Use of portable devices and confocal Raman spectrometers at different wavelength to obtain the spectral information of the main organic components in tomatoes (*Solanum Lycopersicum)* fruits

Josu Trebolazabala, Maite Maguregui, Héctor Morillas, Alberto de Diego, Juan Manuel Madariaga

PII: S1386-1425(12)01272-3
DOI: http://dx.doi.org/10.1016/j.saa.2012.12.047
Reference: SAA 10099

To appear in: *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*

Received Date: 16 October 2012
Revised Date: 12 December 2012
Accepted Date: 14 December 2012

Please cite this article as: J. Trebolazabala, M. Maguregui, H. Morillas, A.d. Diego, J.M. Madariaga, Use of portable devices and confocal Raman spectrometers at different wavelength to obtain the spectral information of the main organic components in tomatoes (*Solanum Lycopersicum)* fruits, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* (2013)Volume 105, Pages 391-399, doi: http://dx.doi.org/10.1016/j.saa.2012.12.047

Use of portable devices and confocal Raman spectrometers at different wavelength to obtain the spectral information of the main organic components in tomatoes (*Solanum Lycopersicum)* fruits

Josu Trebolazabala[1,*], Maite Maguregui[2], Héctor Morillas[1], Alberto de Diego[1] and Juan Manuel Madariaga[1]

[1]*Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), P.O. Box 664, 48080 Bilbao, Basque Country, Spain, Phone: +34 946018298, *E-mail address: josugotzon.trebolazabala@ehu.es*
[2]*Department of Analytical Chemistry, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), P.O. Box 450, 01006 Vitoria-Gasteiz, Basque Country, Spain*

Abstract

Tomato (*Solanum lycopersicum*) fruit samples, in two ripening stages, ripe (red) and unripe (green), collected from a cultivar in the North of Spain (Barrika, Basque Country), were analysed directly, without any sample pretreatment, with two different Raman instruments (portable spectrometer coupled to a micro-videocamera and a confocal Raman microscope), using two different laser excitation wavelengths (514 and 785nm, only for the confocal microscope). The combined use of these laser excitation wavelengths allows obtaining, in a short period of time, the maximum spectral information about the main organic compounds present in this fruit. The major identified components of unripe tomatoes were cutin and cuticular waxes. On the other hand, the main components on ripe tomatoes were carotenes, polyphenoles and polysaccharides. Among the carotenes, it was possible to distinguish the presence of lycopene from β-carotene with the help of both excitation wavelengths, but specially using the 514 nm one, which revealed specific overtones and combination tones of this type of carotene.

Keywords: *Portable Raman spectroscopy, Confocal Raman spectroscopy; tomato; carotenoids; cuticular waxes; polyphenols*

1. Introduction

Tomato (*Solanum lycopersicum*) is a species originally grown in South America [1]. This species is widely cultivated all over the world and it is the second most consumed vegetable nowadays[2]. The fruit of this plant has lots of benefits for human health as many studies have described. These benefits are attributed to the antioxidant compounds present in tomatoes, mainly to the organic polyenic molecules called carotenoids [3-6].

Structurally, carotenoids are usually long, aliphatic, C40 tetraterpenoids composed of eight C5 isoprenoid units giving place to a symmetrical molecule. Cyclic hydrocarbon saturated rings can also be linked at the end of the unsaturated aliphatic chains in some specific carotenoids. This natural pigment has an important characteristic attributed to its centrally located, extended conjugated double-bond system, which constitutes the light-absorbing chromophore that gives carotenoids their intense colour [7,8]. This feature could be possible if the carotenoids had at least seven conjugated double bonds in their structures [9,10]. The electron-rich conjugated system of the polyene is responsible for the antioxidant activities of the carotenoids, both by quenching singlet oxygen and scavenging radicals to trigger chain reactions [11].

Chemically, carotenoids are classified into two major groups. The first one, carotenes, are the highly unsaturated hydrocarbon carotenoids such as lycopene, α-carotene, β-carotene, γ-carotene and ζ-carotene, which contain no oxygen and are usually orange and red. These molecules are particularly susceptible to oxidation because they are highly unsaturated. The second one, xanthophylls, are oxygen derivated carotenes and contain one or more oxygenated group on particular sites of the terminal rings. They are responsible for yellow and orange colours in these organic pigments [7,12].

Apart from these biosynthesised vegetable organic pigments, there are other antioxidant compounds in tomatoes. The proportion of them is lower in comparison with carotenoids, but they are also important. Among the mentioned compounds there are ascorbic acid, polyphenolic compounds, tocopherols (vitamin E), flavonoids, phytoene and phytofluene [4,5]. Furthermore, there are other compounds such as chlorophyll [13] and fatty acids, which are also present in these fruits [14].

Apart from these compounds, which are beneficial for human health, there are other organic components, which are essential to maintain the structure of these vegetables. The plant epidermis, which covers all aerial surfaces of plants, fruits and flowers, is mainly composed of a polyester matrix, cutin, and a mixture of soluble waxes. These fatty acids are embedded in the cutin matrix and deposited on the external surface forming an epicuticular layer [15-17]. From a biochemical and biophysical point of view, the two major components of this structure are cutin and waxes. The first one is a singular polyester of mainly C16 and C18 hydroxy fatty acids or diacids. The second one, consist of a heterogeneous mixture of very long-chain fatty acids and their monomeric derivatives, with carbon chain lengths ranging from C20 to C40 [17]. Furthermore, wax esters (primary fatty alcohols esterified to fatty acids) with chain lengths ranging from C36 to C70 are also present. As mentioned before, below this epicuticular wax layer there is a mixture of intracuticular waxes and cutin where polysaccharides such as pectins, cellulose and hemicelluloses are embedded[18]. The cuticle is thought to play important physiological roles in plants such as preventing

water loss from aerial plant organs, protection against pathogens, mechanical damages, UV radiation and pollutants [15,16].

Spectroscopic techniques, especially Raman spectroscopy, are suitable analytical techniques to characterize carotenoids and other natural components (e.g., polysaccharides, lipids, phenolic compounds, etc.) of tomatoes. The use of different laser wavelengths offers the possibility of detecting more compounds, which is important for a better characterization of a complex matrix. Specifically, the Resonance Raman effect, achieved with a good selection of laser excitation wavelength (e.g. 514 nm laser), can be used to enhance the intensity of certain Raman bands in the Raman fingerprint area (900-1600 cm^{-1}) of carotenoids and can also help enhancing the intensity of Raman bands related to overtones and combination-tones (2000-3000 cm^{-1}). Overtones are defined as vibrational bands that are multiples of the fundamental transition modes, and combination bands are either the difference or sum of two fundamental bands [19]. These bands are fundamental vibrational modes that occur in the mid-high spectral region. They are particularly enhanced as a result of the multiplication of the most intense bands of the spectra [20]. Raman spectra, which Resonance effect is visible, could offer many slight changes that allow distinguishing a specific spatial conformation of certain carotenoids. The possibility of *in situ* and direct analysis in a non-invasive way and with no pre-treatment requirements make this technique a fast and easy-to-handle alternative to characterize the main components of fruits and vegetables [7].

