Title: Fluorescence tools for sensing of quality-related phytochemicals in fruits and vegetables

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ABSTRACT

The quality of fruits and vegetables can be related to particular classes of compounds possessing peculiar optical properties. This aspect has been exploited by developing specific spectroscopic techniques for the non-destructive detection of such compounds, which are also suitable for the product quality monitoring. Among these, fluorescence spectroscopy was preferentially employed for a long time in the labs of the authors. By using various tools, partially developed by the authors, this technique proved to be suitable not only in basic research, but also for applied purposes. In this chapter, we are going to recall basic concepts of fluorescence and fluorescence measurements, give a brief description of different fluorescence methods employed in the acquisition of parameters describing the quality of fruits and vegetables and define what are the quality-related phytochemicals detectable by fluorescence. A report on portable fluorescence sensors available for in-field measurements is presented in subsection 4.3. Finally, some recent applications of the various fluorescence techniques and sensors on quality assessment of both fruits and vegetables are outlined.

1. INTRODUCTION

Since the first scientific approach to the fluorescence phenomenon in the middle of the 19th century (Valeur and Berberan-Santos, 2011), fluorescence spectroscopy became a useful analytical tool to characterize molecules.

It is amazing that the first application of fluorescence, dating back to 1565, concerned the identification of a water extract, from a Mexican tree wood, with curative properties (Valeur and Berberan-Santos, 2011). Nowadays, fluorescence is still largely employed to investigate the plant physiological status or to typify phytochemical products.

In principle, several plant constituents could be detected *in vivo* by measuring their fluorescence (Duval and Duplais, 2017; Garcia-Plazaola et al., 2015). However, the overlapping of various emission bands, the low intensity of fluorescence and the localization of compounds into the plant organs significantly restrict the number of detectable phytochemicals.

Because of the limited penetration of the excitation wavelengths into the sample tissues, fluorescence methods can give information only on compounds present in the superficial layers. They are, therefore, useful to detect compounds mostly concentrated into the skin. This may represent a limitation of the technique, however, often the detectable superficial compounds are well correlated to the total compounds. Moreover, indirect correlation between the measured signals and different quality parameters can be found, even with the help of chemometric analyses.

Chlorophyll is the main fluorophore present in vegetable and green fruit samples. Chlorophyll fluorescence (ChlF) has been largely employed to assess the photosynthetic activity of plants (Baker, 2008; Kalaji et al., 2017; Murchie and Lawson, 2013). Senescence processes accompanying fruit ripening include chlorophyll

degradation and a decrease of photosynthetic activity. Therefore, chlorophyll fluorescence can be correlated to fruit quality parameters, such as sugar content and firmness, which change with maturity.

Polyphenols represent an interesting class of compounds that should be worthy to estimate because of their antioxidant health promoting and disease preventing properties (Chiva-Blanch and Badimon, 2017; George et al., 2017; Nabavi et al., 2017; Vanamala, 2017). Some of them possess significant fluorescence quantum yields allowing their direct *in situ* detection by fluorescence techniques. Others, present in leaf epidermises and fruit skins, can be determined indirectly analyzing the chlorophyll fluorescence from the underlying cell layers.

This last technique, called the chlorophyll fluorescence excitation screening (ChlFES) method, is relatively new, being developed within the last 20 years. It is based on the filtering effect of phenolic compounds that reduces the incoming light impinging on chlorophyll molecules.

The description of the method and its application to vegetables and fruits will be the main issue of this chapter, although other suitable fluorescence methods will be mentioned.

Due to the importance in extending the techniques from the lab to the field, particular emphasis is given to the description of portable fluorescence sensors and their use directly on cultivations.

Fluorescence spectroscopy has been also applied to the analysis of products and extracts from fruits and vegetables. We do not consider this application here, limiting to the description of *in situ* determinations.

2. FLUORESCENCE METHODS

2.1. Basic concepts of fluorescence and fluorescence measurements

The term *fluorescence* refers to the physical phenomenon whereby a molecule excited by the absorption of electromagnetic radiation releases part of its energy as photons (Lakowicz, 2006). The molecules that take part to this process are then named fluorophores. As depicted in Fig. 4.1A, following the absorption of one photon, occurring within 10^{-15} s, the fluorophore reaches an excited state of higher energy. This is an unstable and temporary condition lasting for about 10^{-9} s. The interaction of the excited molecule with its environment leads to different processes of energy relaxation such as i) thermal dissipation, ii) production of other molecules (photochemistry) and iii) emission of photons, i.e. fluorescence. Because of the presence of different minor competing relaxation processes already in the excited state, the energy of the emitted photon is usually only a fraction of that of the absorbed photon. Consequently, the wavelength of fluorescence is always longer than that of the excitation light. Such aspect is depicted in Fig. 4.1B where the absorption and fluorescence spectra of malvidin 3-O-glucoside in solution are reported. The shape and width of the spectra are determined by the structure of the vibrational and rotational energy levels, within each electronic level, allowing for different, more or less probable, absorption and emission transitions.

FIGURE 4.1 (A) Schematic representation of the absorption-relaxation processes occurring during the light-fluorophore interaction. (B) Absorbance and emission spectra of malvidin 3-O-glucoside (see structure drawn at the left of panel A) in aqueous/EtOH solution at pH 1.0.

The efficiency of a molecule to emit fluorescence is measured by the fluorescence quantum yield, which is defined as the number of photons emitted per number of photons absorbed:

$$\phi_F = \frac{\# \text{ of emitted photons}}{\# \text{ of absorbed photons}} \quad (1)$$

or alternatively,

$$\phi_F = \frac{k_F}{k_F + k_{NR}} \quad (2)$$

as the ratio between the fluorescence deactivation rate (k_F) and the total of the deactivation rates, that is the sum of k_F and all the non-radiative decays (k_{NR}).

