

# Leaf Analysis in Citrus: Developments in Analytical Techniques

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## Abstract

The complexity and sophistication of instruments for plant tissue analysis continues to advance, both in research laboratory grade instruments as well as for field use. These advances are seen in direct measurement of nutrients and trace elements, as well as indirect measurement, especially as statistical approaches improve through modeling of relationships between direct and indirect methods. This chapter has given an overview of recent advances, often using review articles followed by selected applications to citrus leaf analysis, but also offering other venues for using these analytical tools to aid both research and precision citrus management.

## Keywords

Leaf analysis • High temperature slow oxidation • Microwave digestion • Automated color analysis • Segment flow analysis • Flow injection technology • Discrete analysis • Inductively coupled plasma analysis • Isotope ratios • Sensors • Infrared spectroscopy • Laser-induced breakdown spectroscopy

## 6.1 Introduction

Plant tissue testing is a common tool of agronomists and horticulturalists for diagnosing nutrient requirements for optimum performance/production. Commonly, leaf tissue is the sampled plant part upon which selected analyses are conducted to determine nutrient or trace element concentra-

tions. Subsequently, these results are interpreted, usually based upon research with the crop of interest allowing calibration of nutrient or trace element concentrations into deficiency, sufficiency, or toxicity. Much less common is research that generates crop response to added nutrients based upon plant tissue concentration interpretations during the growing season.

Citrus leaf tissue interpretations are discussed in other chapters of this book; however, publications by Smith (1966) and Embleton et al. (1973) are seminal works. Subsequent research into citrus leaf tissue interpretations has for the most part confirmed these early summaries.

Laboratory analyses leading to the interpretation of nutrient and/or trace elements are controlled by a number of factors, most of which will be covered in detail in other chapters. It is important, however, to review these pivotal results briefly to provide a framework for this analysis. For example, Ontermma et al. (1996) discuss their observed reliability for citrus analyses applied to field production decisions and fertilization recommendations.

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Analysis of any tissue is essentially a measurement of what the plant has integrated through many uptake, distribution, metabolism, and stage of growth hormonal controls since planting. Said another way, tissue analysis will provide an insight into the physiological response of the plant to specific nutrient management throughout the previous 3–6 weeks before tissue sampling. This integration by the plant is influenced by the mobility of the nutrient(s) of interest within the plant. For example, consider quite mobile nitrogen (N) compared with immobile boron (B). One can readily see that the stage of leaf development for citrus is important since newly forming leaves are considered sinks while older leaves may act as sources if roots are unable to take up sufficient concentrations from the soil. For this reason, most diagnostic or monitoring protocols for orange tree leaf tissue samples are based upon newly mature leaves, for example, 4–6 months after a growth flush (Alva et al. 2006), while others have identified 6–7 months after a flush for mandarin (Manivannan and Chadha 2011). Alva and colleagues also reported citrus yield and quality response for a range of N and potassium (K) concentrations, but found no response to phosphorus (P) tissue concentrations from 0.8 to 2.4 g kg<sup>-1</sup> (Alva et al. 2006).

Similarly, human activities can affect the results of the laboratory analyses. For example, the preparation of leaves for analysis has an influence that appears to be inconsistent when leaves are washed by various methods to remove or minimize the effects of previously applied foliar sprays (Futch and Gallaher 1996; Alva and Tucker 1997). Futch and Gallaher (1996) focused on zinc (Zn) due to its affinity for sorption to the waxy cuticle on the upper surface of citrus leaves, finding advantages to specific washing methods, while Alva and Tucker (1997) note no benefits for washing with a detergent/acid/rinse procedure for all macro- and micronutrients. These researchers suggested that the time between micronutrient sprays and normal tissue sampling (approximately 6 months) was sufficient to avoid surface contamination in Florida, except for copper (Cu), a micronutrient known to bind tightly with organic compounds.

