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CHAPTER 6

HIGH-THROUGHPUT MEASUREMENT METHODOLOGIES FOR DEVELOPING NUTRIENT-DENSE CROPS

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ABSTRACT

With the development of nutrient-dense crops comes the need for analytical methodologies to enable rapid and accurate analysis of the micronutrients of interest. The analysis of provitamin A carotenoids (pVACs) and the minerals iron (Fe) and zinc (Zn) are the focus of this chapter with the considerations and commonly employed methods discussed. When analyzing samples there are various considerations to minimise analyte degradation (in the case of provitamin A) and reduce possible contamination from external sources (for Fe and Zn). Spectroscopic and chromatographic analyses are the most common analysis approaches utilised when screening for carotenoids. Spectroscopic analyses including near-infrared spectroscopy (NIRS) and iCheck are rapid and require minimal samples preparation and provide fast analysis times. The carotenoids present in the sample is dependent on the crop analyzed and resulting number and concentration of carotenoids present will impact the final decision on suitable analysis techniques. For example, in crops with high concentrations of non-pVACs, chromatographic analysis is necessary in order to accurately quantify the micronutrients. This process is able to accurately identify and quantify individual carotenoids, but requires extensive sample preparation and often long chromatographic separation analysis. When analyzing the minerals Fe and Zn, these same techniques are not suitable, but it is still important to ensure careful sample preparation to deliver accurate analytical results. Degradation of these micronutrients is not a concern, however, possible contamination from soil/ dust/ insects can lead to inaccurate results. Commonly employed analysis such as atomic absorption spectroscopy (AAS) and Inductively Coupled Plasma-Optical Emission Spectrometry ICP-OES or Inductively Coupled Plasma-Mass spectrometry (ICP-MS) require sample digestion prior to analysis and highly pure reagents and gases. These techniques are able to analyze multiple elements and have high accuracy and sensitivity but require specialised facilities and highly trained staff. The use of high-throughput analyses to complement these high-accuracy methods include colorimetric and X-ray flourescence (XRF) technologies. These approaches enable much higher throughput with simple sample preparation and enable screening for micronutrient concentration without the need for specialised facilities.

Key words: Screening, Analysis, Carotenoid, Provitamin A, Iron, Zinc, Micronutrient analysis

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INTRODUCTION

The advent of biofortification led to a need to provide more cost-effective and rapid analytical techniques for the pre-breeding activities associated with developing nutrient-dense crops. For metal analysis, traditional methods included Atomic Absorption Spectrometry and Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). These analyses can be of high quality but are generally costly to perform and require high technical ability in operating such equipment. Additionally, for carotenoid analysis, very few options other than High-Performance Liquid Chromatography (HPLC) were available. The HPLC has low throughput and is generally expensive. What was needed was high-throughput and relatively inexpensive analysis technologies for breeding programs to analyze thousands of samples in a season. The following chapter outlines strategies and achievements to date in the development of rapid screening techniques for HarvestPlus target metals (Fe and Zn) and the pro-vitamin A carotenoids (pVACs).

CAROTENOID ANALYSIS

Considerations

Various carotenoids are present in plant material, not all of which are carotenoids that are metabolized to produce the essential micronutrient retinol (vitamin A). Therefore, it is important to identify the concentration of the provitamin A carotenoids (pVACs), specifically beta-carotene, α-carotene and beta-cryptoxanthin, in plant material. In some crops the pVACs constitute the majority of the carotenoids present in the plant, whereas others have only a small percentage of total carotenoid present as pVACs. Consequently, understanding the carotenoid composition of the target crops is an important consideration when deciding which analysis method to perform. Specifically, within the HarvestPlus program, carotenoid analysis is essential for sweet potato, cassava and maize. Each of these crops has a different carotenoid make-up, and consequently different approaches are required. The differences in the carotenoid content of these crops are discussed by Kimura *et al*. [1]. Briefly, the majority of carotenoid in sweet potato is present as beta-carotene, and screening with spectroscopy is sufficient to determine the pVAC content. Cassava, however, contains several minor carotenoids and requires chromatographic analysis after initial spectroscopic screening. Finally, maize screening based solely on spectrophotometry is not feasible due to the high proportion of lutein and zeaxanthin present in the material. Liquid chromatography (High performance liquid chromatography (HPLC) or Ultra-performance liquid chromatography (UPLC)) analysis is needed to accurately determine the pVAC concentration in maize samples.