Raman spectroscopy was used in this work to characterize the nature of the main organic components of unripe and ripe tomato fruits in a non invasive way. Moreover, Raman spectra which were obtained with two instruments (a portable device and a confocal microscope) and with different excitation laser excitation wavelengths (785 and 514 nm) were compared in order to evaluate the importance of an appropriate selection of laser excitation wavelengths. This procedure is essential to extract the maximum spectral information of the organic compounds present in these fruits. Additionally, a comparison of the performance between these instruments was carried out to assess if the portable spectrophotometer offers enough spectral information to indentify the analysed organic compounds and to monitor them using an *in situ* strategy in tomato cultivars.

2. Material and Methods

2.1. Samples

Several tomato fruits were collected in a crop located in Barrika, (Basque Country, North of Spain). Some fruits were selected and collected in an unripe stage (green colour) and others in a ripe stage (red colour). These fruits were collected strait from the plant and they were directly carried to the laboratory. After being washed with deionised water they were measured without any sample preparation or pretreatment.

2.2. Instruments

Raman analysis of green and red tomatoes was carried out using two different Raman instruments: a portable or hand-held instrument and a confocal Raman instrument. The portable instrument used was an innoRam® ($B_{\&}WTEK_{INC.}$, Newark, USA) spectrometer which implements a 785 nm laser excitation wavelength. Raman measurements with the portable instruments were performed on a home-made stage (MICROBEAM S.L, Barcelona, Spain) in which the Raman microprobe was assembled together with a micro-videocamera. This stage (of manual movement) allows performing microscopic Raman measurements (similar to a commercial microscope) focusing on the area of interest. In this case a long-range objective lens (Olympus, Tokio, Japan) of 20x was used. The spectral resolution of the instrument is 2 cm^{-1} and spectra were acquired with 25 seconds integration time and 12 accumulations.

As mentioned before, Raman measurements were also performed using another instrument; a Renishaw (Gloucestershire, UK) inVia confocal Raman spectrometer coupled to a DMLM Leica microscope. With this instrument, two different laser excitation wavelengths were used for the measurements: 785 and 514 nm. The power of 785 nm NIR laser excitation at the source (output power) is 350 mW, and about 150 mW (set as 100% of the laser power) at the surface of the analysed area. The power of 514 nm argon ion excitation laser at the source is around 50 mW, and 20 mW (highest power) at the surface of the sample. In order to prevent possible thermal decomposition of carotenoids and further organic compounds of tomatoes the laser power was controlled with neutral density filters which were implemented in both instruments. After each measurement, the focused and measured area was checked. If the analysed microscopic area was burned, the measurement was rejected. In order to perform a proper comparison of the Raman features we obtain with both Raman instruments, it is quite important to use the same objective lens. Thus the same 20x (Olympus, Tokio, Japan) long-range objective lenses were use in both instruments. The spectral resolution of the instrument is 1 cm^{-1} and spectra were acquired with 10 seconds integration time and 5 accumulations.

Most of the spectra were collected in the range 100-3100 cm^{-1}. However, in order to observe possible overtones, combination tones and C-H Raman bands the spectral range was extended up to 4000 cm^{-1} in the measurements performed with the confocal Raman instrument. The portable instrument does not allow acquiring spectra at wavelengths higher than 3100 cm^{-1}. For both instruments a daily calibration was carried out and for that purpose a 520.5 cm^{-1} silicon line was used. The Spectral acquisition carried out with the portable instrument was performed using the BWSpec^{TM} 3.26 software ($B_{\&}WTEK_{INC.}$) and Renishaw WIRE 3.2 software was used with the confocal instrument. Data treatment was performed using GRAMS/AI 7.02 software (Thermo Fisher Scientific Inc., Waltham, USA). For the Raman bands interpretation, pure β-carotene (Sigma Aldrich-Fluka. Pure >97%) and lycopene (Sigma Aldrich-Fluka. Pure >90%) standards were used. Additionally, bibliographic information was also used for spectral assignation of organic compounds in red and green tomatoes.

Raman spectra included in this work and acquired with the confocal (785 and 514 nm) and portable instrument (785 nm) are presented as the average of more than 50 measurements in different points of red (ripe) and green (unripe) tomatoes.

3. Results and Discussion

3.1. Portable spectrometer (785 nm) vs confocal Raman microscope (785 and 514 nm) in the Raman characterization of green tomatoes

Figure 1 shows some of the Raman spectra obtained from different measurements performed on green tomatoes using both instruments and both excitation wavelengths. The spectra obtained with the confocal instrument show more intense bands than those appearing in Raman spectra collected with the portable instrument. Moreover, the confocal instrument allows obtaining some bands which are related to specific vibrational modes and are not visible in the measurements obtained with the portable instrument. Besides, the spectra obtained with the confocal Raman microscope, using both lasers, show almost the same number of bands and similar intensities. Therefore, the use of the confocal Raman instrument on green tomatoes surface allows identifying bands that are not visible in the spectra obtained with the portable instrument.

Most of the bands found in the low wavenumber region of the green tomato can be assigned to chlorophylls, hydrolysable polysaccharides and waxes that are present in the cuticular membrane of this fruit [18,21]. These polysaccharides, which are associated with the plant cell wall and are responsible for the elastic modulus, stiffness and the linear elastic behaviour of the cuticle, are pectins, hemicelluloses and celluloses [18]. The band ca. 853 cm^{-1} is related to C-O-C asymmetric stretching of the glycoside linkage in acidic pectins [22,23]. This band may also have a contribution of a Raman band related to the carotene compounds present in this fruit (see Table 1).

The broad band and the peaks located in the region of 1040 and 1120 cm^{-1} are supposedly associated with the triterpenoids. These compounds are attributed to the cuticular waxes present in the fruit and leaves of the plants. The bands in this region are situated ca. 1047, 1065, 1081, 1112 cm^{-1} [24,25].

The weak bands observed at ca. 744 cm^{-1} and 1325 cm^{-1} are related to the presence of chlorophyll a [23,26,27]. These bands are the strongest peaks in the spectra of chlorophyll [23,26,27]. Moreover, the bands located around 915 and 985 cm^{-1} are also related to the (C-C-C) bending deformation and the CH_3 bending of the chlorophyll, respectively [26,27]. These bands were only observed in the measurements carried out with the 785 nm laser excitation wavelength, because it is quite difficult to identify the presence of chlorophyll using the 514nm excitation wavelength. However, the resonance effect make the 514nm excitation wavelength more appropriate for the detection of orange and yellow colour carotenoids [26,27] (see Figure 1 and Table 1).

To the authors' knowledge, there have been no reports of bibliographic evidence about spectra of cuticular waxes and polysaccharides obtained directly from the cuticular layer of a tomato without any sample pretreatment. The spectra found in bibliographic material of these fatty acids and polymers involve previous extraction and purification methodologies of these compounds in tomato fruit. However, direct measurements without any sample pretreatment and using the confocality of the Raman microscope and the 514nm excitation wavelength permit the visualization of the bands related to cuticular waxes and polysaccharides without doing any treatment or sample preparation of the fruit.