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## 6.2 Intent of This Chapter

It is the intent of this chapter to present new analytical developments and deal specifically with analysis of selected nutrients or trace elements. Where possible, we have cited literature that summarizes earlier work and we then focused on new analytical equipment or innovative uses or combinations of instruments to enhance accuracy and precision. When a new or innovative approach could apply to citrus in our judgment, we have included information on that approach, even though citrus may have not been the research focus.

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## 6.3 High-Temperature Slow Oxidation

The literature contains considerable information concerning leaf tissue preparation. Procedures vary based upon the type of sample processing in the analytical phase. Typically, ashing in a muffle furnace requires grinding to pass either a 1- or 2-mm screen depending on the quantity to be ashed (Campbell and Plank 1998). Dry ashing oxidation is affected by the internal surface of the muffle furnace since the surface either sorbs or releases specific elements, especially boron (B) (Miller 1998a). Other nutrients and trace elements, such as sulfur (S), arsenic (As), mercury (Hg), and selenium (Se), are subject to volatilization and must be processed by other means for quantification (Miller 1998a). Temperature selection is also well documented since other elements respond through potential volatilization, and typically, internal temperatures exceeding 500°C should be avoided. Controllers that allow so-called ramping up and down of internal temperatures are useful, permitting the oxidation to proceed sufficiently fast to ensure laboratory time efficiencies but slow enough to avoid rapid oxidation (burning) with resulting uncontrolled temperatures in the sample.

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## 6.4 Microwave Digestion

As with muffle furnace digestion, abundant literature describes successful options for liquid digestion of plant tissue using microwave heating. Power requirements and the ability to control the input of energy for digestions have advanced through the years. Vessels have been explored that may be sealed or vented, and selected acids have also been tested using a number of plant tissues and compared with muffle furnace digestions (Soon and Kalra 1994; Kalra and Maynard 1998; Miller 1998b).

Different acids and acid/catalysts have been used, but nitric acid or sulfuric acid with hydrogen peroxide appear to be the most frequently tested (e.g., Esslemont et al. 2000).

Samples are heated by an oscillating electromagnetic field having electromagnetic radiation of frequencies 100–100,000 megacycles per second (Kalra and Maynard 1998). Because the radiation energy is applied directly to the digestion mixture, this radiation provides extremely rapid heating and good control of power and time in the process of plant tissue digestion (White and Douthit 1985).

Microwave digestion technique for sample dissolution/digestion uses microwave radiation in high-pressure Teflon PFA vessels, which are transparent to microwave radiation (Sah and Miller 1992). Teflon PFA is chemically inert and maintains physical integrity to a temperature as high as 260°C. The advantage of this technique includes rapid

sample dissolution, minimizes contamination, and does not require  $\text{HClO}_4$ . Microwave digestion has been used successfully to extract organic samples for further analyses, such as atomic absorption, inductively coupled plasma mass spectroscopy (ICP-MS), and chromatography (Rea and Keeler 1998). Microwave dissolution gives excellent recovery of elements when analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (Abu-Samra et al. 1975; Nadkarni 1984; White and Douthit 1985).

However, the speed at which the samples are heated in the microwave may result in exothermic reactions during the digestion process (Berghof Products and Instruments GmbH 2011). To avoid this undesirable result, selected sensors have been developed to monitor the pressure and temperature, which can be regulated by changes in the microwave power output or changes in digestion time.

## 6.5 Instrumentation

### 6.5.1 Automated Color Analysis

As analytical techniques were automated, those instruments based on absorption spectrophotometry were grouped under Automated Color Analysis technique. The basic technique called “continuous flow” invented by Skeggs, a clinical chemist, was commercialized in 1956 and sold using the brand name Technicon, still widely in use. The instrument was earlier also referred to as Sequential Multiple Analyzer or Sequential Multiple Analyzer with Computer and more currently is being sold as AutoAnalyzer. These instruments are capable of multichemistry analyses enabling determinations of nitrate, phosphate, etc., and their species, simultaneously. At least three major variations using this technique are currently available in the laboratories around the world. A survey done in Australia (Handson and Shelley 1993) revealed that at least one colorimetric procedure for plant analysis was employed in 70% of the labs.