Analysis of carotenoids on some matrices, like maize kernels, can be challenging due to a number of factors. Specifically; multiple carotenoids present in maize (carotenes and xanthophyll), which require many detailed laboratory protocols to optimize the extraction and quantification for each; their interactions with other molecules such as starch and proteins; the wide range of concentrations; the presence of geometric isomers; their rapid oxidation and degradation. All challenges can be addressed by careful consideration of the extraction protocol and analytical method. Degradation can be reduced by minimizing sample storage time while ensuring low temperatures (-20 °C or

preferably – 80 °C), grinding the kernels just before the extraction, and analyzing the extracts within 12h of extraction. Working in a room under dim light and low temperature (18 to 20 °C) will also assist to reduce carotenoid degradation of the sample.

Furthermore, it is important to ensure strict adherence to sample preparation and analysis protocols to minimize degradation of the highly reactive carotenoids. Possible causes of carotenoid degradation include: photodegradation, thermal degradation, and oxidation. When samples are cut, ground, or cooked, this increases the potential for carotenoid degradation. Consequently, sample preparation, extraction, and laboratory set up must be optimized to ensure minimal degradation whilst ensuring accurate analysis. The methods used for screening carotenoid content in crops within the HarvestPlus program are summarized below. Detailed laboratory set up along with sampling and analysis protocols are described in the literature [2, 3]. A video on carotenoid extraction and analysis in maize is also available at
https://www.youtube.com/watch?v=H9EdpRTBM4o

The methods employed within the HarvestPlus program for determining the carotenoid concentration in crops are summarized in Table 6.1 below.

As in any carotenoid analysis, there are many sources of errors, including different physiological stages of the ears analyzed, improper storage of samples, incomplete extraction, physical losses during extraction with the organic solvents, faulty measurement and calculation, degradation and isomerization. When the analysis is done by liquid chromatography, incomplete chromatographic separation and incorrect identification are common sources of error. Standard laboratory procedures and interaction with the clients largely decrease the accumulation of errors (Figure 6.1).

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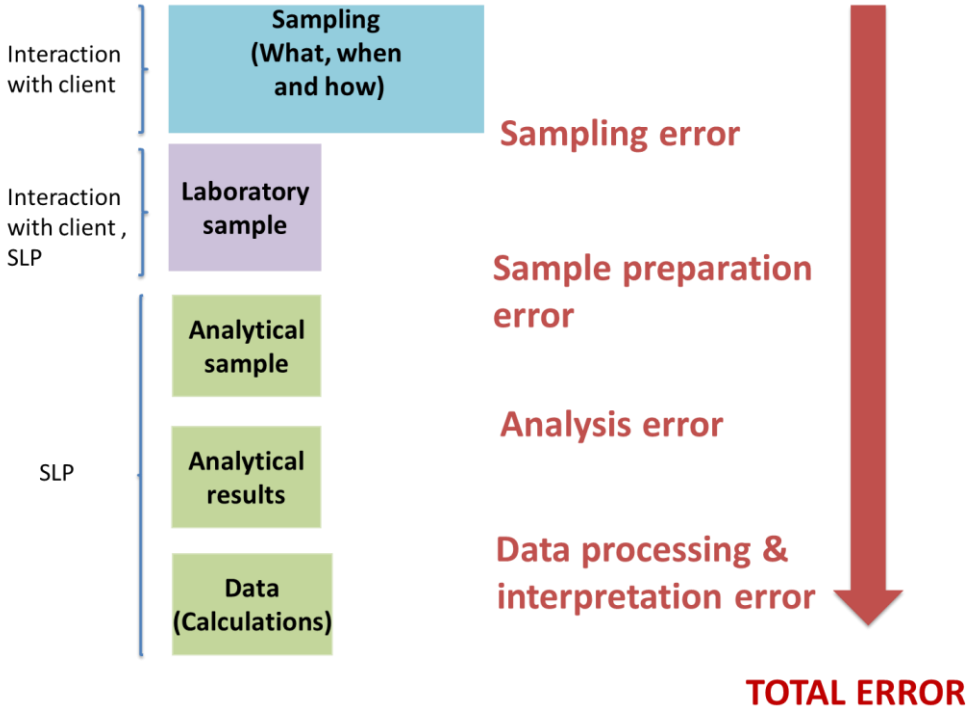