In the Raman spectra from green tomatoes (see Figure 1), the strongest bands belong to cuticular waxes and cutin, which are the major compounds in the cuticular membrane of green tomatoes [18,21,28]. Bands related to these compounds can be associated with two spectral regions. The region between 600-1800 cm^{-1}, which includes information about vibrational modes of the skeletal backbone and different functional groups joined to this skeleton, and the region between 2700-3600 cm^{-1}, which is dominated by C-H, N-H and O-H stretching bands [25] (see Table 1 and Figure 1). The Raman spectra obtained from different parts of the green tomato´s fruit cuticular layer showed spectra of typical long-chain aliphatic compounds. In these spectra, the strong peak at 1303 cm^{-1} represents $(CH_2)_n$ twisting vibration [23,28]. One of the strongest bands of the spectra located ca. 1439 cm^{-1} is related to bending vibrations of the CH_2 [25] and the band at 1371 cm^{-1} represents weak wagging vibrations of these kinds of molecules [25]. The medium intensity band at 1168 cm^{-1} can be assigned to ring in-plane CH deformation [25]. There are also other weaker bands present in this region related to this aliphatic compounds such as 1065 cm^{-1} associated with C-C stretching [25] and, 1267 cm^{-1} assigned to δ_{ip}(=C–H)cis vibration [23,29] (see Table 1). A band around 1720 cm^{-1} is also present in Raman spectra of green tomatoes. This band, also related to cuticular waxes, is usually broad and weak in the case of long acids and esters chains and is associated with the stretching of the C=O [21]. There is also another very weak and broad band located at 2727 cm^{-1} which is also related to these fatty compounds. Concretely, it has been suggested that this band is connected with the stretching of CH and the overtone of this aliphatic compound [30]. In the spectral range 2700-2940 cm^{-1}, groups of stretching vibrational modes of CH_2 and CH_3 are present. These bands are the strongest peaks in the spectra and they are related to the fatty acid and saturated ester compounds located in the epidermis of green tomatoes [21,25]. In the obtained spectra, the broad Raman band between 2830 and 2970 cm^{-1} suggests the presence of more than one band inside this spectral region (see Figure 2). To resolve these peaks, curve-fitting was performed using GRAMS/AI 7.02 software. Prior to this deconvolution process Raman spectra were baselined, in order to minimize the effect of the background (slope). In the curve-fitting process, different algorithms (normally Gaussian, Lorentzian and mixed Gaussian-Lorentzian) were tested at different sensitivity levels (low, medium and high) to obtain all the individual bands. The best results were obtained with Gaussian and mixed Gaussian and Lorentzian. Using this curve-fitting application four Raman bands were observed. The band located at 2853 cm^{-1}, is related to the symmetric stretching of CH_2 [25] and there is another one around 2925 cm^{-1} associated with the asymmetric stretching of CH_2 [29]. Moreover, there is another strong band ca. 2906 cm^{-1} attributed to the CH_3 stretching [25]. Analyzing this region, in the spectra obtained with the portable Raman instrument, a weak and broad band around 2914 cm^{-1} is present. The mentioned band does not show any shoulder to extract additional bands. As in the spectra obtained with the confocal microscope, this broad band can be associated with lipids and cuticular waxes vibrational modes [25].

The spectral region around 1540-1650 cm^{-1} is related to the aromatic ring of phenolic compounds [31,32]. The most intense band in this region at 1605 cm^{-1} is attributed to the phenolic aromatic rings, and the medium band at 1631 cm^{-1} is related to C=C stretching vibrations of isolated unsaturated phenolic compounds [25] (see Figure 1). There is another weak signal ca. 1587 cm^{-1} also associated with phenolic compounds, concretely related to the aromatic ring C-C stretching [31] (see Table 1).

The green tomato also shows weak bands located in the fingerprint area of the carotenoids (1000-1600 cm^{-1}). The three characteristic bands of this tetraterpene appear around 1524 cm^{-1}, 1157 cm^{-1}, 1003 cm^{-1} and they are associated with ν_1 (C=C stretching), ν_2 (C-C stretching), ν_3 (C-CH_3 stretching) vibrational modes respectively [23,30]. Apart from these bands, there are also other secondary bands related to carotenoids, but they are not visible because they are overlapped with strong bands of polymeric cutin and cuticular waxes, such as the band located around 1439 cm^{-1} (see Figure 1). Moreover, it has also been suggested that the very weak band located c.a. 1553 cm^{-1} is associated with carotenoids. This signal is shifted to a higher frequency, which is consequence of a shorter conjugation length in this molecule. Concretely, this band is attributed to phytofluene [33]. However, phytoene could also be associated to this signal because they have a similar conformation, only differentiated by one C=C, thus it is difficult to assure that this band is exclusively related to phytofluene.

3.2. Portable spectrometer (785 nm) vs confocal Raman microscope (785 and 514 nm) in the Raman characterization red tomatoes

In the Figure 3 Raman spectra obtained from the surface of red tomatoes with the confocal and portable instruments using 785 and 514nm excitation lasers can be observed.

The spectra acquired with these instruments show nearly the same bands. In this case, it can also be seen that the intensity of the Raman bands related to carotenes is enhanced using the 785nm laser excitation wavelength. This increase in the intensity of carotenes bands is higher in those bands situated in the fingerprint area (1000-1600 cm^{-1}). Measurements carried out with the confocal instrument using the 514nm laser excitation wavelength show an enhancement of some bands that are hidden or have weak intensity in the spectra obtained with 785nm laser excitation wavelength. The 514 nm laser enabled to make a difference some peaks that are shoulders in the spectra acquired with the 785nm excitation wavelength. Specifically, it can be seen that the main bands related to cuticular waxes and cutin showed stronger bands in the spectra collected with the confocal Raman microscope using the 514nm excitation wavelength than the same bands obtained with portable and confocal microscope using 785nm excitation wavelength.

As was mentioned, compunds such as cutin and cuticular waxes are connected with the superficial cuticular matrix. The spectra acquired with the 514nm laser excitation wavelength enhanced the signal of these compounds. The most characteristic and strong Raman bands related to these compounds are ca. 980, 1167, 1269, 1293 and 1441 cm^{-1} [21,29,34]. Moreover, the very strong band at 1604 cm^{-1} can be attributed to aromatic ring of phenolic compounds and the band at 1623 cm^{-1} is related to C=C stretching vibrations of unsaturated phenolic compounds. These bands are also present in the spectra of green tomatoes, and they are probably related to waxes present in the cuticular membrane. Furthermore, the band ca. 1585 cm^{-1} is related to the C-C stretching aromatic ring probably attributed to phenolic compounds [31]. Raman bands related to these phenolic compounds showed higher intensity in spectra acquired with the 514nm laser excitation wavelength. The most intense bands in Raman spectra obtained with 785nm laser excitation wavelength are those located in the fingerprint area (1000-1800 cm^{-1}) of carotenoids. The main band in this carotenoid