This value can range from almost 0 to close to 1, depending on the molecule's structure and the environment around it. For example, when increasing molecule rigidity or reducing the temperature the fluorescence quantum yield increases, since non-radiative processes, as due to rotational-vibrational motion and collisions, are reduced.

The organic molecules employed in the laser technology possess rather high ϕ_F , so that their fluorescence can be observed by the naked eye, as shown in Fig. 4.2.

FIGURE 4.2 (A) Bright yellow fluorescence from coumarin in ethanol under blue excitation, to be compared to the pale-yellowish appearance under ambient light (B). A stilbene ethanolic solution emits blue fluorescence under UV irradiation (C), while it appears transparent under ambient light (D).

In order to properly measure the fluorescence intensity of a sample, attention must be paid that the detector is not reached by the measuring light strayed by the sample. Stray light can have an intensity comparable to or even higher than that of the fluorescence, affecting significantly the signal-to-noise ratio of the measurement. Fig. 4.3 shows the basic set-up for fluorescence measurements. The excitation source should preferably emit in a narrow spectral band, to reduce tails overlapping to the detector spectral window. A short pass filter in the excitation beam and a long pass filter in front of the detector, which avoid any spectral overlap of stray light, guarantee that the detector senses only fluorescence. The 90° detection with respect to the irradiation direction further reduces the entrance of excitation into the detector device.

Yet, a narrow-wavelength excitation can have the advantage of a more selective excitation of specific fluorophores of interest.

FIGURE 4.3 Basic set-up for fluorescence measurements. Samples are excited by monochromatic (laser) or narrow-band radiation. Fluorescence is detected by a photodiode or a photomultiplier at 90° with respect to the irradiation direction. The detector is preceded by a band-pass filter to limit stray light.

Fluorescence is usually weaker than ambient light, therefore its detection must be performed under darkness. Alternatively, under natural conditions, the fluorescence signal can be derived by using pulsed or modulated excitation sources and synchronized detectors. Electronic filtering will remove the noisy contribution of non-modulated ambient light.

2.2. “Pulse-Amplitude-Modulation” (PAM) method

Particularly in the measurement of fluorescence of chlorophyll, the main fluorophore in green vegetables, techniques using a modulated excitation beam have been applied. In 1986, the so-called Pulse-Amplitude-Modulation (PAM) technique was introduced (Schreiber, 1986). While other fluorophores present in plants such as coumarin emit fluorescence with a constant yield, chlorophyll fluorescence emitted from photosynthesizing chloroplasts is highly variable. Since photosynthesis is consuming the absorbed excitation energy photochemically (process 3 in Fig. 4.1.A) it directly affects fluorescence yield. Furthermore, processes regulating the photosynthetic light reactions are affecting chlorophyll fluorescence yield non-photochemically (process 2 in Fig. 4.1.A). Combining the PAM technique with short pulses of strong light saturating the photochemical reactions allows to monitor photosynthetic activity in intact plant organs, even under natural conditions in full sunlight. A deeper explanation of this technique is beyond the scope of this

article and the reader is referred to exhaustive reviews (Maxwell and Johnson, 2000; Schreiber, 2004). However, as the function of photosynthesis is intimately related to the function of the whole plant cells, chlorophyll fluorescence measurements are a potent means to report the progress of senescence or to indicate the effects of stress on leaves or green fruits.

2.3. Spectral measurements

The concentration and number of different phytochemical compounds in fruits and vegetables affects the size of the fluorescence signal. Their identity or at least the class of chemical compounds to which they belong can be elucidated by recording their fluorescence emission and excitation spectra.

As mentioned before, the dominating fluorophore in plants is Chl. The typical *in situ* ChlF emission spectrum consists of two bands with maxima in the red (685–690 nm) and far-red (730–740 nm) regions. In intact plant organs, its shape depends on the Chl content. In fact, as depicted in Fig. 4.4, the Chl absorption spectrum is partially overlapping the fluorescence spectrum. Therefore, the photons at around 690 nm emitted inside the leaf tissues, in their path to the detector will be partially re-absorbed by Chl itself. The re-absorption is proportional to the Chl concentration.

Because of this, the red/far-red Chl fluorescence ratio has been adopted as non-destructive index of Chl content (Buschmann, 2007). The use of the inverted far-red/red Chl fluorescence ratio (Gitelson et al., 1999) is even more practical because it is linearly related to Chl content and increasing with the increase in Chl. It is named Simple Fluorescence Ratio (SFR) in the recent literature.

The re-absorption effect explains also the observed dependence of the ChlF emission spectrum on the excitation wavelength (Lichtenthaler and Rinderle, 1988). Radiation around the absorption maxima of Chl (blue and red) penetrates less into the leaf tissues in comparison to radiation at wavelengths (green-orange) which are less efficiently absorbed. In the first case, excited ChlF originates mainly from the upper mesophyll cells, therefore only little re-absorption takes place.

FIGURE 4.4 Schematic representation of the fluorescence reabsorption effects inside a leaf. On the right, the case of lower chlorophyll concentration is represented: the fluorescence emitted (red area) inside the leaf (*at the chloroplast level*) undergoes a moderate reabsorption (green area) leading to a reduced red/far-red ChlF ratio in the detected spectrum. On the left, the Chl concentration is higher, determining a greater reabsorption of ChlF and then a larger change in the ChlF spectrum.