Figure 6.1: Sources of error in carotenoid analysis and how to mitigate them
(SLP: Standard laboratory procedures)

Color Analysis

One of the simplest approaches for screening crops with high levels of carotenoid is based on the color of the tissue, because color intensity is closely correlated with the carotenoid concentration in cassava roots [12]. The ability to use a visual technique to identify high carotenoid cassava without the need for comprehensive analytical techniques is beneficial, particularly when sampling crops in remote areas. However, as breeding programs have resulted in higher numbers of high carotenoid genotypes, distinguishing between the subtle color differences in deep yellow roots becomes problematic. Consequently, an ongoing development into rapid and accurate analytical techniques has been a focus for the HarvestPlus program. One such method includes the use of a digital Chromameter to quantify color intensity [10]. The benefits of this technique include both its portability, ease of use, and relatively low cost, enabling rapid screening on fresh root with results validated against standard spectroscopy with $r^2 > 0.7$ for both total beta-carotene and total carotenoid concentration determinations. In the case of maize, kernel color is not correlated with pVAC content, as it also includes the color of the two major carotenoids, lutein and zeaxanthin, that are not pVAC. Therefore color analysis, is not recommended for pVAC biofortification efforts in maize.

Spectroscopy

Carotenoids contain extensive conjugated double bonds, which function as a chromaphore to give the strong yellow to red color synonymous with foods high in carotenoid [13]. It is this light absorbing feature that is exploited to determine the concentration of carotenoids in a sample when using spectroscopic techniques. In order

to determine the concentration, carotenoids are extracted from the plant material using an organic solvent liquid extraction. The extracted sample is then exposed to light and the amount of light absorbed at the absorbance maxima (~450nm) is directly proportional to the concentration of carotenoid in the sample, as per the Beer-Lambert Law [13]. Due to the strong chromaphore in the carotenoids and the sensitivity of spectroscopic analysis, this results in a suitably sensitive detection. Spectroscopic techniques work particularly well when determining total carotenoid content and thus particularly useful when analyzing samples containing the majority pVACs. However, in samples containing a complex mix of carotenoids including both pVAC and non-pVACs, as in maize, this complicates the analysis as the absorption maxima is a similar wavelength region for all carotenoids. It is not possible to accurately quantify the concentration of the individual carotenoids present with this technique.

iCheck CAROTENE

Recently, the iCheck CAROTENE, designed by BioAnalyt, GmbH, Germany, has been released as a quick spectrophotometric method for quantifying carotenoid concentration. Unlike the previously discussed spectroscopic methods, the iCheck system extracts and quantifies the total carotenoids in one step [14]. As mentioned above, this method is suitable for crops containing the majority of pVACs like in cassava. However, in maize samples, for which the most abundant carotenoids are not pVACs, more complex analysis is required in order to accurately determine the pVAC levels in the crop.

NIRS

The use of near-infrared spectroscopy (NIRS) has been trialled as a non-destructive rapid screening technique with cassava samples [10]. Sample preparation for this technique has the advantage of only requiring cassava samples to be peeled and chopped or ground prior to analysis with NIRS. The resulting validation with spectroscopy analysis resulted in $r^2 > 0.88$ for total beta–carotene and total carotenoid concentrations. In order to establish a suitable prediction model for robust analysis with this method, an extensive database of quantified samples is required with significant data processing to ensure accurate results. Given the wider range of pVAC content in biofortified maize, very recently robust NIR models for pVAC quantification have been developed (Palacios N, forthcoming). This will facilitate the rapid screening of breeding populations.