fingerprint area at 1520 cm^{-1} is associated with C=C stretching vibration (ν_1), the second strongest band at 1156 cm^{-1} is related to C-C stretching (ν_2) and a weak band ca. 1007 cm^{-1} is attributed to C-CH_3 stretching (ν_3) (see Table 2). In the Figure 3B, two bands at 958 and 964 cm^{-1} are also present. These bands can be assigned to CH_3 in-plane rocking vibrations (ν_4) of carotenoids [35]. This doublet is only observable in the measurements carried out with the confocal Raman microscope using the 785 nm excitation laser excitation wavelength. If the same instrument with 514 nm laser and the portable instrument (785 nm) are used for this purpose, only a band ca. 960 cm^{-1} can be indentified (see Table 2). Considering the Raman features of β-carotene and lycopene standards (see Figure 4), the bands at 958 and 964 cm^{-1} obtained with the confocal Raman microscope (785 nm laser) or the band at 960 cm^{-1} with the confocal Raman microscope (514 nm laser) and the portable Raman spectrophotometer can only be observed in the lycopene standard. Therefore, the band at 960 cm^{-1} (or 958 and 964 cm^{-1}) cannot be assigned to β-carotene. However, apart from lycopene, this/these Raman band/s can be related to other carotenoids such as lutein, zeaxanthin and/or β-cryptoxanthin which can be also present in red (ripe) tomatoes [35].
As it is seen in red tomato Raman spectra (see Figure 3), there are also other secondary Raman bands attributed to the carotenes such as 445(vw), 517(vw), 870(vw), 889(vw), 1209(w), 1251(vw), 1259(vwsh), 1281(w), 1311(w), 1351(vw), 1389(vw), 1441 (w) cm^{-1} (see Figure 3 and Table 2). These weak bands were also identified thanks to the spectra obtained from pure lycopene and β-carotene standards (see Figure 4). Moreover, there is an additional band c.a. 1184 cm^{-1} also related to carotenoids, which is believed to be associated to the β-carotene [25]. As it is observed in the spectra of green tomato, in red tomatoes the band ca. 1554 cm^{-1} that can be attributed to phytofluene and/or phytoene is also present. However, the intensity of this band in red tomatoes spectra is higher than in green tomatoes (see Figure 1, Figure 3 and Table 2) [33].
The weak and broad bands situated in the region of 1040-1080 cm^{-1} are suggested to be associated to cuticular waxes. Specifically, the bands around 1050, 1063 and 1078 cm^{-1} are attributed to these waxy compounds present in the fruit and leaves of tomatoes [24,25]. Moreover, the band around 1720 cm^{-1} is related to the stretching of the C=O of these cuticular waxes [21].
Apart from these bands, there are also other bands related to the cuticular waxes in the 2850-2950 cm^{-1} spectral region. As in the green tomatoes' spectra, it is difficult to specify the bands that are present in this region because there is a very broad and flat band, which includes additional bands. Therefore, this spectral region was subjected to a curve-fitting treatment (see Figure 5). The fact that this broad band is placed in the same wavenumber region and has similar shape as the broad one on the green tomatoes suggests that the same bands extracted in the green tomatoes are present in the spectra of red tomatoes. Moreover, there is also another very weak and broad band located at 2723 cm^{-1} which is also related to these fatty compounds.
In the Figure 5, the result of the curve-fitting application to perform the deconvolution of broad Raman bands between 2870-2970 cm^{-1} can be observed. The curve-fitting application in this spectral range shows the presence of five bands related to the lipid layer of the cuticular membrane. These bands are located at 2963 cm^{-1} [36], which is usually attributed to the CH_3 asymmetric stretching mode; at 2920-2930 cm^{-1}, which is related to CH_2 asymmetric stretching mode [29]; at 2901-2910 cm^{-1}, that is associated

with the CH_3 symmetric stretching mode [29], and at 2854 cm^{-1}, assigned to the CH_2 symmetrical stretching mode [25]. The presence of cellulosic compounds is also possible in this region, thus some of the bands related to this polysaccharide could be overlapped by the strong bands of these fatty compounds. Another possibility is that the bands of waxes and celluloses could be added resulting in a higher intensity band. In the bibliography, the band around 2898 cm^{-1} is associated to the CH-CH_2 stretching of the cellulose [28].

There is also another characteristic band in these spectra that is related to the polysaccharides. Specifically, the weak band at 853 cm^{-1}, attributed to the main band of pectin, is present in the spectra of ripe tomatoes. This compound is not visible with the 514nm excitation laser excitation wavelength, but it is weakly seen in the spectra achieved with the 785nm laser excitation wavelength. This band could be overlapped by the effect of another band related to carotene, which is situated in the same position (see Figure 5).

It is also important to highlight that the 514 nm excitation laser allows observing some bands that cannot be observed in the spectra obtained with the 785 nm excitation laser wavelength, like overtones and combination tones. These Raman bands increase their intensity due to the Resonance Raman effect achieved with this green laser. With the 785 nm excitation laser it is also possible to obtain Raman vibrational modes, which are normally difficult to observe or are hidden. In these spectra there are some Raman bands related to the carotenes out of the Raman fingerprint area of carotenoids (1000-1600). These Raman bands attributed to overtones and combination-tones are usually visible with the 514 nm wavelength laser in the spectral region ca. 2000-2700 cm^{-1}. According to the literature assignations [37,38,39], in these specific spectra two overtones (at 2308-2316 ($2\nu_2$) cm^{-1} and 3023 ($2\nu_1$) cm^{-1}) and four combination-tones (at 2158-2167 ($\nu_2+\nu_3$), 2518-2524 ($\nu_1+\nu_3$), 2660-2670 ($\nu_1+\nu_2$) cm^{-1} and 3670 ($\nu_1+\nu_2+\nu_3$) cm^{-1}) related to carotenes were identified. As it was mentioned above, ca. 2464 cm^{-1} an overtone or combination-tone, which is a characteristic tone of lycopene spectrum but is not present in β-carotene spectrum, can be observed in red tomato spectra (see Figure 4). Moreover, an overtone of a combination-tone ($2\nu_2+\nu_3$) related to carotenes is also present at 3320 cm^{-1}.

Comparing the Raman spectra obtained from red tomatoes and Raman spectra obtained from lycopene standard, the overtones and/or combination-tones at 2564 and 3619 cm^{-1} are characteristic of lycopene (see Figure 4 and Table 2). Therefore, it can be said that these two overtones and/or combination-tones are the markers of lycopene presence in red tomatoes. Furthermore, these bands can be used to distinguish its presence from the presence of other carotenoids which have similar Raman features especially in their fingerprint area (900-1600 cm^{-1}). But the presence of lycopene does not preclude the presence of other carotenoids.

It should be noted that there is a variation of 10 cm^{-1} in some of the Raman bands of lycopene and beta-carotene standards comparing with the ones observed in the tomatoes. For example, the band located at 1518 cm^{-1} which is attributed to the lycopene is located around 1508 cm^{-1} in the lycopene standard. This variation could be related with the molecular interaction between the carotenoids and the cellulosic moiety presents in the tomato fruits, which difficulties the assignment of the different carotenoids [40]. There is also a small variation in the same bands, observed in the Table 1 and 2. These variations, in the range of three cm^{-1}, are not attributed to the before

mentioned effect. This small shift in the position of the same band could be explained by the different physical characteristics of the different spectrometers or the spectral recording conditions used, such as spectral resolution or the time to acquire a measure[40].

4. Conclusions

The two Raman instruments (portable and confocal microscope) considered in this work for green and red tomatoes measurements offer complementary information. The portable instrument can be used as a good alternative to the confocal microscope for analyses of ripe tomatoes. However, the best alternative to measure the green tomatoes is the confocal Raman microscope, because it gives the maximum spectral information of the organic composition of unripe tomatoes. The Raman spectra obtained with the portable instrument on green tomatoes offer low resolution spectra. This could be attributed to the sensibility of the instrument; the portable instrument has a lower sensibility (around 3.5 cm^{-1}) than the confocal instrument (around 1 cm^{-1}). For that reason, the confocal Raman microscope can offer better results in the monitorization of cuticular waxes in green tomatoes than the portable device.