#### 6.5.1.1 Segmented Flow Analysis (SFA)

The underlying technique of AutoAnalyzers is called “segmented flow analysis,” which has revolutionized laboratory analyses by significantly increasing the number of samples processed from a few dozen to a few hundred per day. A continuous stream of samples is separated by air bubbles into discrete segments, which are then transported into mixing coils through microtubings to various optional modules. The modules may include distillation, dialysis, extraction, heating, signal detector, etc., as appropriate. The introduced air or nitrogen gas (in some versions) bubbles act as barriers between the sample segments preventing cross-contamination or intersample dispersion during their transport and forming

unique reaction segments. The bubbles also aid in chemical mixing of the reagents and sample in glass coils. Glass is generally preferred as it is inert, allows for visual checks, and is easy to clean. The diameter and the length of the coil control the rate and time of the reaction. Newer instrument models employ 1-mm coils, thus increasing the throughput of the samples. The unique feature of the SFA technique is that all the reactions are allowed to reach a completion or a steady state before the final measurement is recorded, thus increasing the sensitivity of the measurement as the chemical strength will be highest at the completion of the final reaction.

Ceballos et al. (2006) successfully used the SFA technique to determine total nitrogen in cassava root samples to estimate crude protein content. No significant difference ( $p=0.05$ ) was found when the cadmium column in the air-segmented continuous flow system was replaced by an enzymatic reduction method using soluble nitrate reductase purified from corn leaves for determination of nitrate+nitrite in natural water samples, providing a nontoxic alternative to cadmium (Patton et al. 2002).

#### 6.5.1.2 Flow Injection Technology (FIA)

The second type of “continuous flow” instruments is built using a technique called the “flow injection analysis,” invented in 1974 (Růžička and Hansen 1980). These systems use a design similar to that of SFA, but instead of air bubbles, the samples are separated by a carrier agent. The sample, carrier agent, and reagents are mixed to obtain a concentration gradient. By removal of air bubbles used in the SFA system, FIA instruments can be miniaturized considerably. In the FIA systems, the reactions are not required to reach completion or a steady state since both samples and reagents are allowed the same time to react. Dispersion of the sample during analysis renders the FIA systems unsuitable for kinetically slow reactions (Gardner and Malczyk 1983). In the FIA systems, precise timing of the reaction is crucial to the accuracy of the measurement.

The FIA technique has been further combined with other instruments to prepare the samples and to remove interferences with resulting improved sensitivities. Carrasco et al. (2007) compared colorimetric analysis of ammonium, phosphate, and nitrite in wastewater using multicommutated peristaltic and solenoid FIA systems. These researchers determined that the peristaltic system had more versatility, potential for miniaturization, and flexibility requiring no physical reconfiguration of the flow manifold (Carrasco et al. 2007).

#### 6.5.1.3 Discrete Analysis

Use of discrete analyzers in soil and tissue testing started circa 2002, and the instruments have quickly become an attractive alternative in the automated color analyses arena

due to their unique ability to offer hassle-free change among chemistries for multiple analytes, consistent and reproducible results, and reduced sample and reagent volume requirements. Analysis in these instruments is carried out in separate reaction chambers and does not include coil assemblages. This discrete setup allows for multiple tests on individual samples and/or different tests on different samples. Also, reagent addition and mixing for new samples are automated and can be performed simultaneously as analysis of the previous sample is being performed. This technique measures the concentrations of the analytes after the reaction reaches a steady state.

### 6.5.2 Atomic Absorption and Inductively Coupled Plasma Spectroscopy

Evans et al. (2003) and Husted et al. (2011) provide reviews for the use of atomic absorption spectrometry (AAS), which is a mature analytical method for plant tissue analyses. The functionality of AAS has been greatly increased by additions of gas selection, hydride generation methods, and automation of multiple light sources.