HPLC & UPLC

Unlike spectroscopic techniques, which are only able to determine the total carotenoid concentrations, liquid chromatography analysis (HPLC and/or UPLC) is able to both identify and quantify the carotenoids present [15].This is particularly useful in crops such as maize and cassava, which contain various carotenoids, with non-pVACs often being the most abundant. Unfortunately, with this added analytical power comes increased analysis time and cost. In comparison to fairly rapid screening with spectroscopic techniques, HPLC analysis requires extensive sample preparation, long analysis times, experienced technicians, and relatively expensive equipment. Consequently, liquid chromatography is generally used for a more accurate reference method.

High-performance liquid chromatography (HPLC) analysis for sweet potato, cassava and maize requires extensive sample preparation [1]; specifically: extraction, filtration,

solvent extraction, water removal, and drying. Immediately prior to injection onto the HPLC column, the carotenoid sample is re-dissolved in HPLC grade acetone and filtered. The chromatographic method utilizes either a C18 or C30 column and requires an isocratic or gradient elution for the respective columns. Both methods require a 60-minute elution with column selection and mobile phase composition dependant on the material analyzed. The UPLC analysis is currently being used for pVAC analysis in maize. As compared to HPLC, UPLC uses less mobile phases and has much lower elution time (9 min) and therefore more samples can be analyzed per day[8].

FE AND ZN ANALYSIS

Analysis of inorganic micronutrients such as iron (Fe) and zinc (Zn) requires a different approach when compared to that discussed previously for organic carotenoid analysis. However, the simplicity of these micronutrients requires a different analytical approach in order to accurately determine the concentrations of Fe and Zn in plant material. Unlike organic micronutrients, degradation is not an issue when considering these elements, but the potential for contamination is greatly increased due to the high abundance of these elements in the environment.

Considerations

When planning analysis of elemental micronutrients (Fe and Zn), one of the important considerations is to ensure the samples and analysis processes are free from contamination. Zinc contamination can occur due to contamination in the sample processing (grinding, polishing, and others). Many sample preparation processes contain plastics (i.e. with equipment), which can contain Zn. Consequently, this can contaminate the sample during the pre-analysis preparation. As a result, within the HarvestPlus program, various commonly used polishing and grinding devices have been scrutinized to ensure they are non-contaminating when used for micronutrient analysis. In some cases it is also possible to modify the equipment to ensure any contaminating plastics are removed and replaced with a suitable non-Zn containing alternative (for specific details refer to Stangoulis and Sison [16]).

Environmental contamination is more likely to affect the Fe results [17, 18]. As reported previously in wheat [19], the presence of dust and dirt contamination on grain can result in significant increases in the Fe determined during the analysis. Maize is manually harvested and the husk is not removed in the field. Ears are collected in bags avoiding contact with soil, once in a clean area where they are shelled manually. Aluminium (Al) is not present in plant material but is present in soil; consequently, a high level of Al is used as an indicator of soil contamination. Thus when the detected levels of Al are greater than 5 mg kg^{-1}, the Fe results are unlikely to be an accurate representation of the Fe concentration in the grain. Due to the prevalence and likelihood of soil/ dust contamination, it is highly useful to detect Al. This is one of the major advantages of a broader elemental analysis, made possible with ICP-OES, as it is possible to quantify Fe, Zn and Al (among other elements) in a single analysis run.

The methods employed within the HarvestPlus program for determining the micronutrient concentration in crops are summarized in Table 6.2 below.

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ICP/AAS

Analytical tools for the determination and quantification of micronutrients in crops are essential when breeding biofortified crops. Spectroscopic methods such as inductively coupled plasma optical emission spectroscopy (ICP-OES) and atomic absorption spectroscopy (AAS) are well established and provide accurate and sensitive results for a range of elements. Limits of detection span a wide analytical range from percent to ppb levels depending on the element of interest and the matrix. Samples require digestion, and extensive pre-analysis preparation is required prior to liquid introduction for analysis. This can include: sample drying, cleaning, grinding, weighing, digestion, and dilution. Each of these steps is time consuming and if not performed diligently and without contamination, can result in significant analytical errors. Furthermore, due to the sensitivity of these methods, high purity reagents are required in order to achieve consistent and accurate results. It is also important to ensure a suitable digestion method is employed to consistently enable maximum extraction efficiency. A number of digestion methods have been reported in the literature for micronutrient analysis of plant samples, and most of these require strong acids and oxidants in combination with high temperatures and/or high pressure in order to completely digest the plant material. When planning a suitable digestion method, it is important to consider which elements are required for quantification and the approximate range of concentrations likely to be expected, as well as understanding the plant matrix and mass of sample likely to be available for digestion, as each of these factors will impact on the final digestion methodology.