The analyses carried out on red and green tomatoes demonstrate that Raman spectroscopy is an appropriate technique to identify the main organic components (carotenoids, cuticular compounds, polysaccharides, polyphenols, etc.) of the two ripening stages in this fruit. The major compounds in unripe tomatoes detected with Raman spectroscopy are cutin and cuticular waxes. On the contrary, in ripe tomatoes the major compounds detected with this technique are carotenes, polyphenols and polysaccharides. Lycopene is the major compound on red tomatoes. Furthermore, the reduction of the waxes and cutin in this ripening stage was clearly seen.

The confocal Raman microscope using the 514 nm excitation laser wavelength is a good alternative to observe Raman bands related to cuticular compounds and other vibrational modes such as overtones and combination-tones related to carotenoids. The enhancement of these Raman bands could take place thanks to the Resonance Raman effect achieved with the 514 nm excitation wavelength. This specific effect makes possible the identification of two overtones or combination-tones exclusively related to lycopene (2464 and 3619 cm^{-1}). Thanks to these two bands, it is possible to assure that lycopene is present in red tomatoes, and its presence can be distinguished spectroscopically among β-carotene. The measurements carried out in these tomatoes offered high resolution spectra that showed many low intensity bands which are not easily visible in previous referenced Raman spectra.

Considering the different Raman features observed for both ripe and unripe tomatoes, portable Raman spectrometers can be proposed as promising devices to monitor this kind of fruit maturation following *in situ* analyses in tomato cultivars.

Acknowledgements

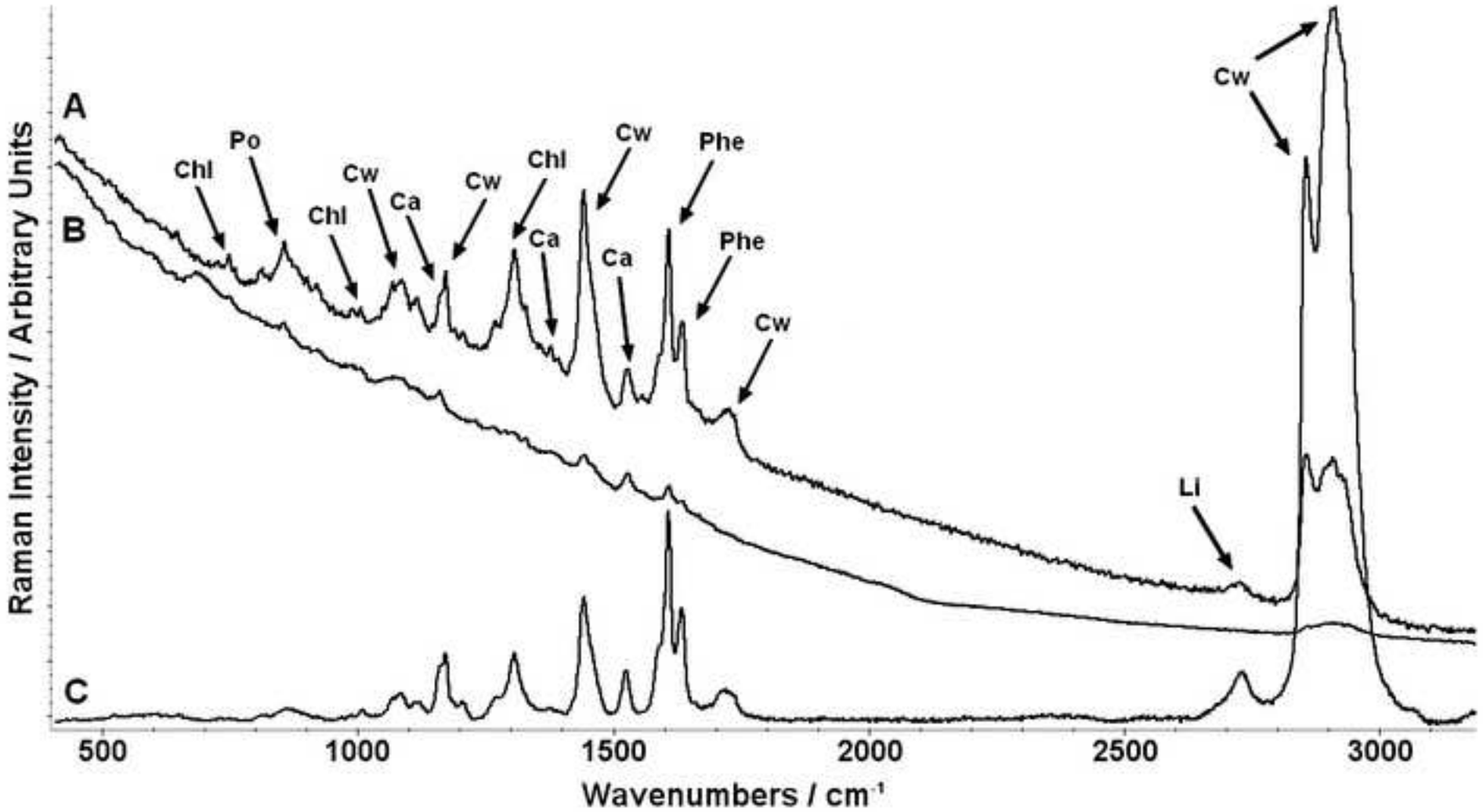
This work has been financially supported by Research Project S-PE11-UN128 of the Basque Country government. Technical and support provided by SGIker (UPV/EHU, MICINN, GV/EJ, ESF) is gratefully acknowledged. The authors also acknowledge the