When UV excitation is used, blue-green fluorescence between 400 and 600 nm is recorded, in addition to the red/far-red Chl bands.

Assignment of blue-green fluorescence is complex since many different fluorophores can contribute to it. Lists of possible natural compounds showing UV-induced blue-green fluorescence are reported elsewhere (Cеровic et al., 1999; Lagorio et al., 2015). It is important to recall that localization of fluorophores in the sample tissues and the fluorophore's micro-environment are two fundamental factors determining the *in situ* detection of compounds. In fact, compounds located into the deeper sample tissues are likely not receiving a sufficient intensity of radiation to be significantly excited. On the other hand, the interaction of the fluorophores with their environment (viscosity, pH, presence of quenchers) can activate more or less deactivation channels (Fig. 4.1A) and then significantly change their fluorescence quantum yield. For this, phenolic and flavonoid compounds bound to cell walls and present in the vacuoles of epidermal cells are the most likely molecules to contribute to the measurable blue-green fluorescence in plants. Spectral measurements can be performed by rather different devices.

Bench laboratory spectrofluorimeters provide both emission and excitation spectra by using a xenon lamp as excitation source and monochromators to select wavelengths. Solid samples can be measured on a front-face geometry, also with the help of bifurcated optical fiber bundles (Pfündel et al., 2006).

Pulsed laser-excitation systems coupled with synchronized high-sensitivity detectors allowed to improve the signal-to-noise ratio even under ambient light. They have been used in proximal sensing laboratory

measurements as well as in the outdoors remote sensing of vegetation, embedded in a LIDAR device (Ounis et al., 2001; Rascher et al., 2009). On the opposite microscopic scale, fluorescence spectra can be recorded at the cellular and sub-cellular level. This is possible by using a standard wide-field epifluorescence microscope connected to a diode-array or charge-coupled-device spectrometer (Raimondi et al., 2009). Most of confocal microscopes can also record fluorescence spectra, but usually at low spectral resolution (≥ 5 nm). In Fig. 4.5, three examples of fluorescence spectra recorded by three different devices are reported.

FIGURE 4.5 (A) Fluorescence spectrum of a corn (*Zea mays* L.) leaf attached to the plant recorded in the field by means of a hyperspectral Fluorescence LIDAR (FLIDAR) imaging system with a pulsed Nd YAG laser excitation at 355 nm. (B) Fluorescence spectra, under 365 nm excitation, of the adaxial layers from a 70 mm thick *Olea europaea* L. leaf cross-section recorded by a diode array multichannel spectral analyzer connected to an inverted epi-fluorescence microscope. (C) Emission and excitation spectra of an intact plum (*Prunus domestica* L.) fruit recorded by means of an optical fiber bundle connected to a bench spectrofluorimeter. All spectra were measured by the research group of the first author.

2.4. Fluorescence imaging

The spatial distribution of fluorophores over the sample can be obtained by fluorescence imaging.

This technique first appeared as application in epifluorescence microscopy to image leaf tissues by high-pressure mercury lamps coupled with band-pass interference filters. Later on, it was employed in confocal microscopy mainly for the detection of exogenous fluorophore markers. In this way, intact leaf or fruit parts can be observed down to a depth of about 100 μm and 3D images of the fluorophore distribution can be obtained.

Multispectral fluorescence imaging is aimed to acquire several pictures of the sample at different emission bands. The elaboration of the digitized images is used to improve discrimination and sensitivity of the technique. Co-localization of different compounds can be presented merging separate pictures in a single image.

At the macroscopic level, the technique employs a pulsed excitation source (laser, LED or flash lamp) and an intensified CCD monochromatic camera with a filter wheel to select the acquisition spectral band. Usually for leaves and fruits, under UV excitation four emission bands in the blue, green, red and far-red regions are acquired.

A comprehensive description of the technique, the needed instrumentation and applications was reported by Buschmann et al. (Buschmann et al., 2008).

Imaging-PAM systems have been developed to study the light-induced ChlF kinetics at the entire leaf level. This is the extension of the PAM technique applied as punctual measurements by using as detector a CCD camera synchronized with the pulse modulated excitation light.

The application of ChlF imaging in horticultural research to detect plant stresses and assess postharvest quality of fruits and flowers has been reviewed by Gorbe and Calatayud (Gorbe and Calatayud, 2012).

2.5. The Chlorophyll fluorescence excitation screening method (detection of non-fluorescing compounds)

Non-fluorescing compounds present on the sample surface can be still indirectly detected by measuring chlorophyll fluorescence. This technique developed for the *in vivo* detection of polyphenols in leaves (Bilger et al., 1997) uses chlorophyll located below the compounds of interest as a sensor of light transmitted through the upper layers. Compounds absorbing fluorescence excitation light will cause a reduced emission of chlorophyll fluorescence.

The *in vivo* ChlF excitation spectrum represents the convolution of the absorption spectra of Chl *a* and of the accessory pigments, carotenoids and Chl *b*, which transfer the absorbed energy to Chl *a*. It contains also the negative contribution of compounds located in the epidermal layers above Chl that attenuate the excitation light reaching the chloroplasts (Figure 4.6, left side). (The absorption spectra reported in Fig. 4.6 refer to *in vitro* conditions, while *in vivo* band broadening and a shift to longer wavelength is expected.)