Once the sample has been suitably digested, the resulting liquid extract can then be analyzed with ICP-OES and/or AAS. The principle behind both of these methods is similar and based on the signature spectral absorption/emission of individual elements. With AAS, the liquid sample is passed through a flame at more than 2000°C and volatilized. A light of wavelength specific to the element of interest is passed through the flame, and the higher the concentration of the specific element in the sample (and consequently the flame), the more light is absorbed. The light that passes through the flame is recorded and this can be used to determine the concentration of that element in the digested sample. Similarly, with ICP-OES, liquid digests are exposed to temperatures of up to 10,000 °C. When exposed to these temperatures, this results in excitation of the constituent atoms in the samples and results in emission of light. The wavelength of the emitted light is specific to the element, thus enabling elemental identification. Furthermore, the intensity of the emitted light is proportional to the elemental concentration. With the use of suitable calibrations, it is possible to determine the concentration of the element in the digested sample and consequently the micronutrient(s) concentration in the original sample of plant tissue.

Inductive coupled plasma-optical emission spectroscopy (ICP-OES) has been the “gold standard” for micronutrient analysis due to the high accuracy, wide analytical detection range, and expansive elemental analysis possible. However, this high quality analysis comes at a cost both in terms of expense (such as.: equipment, high purity reagents required, and consumables) and time (sending samples for analysis, possible quarantine delays, and pre-analysis preparation). There are many considerations when producing

high quality micronutrient analysis with ICP techniques. This includes ensuring samples are clean and no contamination occurs during sample preparation, digestions, and analysis.

Atomic absorption spectroscopy (AAS) is a less expensive analysis method both in terms of instrument outlay costs and analysis costs. However, this method requires greater volumes of digested plant material when compared with ICP-OES, and is generally limited to only one element analyzed in a single run. While multiple elements can be analyzed simultaneously, this generally comes at the expense of sensitivity and accuracy.

Colorimetric

An alternative to ICP and AAS analysis is the use of colorimetric approaches to quantify elemental concentrations. The basis of this approach is exploiting the color change observed when specific reagents chelate with a metal ion of interest. The use of this technique is able to detect ppm levels of specific elements and has the added benefit of not requiring expensive equipment or pre-analysis digesting. Unlike ICP and AAS, each colorimetric reagent is specific to a single element, which is particularly useful when screening for biofortification trials as most trials are focussed on breeding for high levels of a specific micronutrient (that is Fe or Zn). Consequently, staining techniques have been used widely within the HarvestPlus program to screen for genotypes with high levels of micronutrients. For Fe screening, Perl's Prussian Blue (PPB) and 2,2' dipyridyl stain have been reported, with Zn screening achieved by staining with Dithizone (DTZ, diphenyl-thio-carbazone) and Zincon® (2-carboxy-2-hydroxy-5-sulfoformazyl benzene). Each of these reactions results in the formation of a colored chelate in the presence of the specific metal ion. The intensity of the color change is proportional to the concentration of the metal (under optimized conditions). Consequently, it is possible to identify the genotypes with high levels of the micronutrient of interest from those with low levels based on a visual inspection. The method was further improved to enable semi-quantitative analysis of micronutrient concentrations with the use of image analysis software such as Adobe Photoshop® and ImageJ as demonstrated by Choi *et al.* [23] and Duarte *et al.* [24], respectively. By using this combination of staining and image processing, it was possible to achieve results correlating color intensity with reference micronutrient analysis (ICP-OES) with $r^2 > 0.8$ for both Fe and Zn [23]. This enables semi-quantitative analysis without the need for expensive analytical equipment, and with only a handful of readily available chemicals and computer software, enabling high-throughput screening even in basic laboratories.