Advances using inductively coupled plasma (ICP) have allowed this technology to see widespread use for plant tissue analyses. While AAS is often limited to sequential analysis as a function of the light source(s), ICP can provide sequential or simultaneous analyses of multiple elements due to the higher energy associated with the plasma and the design of light bench/sensor array technology (Husted et al. 2011).

ICP spectrometry includes combinations of analytical techniques including ICP-optical emission spectrometry (ICP-OES) and ICP-mass spectrometry (ICP-MS), among which ICP-MS is the preferred analytical technique for routine analysis of plant tissues (Laursen et al. 2009). This technique has been successfully used in numbers of studies for analyzing citrus leaf tissues for different elements (Boaretto et al. 2008; Menesatti et al. 2010; Kraska and Breitenbeck 2010). However, full-quantitative ICP-MS analysis is time as well as labor consuming, and its capacity is not fully utilized. Therefore, semiquantitative ICP-MS has been recommended to be the strong alternative of the full-quantitative ICP-MS, which relies on a simple calibration procedure (Taylor 2001), for analyzing leaf tissues of various plants including citrus (García-Alonso et al. 1997).

Mass spectrometry is based on the physical properties of the atomic nucleus consisting of protons and neutrons (Becker 2008). By means of spectrometry, the mass of atoms and molecules can be determined by measuring the mass to charge ratio. The mass of an atom or molecule is measured in a multiple of the atomic mass constant, not in g or kg (Becker 2008). Samples are analyzed after microwave-induced

digestion using closed vessels and acid/catalysts such as nitric acid, hydrogen peroxide, and fluoric acid. An ICP typically has five different components: sample introduction system (nebulizer), ICP torch, high-frequency generator, transfer optics and spectrometer, and computer interface. The torch assembly consists of three concentric tubes, usually made of silica, that are termed as outer loop, intermediate loop, and inner loop. The torch is situated within a water-cooled coil of a radio frequency (r.f.) generator. As flowing gases are introduced into the torch, the r.f. field is activated and the gas in the coil region becomes electrically conductive; this sequence of events results in formation of plasma (Bradford and Cook 1997).

### 6.5.3 Linking Together of Instrumentation for Greater Specificity

With linkages between or among selected instrumentation types, often referred to as multiple hyphenated instrumentation, for example, ICP-MS, the ability to analyze nutrients and/or trace elements has expanded. ICP-MS phosphorus (P) determination from a low-volume microwave digestion was compared with colorimetric flow injection analysis or aluminum block digestion and vanadomolybdate P colorimetric analysis (Esslemont et al. 2000). In addition to the fully quantitative methods that most laboratories use, Laursen et al. (2009) have employed a simultaneous ICP-MS (mass spectrometer) method for a reliable semiquantitative analysis of plant tissue digestions. The MS is used to estimate elements throughout the mass scan range, a small set of elements compose a suite of internal standards, and algorithms are used to estimate tissue concentrations. Determining concentrations for up to 70 elements at speeds fivefold that required for full quantification using traditional ICP-MS procedures, this process is useful for a number of field-scale management decisions and has been tested with a number of different plant species, including citrus (Laursen et al. 2009).

When high-pressure liquid chromatography (HPLC) is combined with hydride gas generation (HG) and atomic fluorescence spectroscopy (AFS), the resulting HPLC-HG-AFS system is useful for selected trace element analysis. Nash et al. (2002) produced a review of this linkage focusing on antimony (Sb). Antimony (Sb) is of great health concern for human and animal food chains and is an excellent example of this linkage system. The use of HPLC-HG-AFS instrumentation to determine Sb has been refined for work with both solids (marine sediments, Potin-Gautier et al. 2005) and liquids (sea water, Gregori et al. 2005). The process uses the liquid column to create separation of the Sb from other constituents and among Sb species/compounds. The HG portion creates stibine gas, which is then introduced into the AFS detector. These two groups of researchers identified EDTA as

an important chelate in the HPLC phase to stabilize trivalent Sb, the most reactive species. The chemistry and analytical technique should apply to citrus leaf tissue as well for other selected elements.