FIGURE 4.6 Left side, excitation, and emission spectra of a Chl containing sample (full lines) along with the positive spectral contributions due to absorbance of Chl and carotenoids located into the chloroplasts (dashed lines) and the negative spectral contributions due to absorbance of compounds (Flavonoids, Anthocyanins) (top of the figure) located into the leaf epidermis. The reported absorption spectra refer to *in vitro* conditions, while *in vivo* band broadening and a shift to longer wavelength are expected. Right side, schematic representation of the chlorophyll fluorescence excitation screening (ChlFES) method using the three-dimensional view of the chloroplast distribution (green color) in the adaxial portion of a *Phillyrea latifolia* leaf cross-section. Sixty Chl fluorescence images were recorded (at 0.3-mm-steps) along the z-axis in a Confocal Laser Scanning Microscope, with $\lambda_{exc} = 488$ nm and λ_{em} over the 687–756 nm waveband. The yellow coloration of the epidermis indicates the presence of compounds absorbing at λ_1 , but not at λ_2 . The attenuation at λ_1 leads to less chlorophyll fluorescence (less ChlF) with respect to excitation at λ_2 that fully penetrates to the mesophyll resulting in higher chlorophyll fluorescence (more ChlF).

Therefore, the intensity of ChlF at each excitation wavelength is given by

$$ChlF = I T_{ep} A_{chlo} \phi_F \quad (3)$$

where I is the intensity of the excitation beam, T_{ep} is the transmittance of the epidermal layers, A_{chlo} is the absorbance of chloroplasts and ϕ_F is the ChlF quantum yield.

Let's consider two excitation wavelengths, λ_1 inside the absorption band of an epidermal compound and λ_2 outside it so that $T_{ep}(\lambda_2) = I$, with the same intensity [$I(\lambda_1) = I(\lambda_2)$] and similar chloroplast absorbance.

According to eq. 3, the ratio of the two ChlF will be

$$\frac{ChlF(\lambda_1)}{ChlF(\lambda_2)} = T_{ep}(\lambda_1) \quad (4)$$

The fluorescence excitation method can then provide a quantitative estimate of epidermal transmittance on intact samples (Markstädter et al., 2001).

And, according to Beer-Lambert's law ($A = \log T^{-1}$),

$$\log \frac{\text{ChlF}(\lambda_2)}{\text{ChlF}(\lambda_1)} = A_{ep}(\lambda_1) \quad (5)$$

That is proportional to the concentration of the epidermal compound taken into account (see Fig. 4.6, right side).

On the other hand, comparing the ChlF excitation spectrum from two samples with different epidermal compound concentrations, 1 and 2, allows to derive the spectrum of the difference in the epidermal absorbance between samples 2 and 1:

$$\Delta A_{ep,2-1}(\lambda) = \log \frac{\text{ChlF}_1}{\text{ChlF}_2}(\lambda) \quad (6)$$

The ChlFES method works properly when the compound to be detected is spatially well separated from the first Chl layer. In leaves, the sub-epidermal localization of Chl allows for detection of transmittance (absorbance) of compounds in the epidermis. Although phenols present in the epidermis have the strongest influence on chlorophyll excitation, it has been shown that also compounds present in sub-epidermal layers can be detected by the ChlFES method (Nichelmann and Bilger, 2017).

This is the case reported in Figure 4.7, where the 3-D dual-band fluorescence image of a purple basil (*Ocimum basilicum*) leaf adaxial surface shows the epidermal distribution of anthocyanins (purple color) above the first Chl layer of the mesophyll (green color).

FIGURE 4.7 Three-dimensional view of a purple basil (*Ocimum basilicum*) leaf adaxial surface built with 127 fluorescence images recorded (at 0.3-mm-steps) along the z-axis in a Confocal Laser Scanning Microscope, with $\lambda_{xc} = 488$ nm and λ_{em} over the 571–646 nm and 676–732 nm wavebands for the anthocyanins and Chl acquisition, respectively.

In grapevine leaves, the localization of phenolic compounds assessed by means of multispectral fluorescence microscopy showed that flavonols but not hydroxycinnamic acids can be detected non-destructively by the fluorimetric method (Agati et al., 2008a).

In fruits, flavonoids are mostly localized above Chl (Hagen et al., 2006; Kolb et al., 2003) or they are partially co-localized with Chl, as is the case of anthocyanins in olives (Agati et al., 2005) or grapevine berries (Agati et al., 2007). Since the co-localized pigments will screen chlorophyll excitation with lower efficiency than the epidermal pigments the ChlFES technique will quantify the total pigment content in a non-linear manner.

In the practical situation, it is difficult to achieve an identical intensity of the measuring beam at λ_1 and λ_2 (equation 4). In addition, chloroplast absorption at both wavelengths may not be the same. In that case, referring the data to values determined with an epidermis free leaf will allow to calculate UV transmittance and absorbance (Bilger et al., 1997; Cerovic et al., 2002). Alternatively, the lower epidermis of bifacial leaves or basal segments of monocotyledonous leaves, which have usually much reduced contents of screening compounds, can be used as a reference (Cerovic et al., 2002). The identification of the UV-screening compounds present in the leaf epidermis can be facilitated by the spectral application of the method (Cerovic et al., 2002).

3. SENSORS DESCRIPTION

Several fluorescence sensors for applications on vegetables and fruits have been developed. Table 4.1 reports a list of the most common commercially available devices for ChlF detection. Most of them are portable and accordingly usable under *in field* conditions. The description of the sensors can be found at the producer web page and in the reported references.