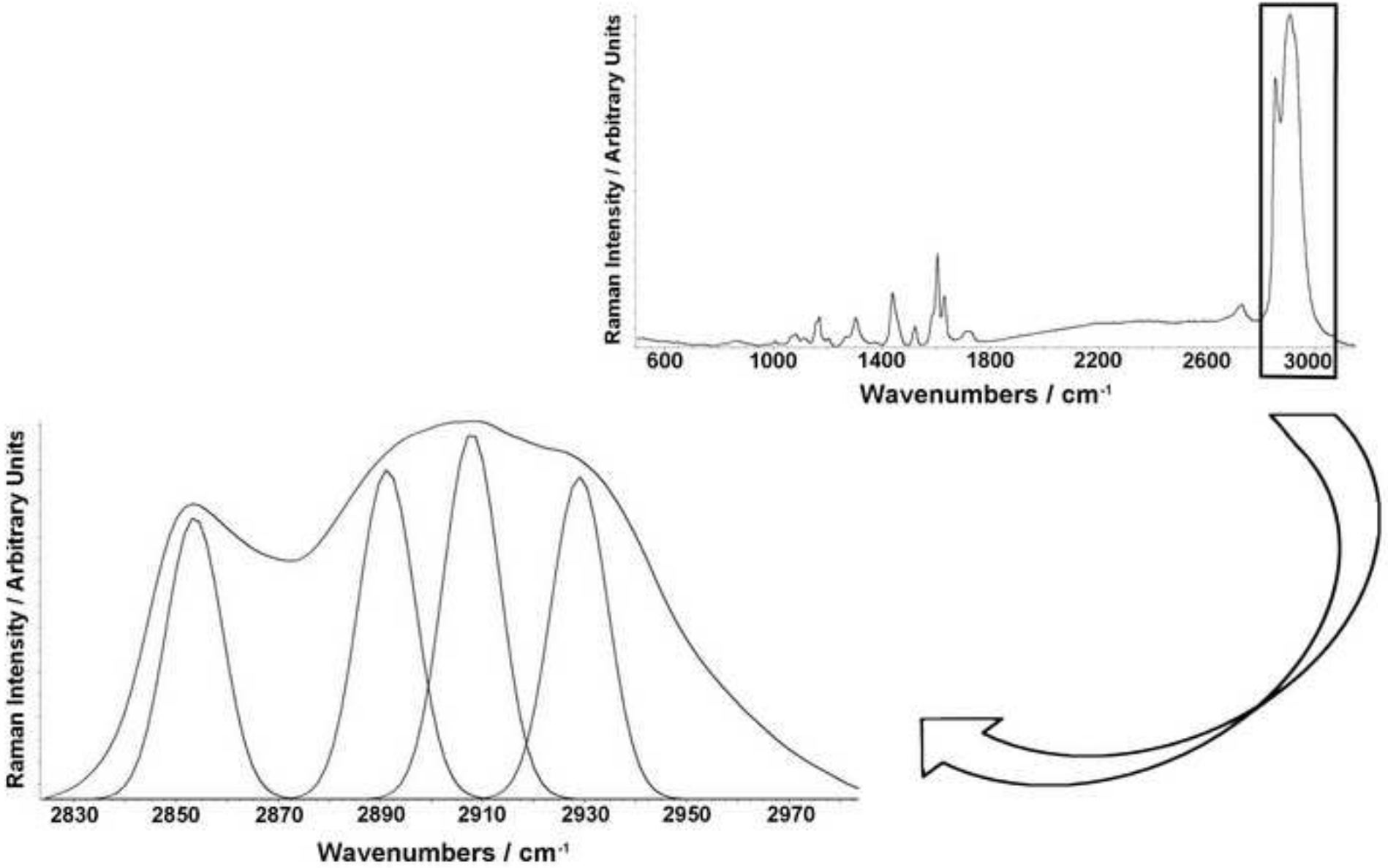
provided language help of Ira Ortigosa graduated in translating and interpreting and English studies degree.

