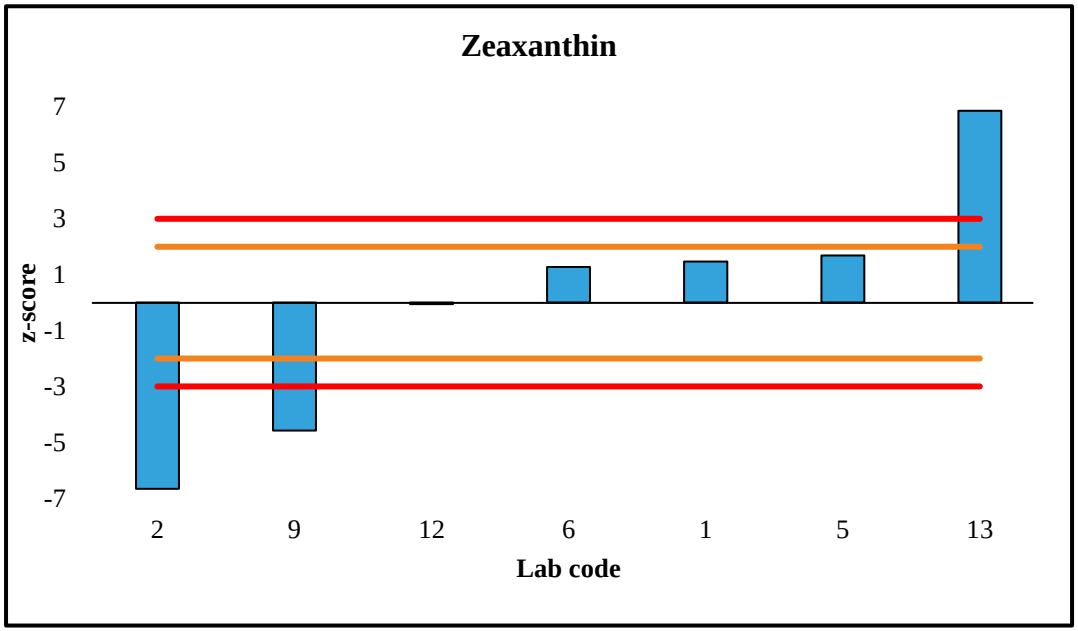

Figure SS.1. Z-score plot for α-carotene (a), β-carotene (b), (*9Z*)-β-carotene (c), β-cryptoxanthin (d), lutein (e), zeaxanthin (f). The orange bar represents a Z-score of 2, the red bar represents a Z-score of 3