TABLE 4.1 Commercially Available Sensors for the Detection of Chlorophyll Fluorescence Parameters

Based on	Type	Company/Web Site	Descriptive Features
ChlF kinetics parameters			
	Plant efficiency analyzer (PEA); FMS1; FMS2	Hansatech, King's Lynn, UK www.hansatech-instruments.com	Mouget and Tremblin, 2002; Öquist and Wass, 1988
	FluorPen	Photon Systems Instruments, Brno, Czech Republic, www.psi.cz	
	OS-500	Opti-Sciences Inc., Tyngsboro, MA, USA www.optisci.com	
	MINI-PAM; PAM-2000; PAM-2500	Walz, Effeltrich, Germany www.walz.com	Walz, Effeltrich, Germany www.walz.com
	HarvestWatch™	Satlantic Inc., Halifax, NS, Canada www.harvestwatch.net	DeLong et al., 2004; Prange et al., 2002
Imaging			
	Handy FluorCam FC 1000-H	Photon Systems Instruments, Brno, Czech Republic, www.psi.cz	Nedbal et al., 2000a
	IMAGING-PAM	Walz, Effeltrich, Germany www.walz.com	Ralph et al., 2005
Fluorescence excitation ratios			
	DUALEX®	Force-A, Orsay, France www.force-a.com	Goulas et al., 2004
	MULTIPLEX®	Force-A, Orsay, France www.force-a.com	Ben Ghazlen et al., 2010a
	UVA-PAM*	Gademann Messgeräte, Würzburg, Germany	Kolb et al., 2005
	Xe-PAM	Walz, Effeltrich, Germany	Bilger et al., 1997

* no longer available.

The portable Handy Plant Efficiency Analyser (PEA) (Hansatech, UK) sensor uses a continuous excitation by an array of ultra-bright red LEDs at 650 nm (22 nm half width) with NIR short pass cut-off filters. The maximal intensity can be up to 3,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The detector consists of a fast response PIN photodiode with a RG9 long pass filter (fluorescence measuring range above 700 nm). The Hansatech

Fluorescence Monitoring System FMS2 is a field portable pulse modulated (at 4 different frequencies) device exciting with a 594 nm amber LED or optionally with a 470 nm blue LED. Actinic, up to $3,000 \mu\text{mol m}^{-2} \text{s}^{-1}$, and saturating, up to $20,000 \mu\text{mol m}^{-2} \text{s}^{-1}$, lights are provided by a halogen lamp. A PIN photodiode detects fluorescence above 700 nm.

The FluorPen (PSI, Czech Republic) sensor uses a pulsed optically filtered blue LED at 470 nm and detects fluorescence between 667 to 750 nm, through a bandpass filter, by a PIN photodiode. Actinic and saturating lights are up to $1,000$ and $3,000 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. The Handy FluorCam FC 1000-H (PSI, Czech Republic) imaging system acquires Chl fluorescence by a CCD camera with a RG695 optical filter (measuring range above 700 nm). The pulsed measuring LED emits at 620 nm. Three different actinic / saturating lights, white, blue (455 nm) or orange (620 nm) can be set up.

The Opti-Sciences chlorophyll fluorimeter has a modulated LED light probe at 660 nm (optional at 450 nm) with a 690 nm short pass filter. A PIN photodiode with a 700 - 750 nm bandpass filter acts as detector. Saturation pulses and actinic light with white LEDs provide $15,000$ and $5,800 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively.

The MINI-PAM (Walz, Germany) fluorimeter exists as a blue (470 nm) and a red (655 nm) LED version measuring fluorescence above 630 and 700 nm, respectively. Actinic and saturation light reaches $3,000$ and $6,000 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. In the PAM-2500 (Walz, Germany), detection by a PIN photodiode occurs above 715 nm with a measuring red LED at 630 nm. Actinic light can reach $4,000 \mu\text{mol m}^{-2} \text{s}^{-1}$, while saturation pulses can be up to $25,000 \mu\text{mol m}^{-2} \text{s}^{-1}$.

The light sources for the Imaging PAM (Walz, Germany) consist of 44 blue (450 nm) or red (650 nm) 3 W LEDs providing an actinic intensity of $1,900 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a saturation intensity of $4,000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Detection is through a CCD camera protected by a long-pass filter (RG 645) and a short-pass filter (<780 nm).

Multispectral fluorescence imaging is a common tool present in commercial confocal or deconvolution microscopes. For wide-scale detection, it is not easy to find complete multispectral fluorescence imaging systems on the market. Often, researchers adopt self-assembled instruments (Buschmann et al., 2008). One may mention the customized versions of FluorCam (PSI, Czech Republic) that allow for different combination of LED panel excitation wavelengths, from UVA to red, and up to 7 acquisition fluorescence bands.

Several instruments applying the ChlFES method to detect UV transmittance of leaf epidermis have been developed during the last decades. The Xe-PAM fluorimeter (Xe discharge lamp and Pulsed Amplitude Modulation, Walz, Effeltrich, Germany) provides three bands of excitation, in the UV-B (λ_{max} at 314 nm), UV-A (λ_{max} at 366 nm) and blue-green (400–550 nm) spectral ranges, and detects ChlF above 700 nm (Bilger et al., 1997).

While the Xe-PAM fluorimeter operates on detached leaves, the UV-A PAM fluorimeter (Gademann Instruments, Würzburg, Germany) can perform in situ measurements for assessing epidermal compounds on

attached leaves in the field (Bilger et al., 2001). It provides two bands of excitation in the UV-A (peak 375 nm) and blue (peak 470 nm) spectral regions and detects ChlF above 650 nm.