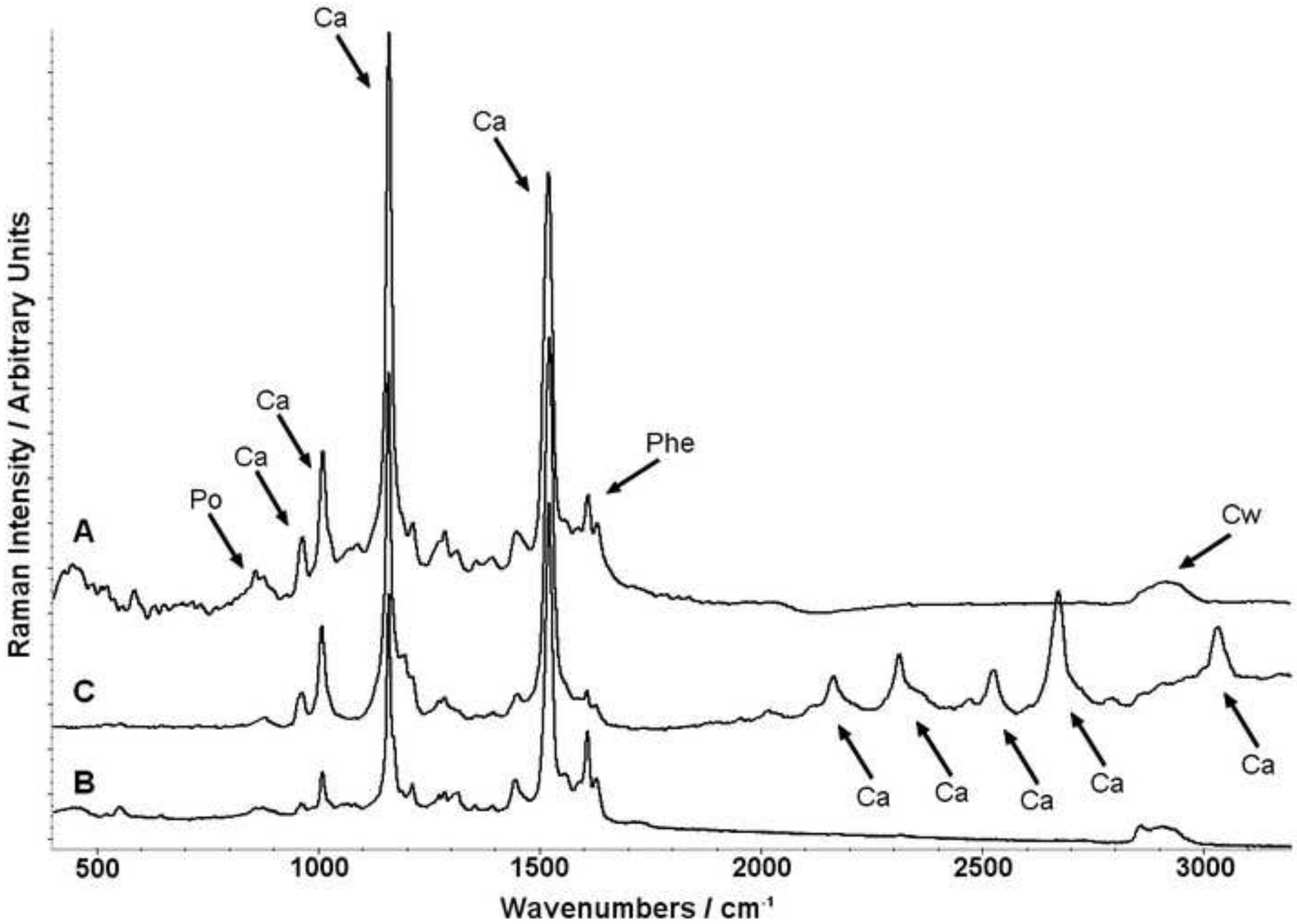
References

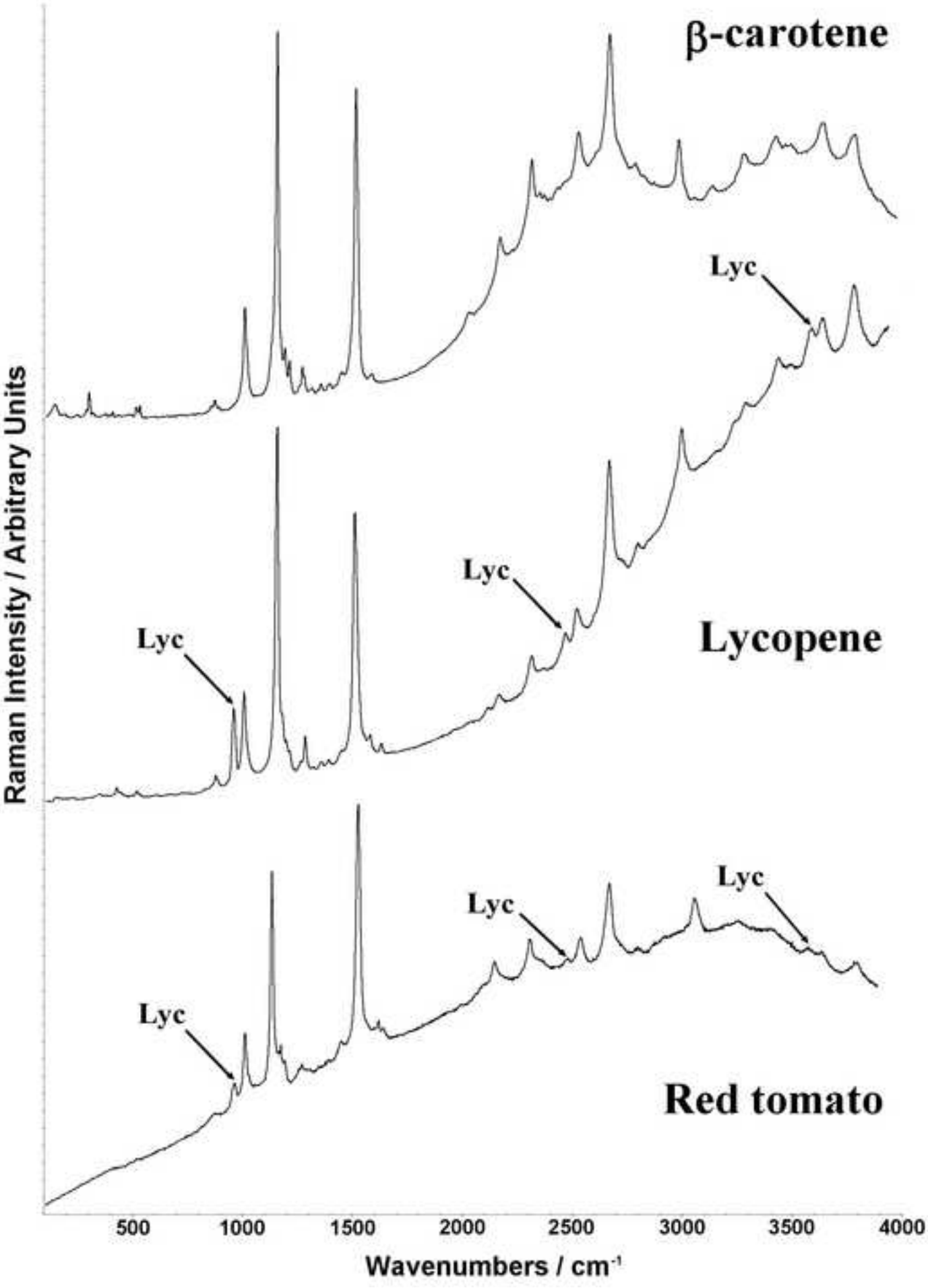
[1] F. Nuez, El cultivo del tomate, Ediciones Mundi-Prensa. Bilbao, Spain, 1995.
[2] A.M.K. Pedro, M.M.C. Ferreira, Anal. Chem. 77 (2005) 2505-2511.
[3] A.V. Rao, S. Agarwal, Nutr. Res. 19 (1999) 305-323.
[4] L. Frusciante, P. Carli, M.R. Ercolano, R. Pernice, A. Di Mateo, V. Fogliano, N. Pellegrini, Mol. Nutr. Food Res. 51 (2007) 609-617.
[5] C. Ulrichs, G. Fischer, C. Büttner, I. Mewis, Agron. Colomb. 26 (2008) 40-46.
[6] A.A. Abushita, H.G. Daood, P.A. Biacs, J. Agric. Food Chem. 48 (2000) 2075-2081.
[7] L. Feltl, V. Pacáková, K. Stulík, K. Volka, Curr. Anal. Chem. 1 (2005) 93-102.
[8] D.B. Rodriguez-Amaya, M. Kimura, Carotenoids in Foods. In *HarvestPlus Handbook for Carotenoid Analysis*, HarvestPlus, Wahington, USA, 2004, 2-4.
[9] D. Dutta, U.R. Chaudhuri, R. Chakraborty, Afr. Jour. of Biotech. 4 (2005) 1510-1520.
[10] A. Mortensen, Pure Appl. Chem. 78 (2006) 1477-1491.
[11] H.P. McNulty, J. Byun, S.F. Lockwood, R.F. Jacob, R.P. Mason, Biochim. Biophys. Acta 1768 (2007) 167-174.
[12] S. Xianquan, J. Shi, Y. Kakuda, J. Yueming, J. Med. Food 8 (2005) 413-422.
[13] G. Polder, G.W.A.M. van der Heijden, H. van der Voet, I.T. Young, Postharvest Biol. Tec. 34 (2004) 117-129.
[14] S. Bauer, E. Schulte, H.-P. Thier, Eur. Food Res. Technol. 219 (2004) 223-228.
[15] T. Issacson, D.K. Kosma, A.J. Matas, G.J. Buda, Y. He, B. Yu, A. Pravitasari, J.D. Batteas, R.E. Stark, M.A. Jenks, J.K.C. Ros, The Plant J. 60 (2009) 363-377.
[16] J.J. Reina-Pinto, A. Yephremov, Plant Physiol. Biochem. 47 (2009) 540-549.
[17] A. Heredia, Biochim. Biophys. Acta 1620 (2003) 1-7.
[18] G. López-Casado, A.J. Matas, E. Domínguez, J. Cuartero, A. Heredia, J. Exp. Bot. 58, (2007) 3875-3883.
[19] W. Barnard, D. de Waal, J. Raman Spectrosc. 37 (2006) 342-352.
[20] M. Baranska, W. Schütze, H. Schulz, Anal. Chem. 78 (2006) 8456-8461.
[21] D.S. Himmelsbach, D.E. Akin, J. Agric. Food Chem. 46 (1998) 991-998.
[22] C.F.B. Séné, M.C. McCann, R.H. Wilson, R. Grinter, Plant Physiol. 106 (1994) 1623-1631.
[23] H. Schulz, M. Baranska, Vib. Spectrosc. 43 (2007) 13-25.
[24] M.M.L. Yu, H.G. Schulze, R. Jetter, M.W. Blades, R.F.B. Turner, Appl. Spectrosc. 61(1) (2007) 32-37.
[25] L.C. Prinsloo, W. Du Plooy, C. Van der Merwe, J. Raman Spectrosc. 35 (2004) 561-567.
[26] S.E. Jorge-Villar, H.G.M. Edwards, Anal Bioanal Chem. 384 (2006) 100-113.
[27] S.E. Jorge-Villar, L.G. Benning, H.G.M. Edwards, Geochem Trans. (2007) 8.
[28] N. Gierlinger, M. Schwanninger, Plant Physiol. 140 (2006) 1246-1254.
[29] C.E. da Silva, P. Vandenabeele, H.G.M. Edwards, L.F. Cappa de Oliveira, Anal. Bioanal. Chem. 392 (2008) 1489-1496.
[30] M. Wegener, R. Neubert, W. Rettig, S. Wartewig, Chem. Phys Lipids 88 (1997) 73-82.
[31] M. Nuopponen, S. Willför, A.S. Jääskeläinen, T. Vuorinen, Spectrochim. Acta A 60 (2004) 2963-2968.
[32] M. Schmidt, A.M. Schwartzberg, P.N. Perera, A. Weber-Bargioni, A. Carroll, P. Sarkar, E. Bosneaga, J.J. Urban, J. Song, M.Y. Balakshin, E.A. Capanema, M. Auer, P.D. Adams, V.L. Chiang, P.J. Schuck, Planta 230 (2009) 589-597.

