Ensuring the Reliability of Analytical Data for Carotenoids and Flavonoids: Challenges and Progress

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PREMISES

- The quality of a database depends on quality of the data generated.
- The quality of the data generated depends on the quality of the sample submitted to analysis.
REQUIREMENTS FOR RELIABLE RESULTS

- Representative sampling
- Validated method
- Quality assurance system
- Adequately trained analyst

Determination of bioactive compounds is a challenging task because:

- They are generally present at very low levels together with other compounds, at much higher concentrations, that may interfere in the analysis.
- There are many classes of compounds now considered to be health promoting, each group consisting of many members with differing biological activities or efficacies, thus requiring separation, identification and quantification of individual compounds.
- Many are unsaturated, susceptible to degradation during analysis, with members of a group capable of degrading at varying degrees.
Difficulties in carotenoid analysis are due to:

- Marked qualitative and quantitative variation of the carotenoid composition of foods
- Wide concentration range among carotenoids in a given food
- Uneven distribution of carotenoids within a sample and between samples of a given food
- Variation in the nature of the matrix
- Susceptibility of carotenoids to isomerization and oxidation during analysis and during storage of samples and standards.

Critical steps and sources of errors have been identified.

Certified reference materials have been developed.

Interlaboratory studies have been conducted.
ILLUSTRATION OF THE TOTAL ERROR IN DATA GENERATION

TOTAL ERROR

- Sampling error
- Analytical sample preparation error
- Analysis error
- Data processing & interpretation error

Lot (population) → Laboratory sample → Analytical sample → Analytical results → Data (information)

Non-representative sampling.

Representativity not maintained in the analytical sample, enzymatic and non-enzymatic oxidation of carotenoids, geometric isomerization.

Incomplete extraction, physical losses, oxidative degradation.

Incomplete transfer to the solvent, formation of emulsion.

Degradation or artifact formation during saponification, loss of carotenoids with the washing water.

Loss of carotenoids by degradation and/or adherence to wall of container when carotenoid is brought to complete dryness.

Coelution, overlapping peaks, low recovery from column.

Erroneous identification, unaccounted impurity and degradation of standards, calibration errors.

General procedure for carotenoid analysis and sources of errors in each step. Exposure to light at any step from extraction will enhance photoisomerization and photodegradation.

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Bangkok, Thailand
Results: The carotenoid content was higher in the part of the root closest to its attachment to the stem (proximal section), gradually decreasing toward the opposite end (distal section). Across the root, carotenoid content was higher in the core, lower towards the periphery.

For carotenoids, two certified reference materials have been developed:

- The Community Bureau of Reference BCR485 (freeze-dried mixed vegetables).
- NIST SRM 2383 (baby food composite).
EUROPEAN INTERLABORATORY STUDY

Scott et al. (1996)
17 European laboratories
Lyophilized vegetable mix used as potential reference material
Carotenoids measured – lutein, zeaxanthin, lycopene, α-carotene, β-carotene
Results suggested:
The effect of the chromatographic system is probably not a major variable.
In the more experienced laboratory, variation in the standardization of the carotenoid solution was not thought to be a significant problem.
There were greater variations for lycopene calibration and measurement
The preparation of the extract may account for about 13% of the overall variance of around 23%.

EUROPEAN INTERLABORATORY STUDY

Schüep and Schierle (1999)
14 European laboratories
Carotenoids determined - β-carotene and its Z-isomers
4 commercial products – margarine, vitamin drink, pudding powder, natural mixed vegetables
The supplemented drink showed the best repeatability while the pudding powder gave the worst results.
Excluding the Z-isomers from quantification of β-carotene would result in significant underestimation of β-carotene.
NIST INTERLABORATORY STUDY

Sharpless et al. (1999)
26 US and European laboratories
Baby food composite as reference material
The relative expanded uncertainties were higher than those generally expected for certified values.
Certified concentrations were provided for some carotenoids, but only reference values were obtained for other carotenoids.
Only reference values were provided for lycopene (all-\(E\)- and total) because of its instability.

HARVESTPLUS INTERNATIONAL INTERLABORATORY STUDY

Rodriguez-Amaya et al. (unpublished)
Test materials – sweetpotato, cassava and maize flours.
19 Asian, African, European, Latin American and US laboratories.
Widely varying laboratory conditions and experience in carotenoid analysis.
Results:
1. Incomplete extraction is a major source of error.
2. HarvestPlus had several laboratories that can determine the carotenoid contents of sweet potato and cassava accurately, but more work had to been done with maize.
3. Training of analysts was very important.
Quantitative determination of individual glycosides is difficult because:

- Several glycosides can occur for each flavonoid.
- Standards are not commercially available for most of these compounds.

DETERMINATION OF FLAVONOL

Critical step – hydrolysis / extraction

Hertog et al. (1992)
Refluxing for 2 h at 90°C with 1.2 M HCl in 50% aqueous methanol.

Häkkinen et al. (1998)
Using a mixture of flavonol and phenolic acid standards, best results were obtained with 1.2 M HCl at 90°C for 2 h and 0.6 M HCl at 35°C for 16 h. For blackcurrant and strawberry, the method of choice was hydrolysis at 35°C for 16 h with 1.2 M HCl.
DETERMINATION OF FLAVONOL

Critical Step – hydrolysis / extraction

Nuutila et al. (2002)

Compared the methods of Hertog et al. and Häkkinen et al., using individual standards or mixtures of standards (phenolic acids, flavonols, flavones and catechins). The best results for majority of the compounds were obtained with Hertog et al.’s condition.

Response surface diagrams for the determination of quercetin, kaempferol and apigenin in vegetable samples.
Chromatogram of flavonoid standards with the corresponding absorption spectra. M = myricetin, Q = quercetin, L = luteolin, K = kaempferol and A = apigenin, injected in the same concentration. Nova-Pak C18 column, mobile phase starting with 20:80 methanol:water (acidified with 0.3% formic acid), reaching 45:55 in 5 min, 48:52 in 17 min and 20:80 in 20 min, maintaining this proportion for 10-15 min.

DETERMINATION OF QUERCETIN
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