The use of the blue-excited ChlF as reference signal limits the application of both the Xe-PAM and UV-A-PAM fluorometers to sample free of blue-absorbing compounds in the epidermis, as evidenced by Barnes et al. (2000) (Barnes et al., 2000) and Pfündel et al. (2007) (Pfündel et al., 2007) in anthocyanin-containing leaves. Recently, it has been shown that the blue beam of the UV-A-PAM can be used to detect changes in carotenoid contents, when referenced to the ChlF excited with red beam of a Mini-PAM (Nichelmann et al., 2016).

The Dualex Scientific+ (Force-A, Orsay, France) is a leaf-clip sensor, providing excitation in the UV-A (375 nm), in the green (520 nm) and in the red (655 nm) and detecting ChlF above 700 nm. The Dualex and the UV-A-PAM fluorometers were compared on leaves of a wide range of different species (Pfündel et al., 2007).

Applications of the ChlFES technique to relatively large (up to 8-cm in diameter) and thick samples is possible by the Multiplex sensor (Force-A, Orsay, France) (Ben Ghazlen et al., 2010a). It provides excitation in the UV-A (375 nm), blue (450 nm), green (520 nm) and red (630 nm) spectral regions and detects over three channels in the blue-green (or yellow), red and far-red spectral regions, these two last at 680–690nm and 730–780 nm, respectively, corresponding to the two emission peaks of chlorophyll.

As an example of a self-assembled instrument to apply the ChlFES method as imaging technique, it is worthy to mention the system used by Lenk et al. (Lenk and Buschmann, 2006; Lenk et al., 2007). The fluorescence was excited by a Xenon flash lamp filtered at 340 nm (half bandwidth of 75 nm) or at 430nm (half bandwidth of 70nm) for UV- and blue-excitation, respectively. Fluorescence images were taken using a gated intensified video camera with a filter wheel housing 4 narrow-bandwidth (10 nm) interference filters in the blue (440 nm), green (520nm), red (690 nm) and far red (740 nm) spectral regions.

4. APPLICATIONS ON VEGETABLES

Some recent applications of the various fluorescence techniques and sensors are outlined in the following. They are limited to the assessment of quality-related features of products and grouped according to the method used. Detection of the plant physiological status, stress effects, nutrient deficiency and plant diseases are not covered here.

4.1 ChlF kinetics

The non-destructive monitoring of the ChlF dark-light kinetics parameters was related to the quality of broccoli during storage under modified atmosphere (DeEll and Toivonen, 2000). It was also applied to cabbage, green pepper and lettuce (Prange et al., 2003).

Schofield et al. (Schofield et al., 2005) showed that several ChlF kinetics parameters (particularly $F_v/F_m = F_m - F_0/F_m$) were strongly correlated to the overall visual quality and decay of iceberg lettuce during storage. ChlF measured at harvest can predict the storage potential of lettuce.

The storability at 4°C and 10°C of lamb's lettuce *Valeriana locusta* was evaluated by the analysis of the fast rise of the ChlF kinetics (Ferrante and Maggiore, 2007). The authors highlighted the potential of different ChlF indices of the health status of vegetables. Analogously, Qiu et al. (Qiu et al., 2017) found a correlation between ChlF kinetics parameters and the freshness of spinach, lettuce and cabbage leafy vegetables.

4.2 Imaging

ChlF imaging was proved to be useful for monitoring the tissue alteration induced by minimal processing (cutting and washing) of romaine lettuce (*Lactuca sativa* L. var. longifolia) and endive (*Cichorium endivia* L.) during storage (Hägele et al., 2016). Simko et al. elaborated a lettuce decay index based on ChlF imaging for measuring product deterioration and freezing injuries (Simko et al., 2015).

The imaging of the ratio between two fluorescence bands in the red spectral region was able to detect bovine fecal contaminants on Romaine lettuce and baby spinach leaves (Lee et al., 2014).

Other applications to horticulture of ChlF imaging can be found in the review by Gorbe and Calatayud (Gorbe and Calatayud, 2012).

The use of 4-bands multispectral fluorescence imaging to the assessment of quality on green bell peppers (*Capsicum annuum* L.) was reported (Buschmann et al., 2008).

4.3 ChlFES method

A self-assembled apparatus was used to detect non-destructively flavonoids on marketable broccoli heads (Bengtsson et al., 2006).

UV-A-PAM measurements on curly kale leaves were found to be well correlated with the quercetin content determined by HPLC (Hagen et al., 2009). Other recent applications of the ChlFES method by the UV-A-PAM sensor comprised the evaluation of pre-harvest UV-B treatments at 22 °C and 9 °C and controlled storage conditions on epidermal UV-A absorbance in pak choi (*Brassica campestris* L. ssp. *chinensis* var. *communis*) leaves (Harbaum-Piayda et al., 2010).

Zivcak et al. (2017) applied the Multiplex-3 sensor to study the differences in the accumulation of phenolic compounds between genotypes and growing conditions in lettuce (*Lactuca sativa* L.) (Zivcak et al., 2017). The Multiplex sensor was also used to control the synthesis of flavonoids induced by UV-B radiation and white fluorescent lamps in greenhouse produced lettuce (*Lactuca sativa* L.) cv. Lollo Rosso, in order to improve their antioxidant properties (Rodriguez et al., 2014). Furthermore, the kinetics of flavonol accumulation during postharvest temperature and radiation treatments of Broccoli (*B. oleracea* L. var. *italica*) inflorescences was evaluated (Rybarczyk-Plonska et al., 2016).

5. APPLICATIONS ON FRUITS

5.1 ChlF kinetics

The F_m and F_0 ChlF were found to decline with ripening in papaya fruits, due to chlorophyll degradation. They were correlated to fruit firmness and skin color and represent a complementary parameter to evaluate the overall fruit quality (Bron et al., 2004).