XRF

In recent years, the use of X-ray fluorescence spectroscopy has been demonstrated for screening Zn and Fe in crops for biofortification breeding programs within HarvestPlus [22, 25]. The benefits of this method are the lack of hazardous chemicals required along with minimal pre-analysis preparation. Samples can be screened in either whole grain or flour form. The advantages of the former include reduced sample processing time along with reduced risk of contamination. Conversely, flour analysis improves the reproducibility and accuracy, but increases the likelihood of contamination and increases labour in between samples. The aim of this technique within the HarvestPlus program is for rapid screening of Fe and Zn levels in breeding programs, which is followed by more

accurate analysis (that is AAS or ICP-OES) performed on those genotypes identified with high micronutrient levels.

With XRF analysis, the sample is exposed to X-rays and this results in excitation of the elements in the sample. De-excitation occurs and results in the emission of secondary "fluorescent" x-rays. The energy of the secondary x-ray is specific to each element and the abundance proportional to the concentration. Consequently, this technique is both qualitative and quantitative, enabling identification of elements and, with suitable calibrations, determination of elemental concentration.

CONCLUSION

High-throughput analytical methodologies are critical for the integration of biofortification strategies into mainstream plant breeding. Without these technologies in place, plant breeders do not have the tools to efficiently and cost-effectively screen thousands of genotypes in the search for donor parents and for ongoing selection within breeding populations. HarvestPlus has supported the development of fast and accurate analytical expertise, which is cost-effective; whether this be XRF for metal analysis or NIRS for pVACs. The analytical system is not perfect, and as biofortification research continues to be adopted within breeding programs, new high-throughput analytical technologies will be developed to keep pace with a growing demand. Given the advances made in high-throughput screening technologies in the last 10-15 years, the advances in the next 10 years are sure to be significant, strengthening the breeding pipeline for development of biofortified crops.

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Table 6.1: Summary of the effectiveness of methods used for quantification of total carotenoids and provitamin A carotenoids in biofortified crops

	Total carotenoid analysis	Provitamin A carotenoid analysis (in the presence of other carotenoids)	Current application in biofortified crops	References
Color scoring	+	-	None	[4]
Spectrophotometry	+	-	Sweet potato	[1]
HPLC	+	+	Cassava Maize	[5, 6]
UPLC	+	+	Maize	[7, 8]
NIR	+	+	Sweet potato Cassava Maize	[9, 10] (npalacios, 2016)
iCheck	+	-	Cassava	[11]

HPLC – High-Performance Liquid Chromatography
UPLC – Ultra-performance liquid chromatography
NIR – near-infrared spectroscopy

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Table 6.2: Current Fe and Zn analytical methods used by HarvestPlus for target crops

	ICP-OES	AAS	XRF	Colorimetry	
				Fe stain PPB and 2,2'-dipiridyl	*Zn stain* DMZ and Zincon
Grinding	Yes/ No	Yes/ No	Yes/ No	Yes/ No	Yes/ No
Digestion	Yes	Yes	No	No	No
Qualitative	Yes	No	Yes	No	No
Quantitative	Yes	Yes	Yes	Semi-quantitative	Semi-quantitative
Destructive	Yes	Yes	No	Yes	Yes
Fe detection	Yes	Yes	Yes	Yes	No
Zn detection	Yes	Yes	Yes	No	Yes
Al/ Ti detection	Yes	Yes	No	No	No
Other elements	Yes	Yes	Yes	No	No
Simultaneous analysis	Yes	No	Yes	No	No
Analysis time	2.5 min/ sample	2.5 min/ element	1 min/ sample	4 min/ sample	4 min/ sample
Instrument cost	$50 000 – 300 000	$10 000- 50 000	$50 000-100 000	-	-
Gas required	Yes	Yes	No	No	No
Reagents required	Yes	Yes	No	Yes	Yes
Cost per sample (approx.)	$5-10	$1-3	$1	$1	$1
Fe LOD (plant material)	1.2 µg kg^{-1} ^	2.0 mg kg^{-1} #	3 mg kg^{-1} +		
Zn LOD (plant material)	0.9 µg kg^{-1} ^	0.4 mg kg^{-1} #	7 mg kg^{-1} +		

^ Wheal *et al.* [20]
# Motsara *et al.* [21]
+ Paltridge *et al.* [22]

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