#### 6.5.4 Isotope Ratios

Micronutrients are often used as a target for foliar applications. This application method affects, to some extent, the traditional analyses since the element may be sorbed in some fashion to leaf, but not functional with the plant system as a nutrient (see Sect. 6.1). The effectiveness of these foliar sprays is often recorded as the amount applied and subsequent yield improvement compared with a 0 application rate. Boaretto et al. (2011) compared boron (B) use in low-fertility tropical soils of Brazil. Fertigated 4-year-old “Valencia” sweet orange trees on “Swingle” citrumelo rootstock were treated with either foliar spray or soil-applied isotopically enriched  $^{10}\text{B}$ . This technique allowed the researchers to compare the isotope ratios found in fruit that was solely from the foliar or soil applications, and reduce the interference of B from native soil sources. Soil applications allowed 21% recovery of the enriched B, while only 7% B originated from foliar applications (Boaretto et al. 2011). While enriched or depleted sources of nutrients and trace elements are useful for exploring research aspects that affect management decisions, use of this technique in citrus leaf diagnostic programs is unlikely.

#### 6.5.5 Sensors

The development of sensors, often using a light source that impinges on individual plant parts or, more broadly, whole canopies, is an active field of research. Enhanced by state and federal programs in many countries, this research area continues to produce interesting results. Currently, this approach to make field-scale decisions based on representative sampling is not a rigorous analytical essay, but rather an indirect measurement. Since this topic is quite broad, we provide only a couple of examples.

Typical of sensors used for plant parts, Min and coworkers (2008) produced a sensor that indirectly measures nitrogen (N) in a single leaf. A light source impinges on the leaf, which is shielded from other ambient radiation. Filters pass selected wavelengths of reflect light from the leaf surface to the actual sensor. The wavelengths are often chosen for those that are sensitive to known leaf and nutrient properties, in this case, leaf chlorophyll and proteins, which relates well to N (Min et al. 2008). The sensor was measuring reflected light from 620 to 950 nm and 1,400–2,500 nm with a resolution of less than 30 nm. Comparing the calibrated sensor with more

traditional chemical analysis results, the root mean square difference was  $1.69 \text{ g kg}^{-1}$ , which was sufficiently accurate to group citrus N concentrations into low, medium, or high categories with 70% accuracy (Min et al. 2008). This achievement is comparable with specific ion electrodes using sap expression for nitrate-N, but is considerably faster than the electrode method.

Whole grove indices can be developed using the so-called normalized difference vegetation index (NDVI), which compares near-infrared and visible reflectance difference to the sum of both. Fletcher et al. (2004) report on one such study for citrus using a commercially available sensor mounted in an aircraft. Tree health was easily differentiated between the stressed block and the unstressed block. The usefulness of this type of measurement is that entire blocks and groups of blocks can be sampled quickly and results using NDVI calculations are quickly analyzed and interpreted. Depending on the selectivity of the sensor, additional reasons for stress may be predicted.

Combining NDVI measurements with other information from the grove, Mann et al. (2011) produced productivity zones. The primary indicator was found to be canopy volume, which itself was ultrasonically recorded using another type of sensor. NDVI and soil characteristics were all strong indicators of yield. This integration for management of citrus is another indicator of the value of this type of sensor research and its applicability.

#### 6.5.6 Near-Infrared Spectroscopy

Near-infrared (780–2,500 nm) instrumentation is being used throughout the citrus industry. Laboratory-grade instruments can be controlled via filters or equipped to scan through a range of wavelengths, often with a precision diffraction grating. In general, instruments are designed to focus on specific bonds, such as C–H, N–H, or O–H, that can be made to generate oscillations when an NIR source is used to stimulate overtones and combinations (Foley et al. 1998). For solid materials, like finely ground citrus leaves, NIR can penetrate the substance more deeply than visible or mid-infrared wavelengths, allowing a larger volume of material to respond to this energy input. The reflectance energy, either at specific filter settings or a spectrum scan, can be subjected to statistical analyses where the reflectance is related to standardized chemical analyses. For this reason, most NIR results are indirect measurements.