Kolb et al. (2006) found a negative association between F_0 ChlF and the concentrations of fructose, glucose, and total sugars, and the fructose/glucose ratio in white grapes (*Vitis vinifera* L. Cv. Bacchus and Silvaner) at various degrees of ripeness (Kolb et al., 2006).

The HarvestWatch system, based on an estimate of F_0 ChlF, has been applied to the control of quality retention in stored apples (DeLong et al., 2004) and other Chl-containing fruits such as avocado, pears, kiwifruits, banana and mango (Prange et al., 2003; Prange et al., 2002).

In an *in field* study, ChlF was measured directly on mango fruits attached to the tree. F_0 , F_m , and $F_v = F_m - F_0$ were significantly lower on fruits at the top of the canopy with respect to those within the canopy and correlated to fruit maturity (Lechaudel et al., 2010).

ChlF kinetics parameters combined with chemometrics represent a useful nondestructive tool to predict the sugar content in figs at different maturity stages (Jiang et al., 2013).

In mulberry (*Morus alba* L.) fruits, a high correlation was found between ChlF and total flavonoids, total phenols, sugars and antioxidant activity (Lou et al., 2012). ChlF was also used to evaluate the quality and the optimal harvest time of jujube fruit (*Zizyphus jujuba* Mill. cv.) (Lu et al., 2012).

All of the primary ChlF parameters (F_0 , F_m , F_v and F_v/F_m) were found to be correlated to the grape mass loss during postharvest dehydration of 'L'Acadie' and 'Thompson Seedless' grape clusters (Wright et al., 2009).

5.2 Laser-induced Fluorescence

Laser-induced fluorescence spectroscopy with excitation at 337 nm was applied to detect phenolic compounds in strawberry fruits (Wulf et al., 2008) and polyphenols in apples (Wulf et al., 2005).

Laser excitation of ChlF in the blue or the red spectral region allowed monitoring of ripening in Riesling and Cabernet Sauvignon grapevine berries (Navrátil and Buschmann, 2016), as well as senescence- induced changes in peel chlorophyll of 'Jonagold' and 'Golden Delicious' apples during shelf-life (Kuckenberg et al., 2008).

5.3 Imaging

Multicolor fluorescence yield and fluorescence ratio imaging over four distinct bands, from the blue to the far-red, was applied to monitor storage and ripening of Braeburn apples up to 6 months (Buschmann et al., 2008; Lichtenthaler et al., 2012).

ChlF imaging can identify damages to the lemon rind before the appearance of visual symptoms (Obenland and Neipp, 2005). Nedbal et al.(2000b) showed the potential of ChlF imaging in detecting injured areas or infected spots on lemon fruits in postharvest (Nedbal et al., 2000b).

Hyperspectral fluorescence imaging was used to detect defects on cherry tomato (*Lycopersicon esculentum* Mill. cv. Yoyo) fruits (Cho et al., 2013). Blue-green fluorescence on cracked cuticle spots was significantly higher than that of the sound surfaces.

The intensity of ChlF images of fresh apples correlated with the fruit firmness of fresh apples (*Malus domestica* cv. Red Delicious), fresh peaches and nectarines (*Prunus persica* cv. Elegant Lady and Sweet Lady, respectively) (Bodria et al., 2004).

Integrated techniques of multispectral reflectance and fluorescence imaging were found to be accurate tools for recognition of various disorders on apples (Ariana et al., 2006).

Hyperspectral laser-induced fluorescence imaging of apple fruits with the combination of a principal component analysis and neural networks modeling showed the potential for assessing selected quality parameters of apple fruits (Noh and Lu, 2007). An excellent prediction of apple skin hue, relatively good predictions for fruit firmness, skin chroma and flesh hue, and poorer correlations for soluble solids content, titratable acid, and flesh chroma were found.

5.4 ChlFES method

Most of the ChlFES method applications on fruits concerned the use of the Multiplex sensor. Among these, a large number of papers have been published in the viticulture area, with the aim to define a new non-destructive tool for the assessment of the wine grape (*Vitis vinifera* L.) phenolic maturity directly in the vineyards. The Multiplex sensor was used for the measurement of the temporal accumulation of anthocyanins in the grape bunches to predict the best harvest time (Agati et al., 2013; Ben Ghazlen et al., 2010a; Ben Ghazlen et al., 2010b; Diago et al., 2013; Tuccio et al., 2011). It was possible to evaluate the spatial heterogeneity of the anthocyanin berry content and to graph the results on vineyard maps by using the sensor either manually (Agati et al., 2013; Baluja et al., 2012a; Baluja et al., 2012b) or mounted on a harvester for on-the-go sensing (Bramley et al., 2011). Calibration of the Multiplex sensor against wet-chemical destructive analysis of berry extracts is a critical issue and is still in progress (Ben Ghazlen et al., 2010a; Ferrandino et al., 2017; Pinelli et al., 2018).

These in field studies were preceded by in lab investigation defining the basic protocol of the ChlFES method at the single grape berry level (Agati et al., 2007) and whole bunch level (Cerovic et al., 2008). Lately, attention has been devoted to the non-destructive Multiplex detection of flavonols in white wine grape cultivars (mainly Vermentino, Chardonnay and Nascetta) (Agati et al., 2013; Ferrandino et al., 2017). Berry skin UV absorbance in the berries of the Bacchus white grape cultivar were also investigated by the Xe-PAM and UV-A-PAM fluorimetric methods. Comparison of non-destructive data with those from wet chemistry on skin extracts suggested that UV screening in berries depends almost exclusively on flavonol content (Kolb et al., 2003).

Other applications of the Multiplex sensor consisted in the assessment of flavonols in apple (Betemps et al., 2012) and kiwifruit (Pinelli et al., 2013) exocarps, as well as of anthocyanins in olive fruits (Agati et al., 2005) and anthocyanins and flavonoids in sun exposed mango fruits (Sivankalyani et al., 2016).

The Multiplex fluorimeter was as well able to: i) follow the ripening of Thompson Seedless grapes and the change in the color of Crimson Seedless grapes (Bahar et al., 2012); ii) provide complementary information on the effect of cytokinin analogue forchlorfenuron on the ripening of Thompson Seedless table grape (Maoz et al., 2014); iii) classify the maturity stage of oil palm bunches (Hazir et al., 2012a; Hazir et al., 2012b) and iv) monitor the pre- and postharvest ripening of tomato fruits (Hoffmann et al., 2015).

The combination of a UV-A-PAM and a PAM-2000 fluorimeters was used to assess the content of anthocyanins and flavonoids in the apple skin of the Aroma red cultivar (Hagen et al., 2006) and their kinetics during postharvest treatments by visible light and UV-B radiation (Hagen et al., 2007).

The application of the ChlFES method as imaging technique for assessing flavonols in white grape berries (Lenk et al., 2007) and anthocyanins in whole red grape bunches (Agati et al., 2008b) deserves a mention.

6. CONCLUDING REMARKS

The above described optical techniques have several major advantages. They can be applied to intact produce saving time-consuming and costly destructive analyses, thereby providing as a side effect also a positive environmental impact. Repeated analyses of identical samples are possible, which enhances the precision of analyses when time courses need to be followed. A further strong advantage is the rapidity of the measurements allowing large amounts of samples tested in short time. This combined with almost non-existing costs for consumables makes optical techniques very well suited for routine analyses.

The compounds, which can be detected, as e.g., polyphenolic antioxidants, have attained a high importance as so-called nutraceuticals in recent years. A group of the flavonoids, the flavanones have been found to protect vitamin C and were even termed for some time vitamin P (Rice-Evans and Packer, 2003).

The usefulness and the spreading of these tools within the agro-food sector has been proven by the large number of applications of the fluorescence techniques for the assessment of quality features in vegetables and fruits. Whereas the most sophisticated spectroscopic techniques are mainly suitable to quality control laboratories, portable sensors can be used directly in the field (or in greenhouses) to forecast the harvest date of products with the highest level of beneficial phytochemicals. This practice would improve fruit and vegetable qualities, considering their large dependence on the variability of climatic conditions such as rainfall, temperature, and irradiance, even within short periods.

The ChlFES method for the assessment of non-fluorescing superficial compounds is particularly useful to detect the UV-absorbing antioxidants, for which detection by reflectance spectroscopy is limited by the very low reflectance signals in the UV region. These detected compounds are also indicative of plant growth conditions. Flavonoids are readily formed under field conditions but are strongly reduced in plants grown under greenhouse conditions. Hence, detection of flavonoids may also serve to distinguish field grown plants

from those grown in the greenhouse, which not only have different flavonoid contents but contain also different amounts of vitamins (E and C). Due to the dependence of flavonoid biosynthesis on nutrient conditions, they can serve as indicators of nutrient deficiency.

Most of the described techniques are based on the detection of chlorophyll fluorescence. Much less information has been derived from the *in situ* measurement of blue-green fluorescence from beneficial phytochemicals belonging to the group of hydroxycinnamic acid derivatives. Therefore, it would be appealing if the relationship between the blue-green fluorescence and the sample quality could be better investigated in the future.

During postharvest, the rapid optical sensing could improve the control of the whole supply chain from production, through storage and distribution, to consumers in order to guarantee the highest value of products. Fluorescence sensors can be embedded into sorting machines to improve the automated grading system of produce. On-line quality monitoring of postharvest treatments can predict shelf-life of fresh produce, increasing profitability.

Thanks to the ongoing progress in the field of electronic components and to the increasing power of LEDs, miniaturization of devices for ChlFES measurement is possible, and is currently underway. This may lead in the future to a higher spectral resolution also in portable instruments, enabling the determination of more complex indices providing higher information.

It is worth to recall that the ChlFES technique is limited to the detection of compounds located at the sample's superficial layers and that it is as poorly specific as any spectrophotometric method. When the detailed compound composition is sought, the standard extraction and chromatographic procedures are still needed. However, the fluorescence sensors can still be useful for the intentional selection of plants with the most interesting antioxidant content.

There are three major competing techniques for nondestructive sensing of intact fruits and vegetables: colorimetry, near-infrared spectroscopy (NIRS, coupled to chemometrics) and ChlFES. The major problem of the first is the limitation to chromophores absorbing visible light and its saturation at physiological concentration; of the second the omnipresence of water in the sample and the changing matrix effect; and of the third the loss of chlorophyll in very mature fruits. Only samples retaining at least minimal chlorophyll content can be measured.

The absolute intensity of fluorescence signals may be disturbed by unwanted contributions, for example by reflecting surfaces like waxes or hairs, dust or residues of phytosanitary treatments. Fluorescence imaging can be affected by non-uniform contributions due to non-flat surfaces, as in curled leaves or rounded fruits. For this, fluorescence signal ratios should be preferred to minimize the above mentioned problems.

When the mentioned limitations are kept in mind and the available instruments are employed wisely, they have a high potential to lead to further considerable progress in agriculture.

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