Portable equipment may also represent a combination of visual light and NIR. Citrus leaves (*Citrus sinensis* (L.) Osbeck cv. Tarocco) were analyzed individually ( $n=50$ ) using a pen probe and portable spectrophotometer followed by chemical analyses (Menesatti et al. 2010). While a number of statistical comparisons were made, potassium (K,  $r=0.991$ ) and magnesium (Mg,  $r=0.883$ ) were correlated

well while phosphorus (P,  $r=0.481$ ) was poorly correlated (Menesatti et al. 2010). Variations observed in this study are typical of this type of indirect measurement that relies on statistical modeling of the resulting reflectance data to that of chemical analyses.

While the literature contains many references statistically calibrating NIR or NDVI to leaf tissue nutrient concentrations, this indirect technology can also be used in many other ways as both a research tool and as a quality control asset where qualitative information can be useful. For example, the quality aspects of citrus fruit can be approximated by NIR to assess quality-related attributes (Abbott 1999). Using portable NIR equipment, individual readings of citrus fruit were taken on trees and statistically regressed with refractometer readings (Zude et al. 2008). Findings indicated that fruit development and maturity were greatly affected by precipitation and that individual fruit maturity dates could be generated to predict grove harvesting windows for high fruit quality (Zude et al. 2008).

Blasco et al. (2007) expanded fruit-grading line cameras to include NIR and ultraviolet spectra in addition to the normal use of visual spectra. These innovations successfully identified imperfections that would have gone unnoticed if just visual spectra were used. These researchers reported increased profits from high-quality and acceptable-quality fruit being packed and shipped separately, receiving a premium for the high-quality fruit (Blasco et al. 2007)

### 6.5.7 Laser-Induced Breakdown Spectroscopy

Citrus leaves and many other plant parts can be analyzed using laser-induced breakdown spectroscopy (LIBS). After particulate size reduction (grinding), a pellet is lased and the resulting plasma emission is quantified using a spectrophotometer. Gomez et al. (2011) studied the effects of different types of tissue particle reduction. Ball milling and cryogenic grinding were more successful as measured by a 50% emission signal enhancement on LIBS measurements. These researchers found that pellets created with a particle size less than 7.5  $\mu\text{m}$ , somewhat independent of grinding method but dependent on grinding time, proved to be superior. In this study, the laser source produced 25  $\text{J cm}^{-2}$  at 1,064 nm for 25 pulses at 5 ns per pulse laser pulses per pellet site (Gomez et al. 2011). In contrast, other researchers tested a low-power laser that produced <1 mJ on raw citrus (*Citrus unshiu*) leaves (Ohta et al. 2009). The ablation process (dissipating leaf tissue moisture with the laser before taking a reading) was much more successful when metallic colloidal particles were applied to the raw leaf surface. The researchers identified the improvement as localized surface plasmon resonance that could be further enhanced by correctly selecting the size and material makeup of the colloidal particles.

## 6.6 Future Research

Instrumentation continues to both improve and become more automated increasing accuracy and precision while reducing analytical costs. With respect to citrus, analyses have aided in the documentation of plant responses, but the problems of scale are still an issue. For example, chemical and physical models of selected processes among cells and organs within cells do not translate well to whole trees, or from single trees to groves. Instrumentation, including 3-D printing, selected radiation tomography, and advances in computer algorithms, is likely to advance our knowledge regarding these issues of scale as these analyses and others are incorporated into current instrumentation in novel ways. In conclusion, the integration of scales must relate the citrus organism to its changing climate throughout the world. Analyses that aid in the understanding of the chemistry of plant responses to changing conditions are needed to maintain high-quality citrus production for generations to come.

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