[33] I.V. Erkamov, M. Sharifzadeh, M. Ermakova, W. Gellermann, J. Biomed. Opt. 10 (2005) 064028.
[34] H.G.M. Edwards, M.J.P. Flak, Spectrochim. Acta A 53 (1997) 2685-2694.
[35] B. Robert, Photosynth. Res. 101 (2009) 147-155.
[36] L. Shan-Yang, L. Mei-Jane, L. Run-Chu, L. Shui-Mei, Spectrochim. Acta A 54 (1998) 1509-1517.
[37] H. Okamoto, S. Saito, H. Hamaguchi, M. Tasumi, C.H. Eugster, J. Raman Spectrosc. 15 (1984) 331-335.
[38] M. Veronelli, G. Zerbi, R. Stradi, J. Raman Spectrosc. 26 (1995) 683-692.
[39] H. Okamoto, Y. Sekimoto, M. Tasumi, Spectrochim. Acta A 50 (1994) 1467-1473.
[40] V. E. de Oliveira, H. V. Castro, H. G. M. Edwards, L. F. C. de Oliveira, J. Raman Spectrosc. 41 (2010) 642-650.









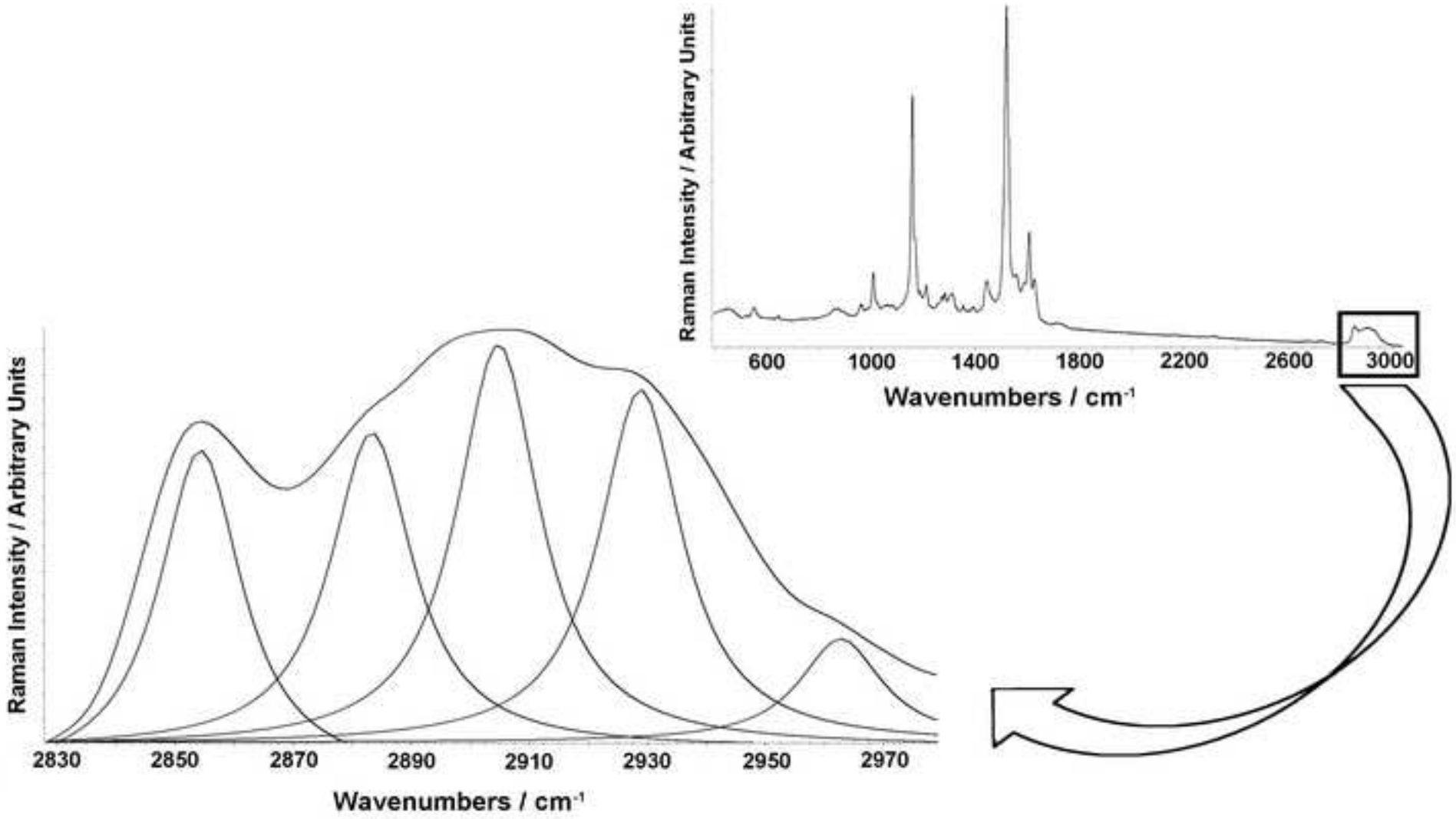


Table 1. Assignation of vibrational modes identified in the spectra of green tomato (unripe) obtained with both instruments and both laser excitation wavelengths (785 and 514 nm).

785 nm Portable	785 nm Confocal	514 nm Confocal	Vibrational assignments	Molecular assignments
743 vw	744 w		δ(N-C-C)	chlorophyll a
852 vwbr	853 m	852 vw,br	ν_{as}(C-O-C)	polysaccharide
				carotene
	915 w		δ(C-C-C)	chlorophyll a
	985 w		$\delta(CH_3)$	chlorophyll a
1002 vw	1002 w	1003 w	$\nu(C\text{-}CH_3)$	carotene
	1047 vw			cuticular wax
	1065 vw	1065 w,sh	ν(C-C)	cuticular wax
	1081 br	1082 w		cuticular wax
	1112 w	1109 w		cuticular wax
1157 m	1157 m, sh	1158 m	ν(C-C)	carotene
	1169m	1168 m	ring δ_{ip}(CH)	cuticular wax
	1266 w	1267w	δ_{ip}(=C-H)	lipids
	1303 m	1303m	$\tau_{ip}(CH_2)$	lipids
1325 vw,br	1325 vw		δ(CH)	chlorophyll a
	1371 vw		$\omega(CH_2)$	cuticular wax
1438 m	1439 s	1439 s	$\delta(CH_2)$	cuticular wax
			$\delta(CH_3)$	carotene
1525 m	1524 m	1521m	ν(C=C)	carotene
	1553 vw		ν(C=C)	phytoene/phytofluene
	1588 vw	1587 m, sh	aromatic ν(C-C)	phenolic compounds
1605 m	1605 s	1605 s	aromatic ring	phenolic compounds
1630(1633) w	1631 m	1630 m	ν(C=C)	phenolic compounds
	1721 m, br	1720 m, br	ν(C=O)	cuticular wax
	2720 vw, br	2727 vw, br	ν(CH) aliphatic + overtones δ(CH)	lipids
2853 vw	2853 s	2853 vs	$\nu_s(CH_2)$	cuticular wax
2914 w, br				cuticular wax
	2904 s	2908 vs	$\nu_s(CH_3)$	cuticular wax
	2921 s	2927 vs	$\nu_{as}(CH_2)$	cuticular wax
		3063 w		

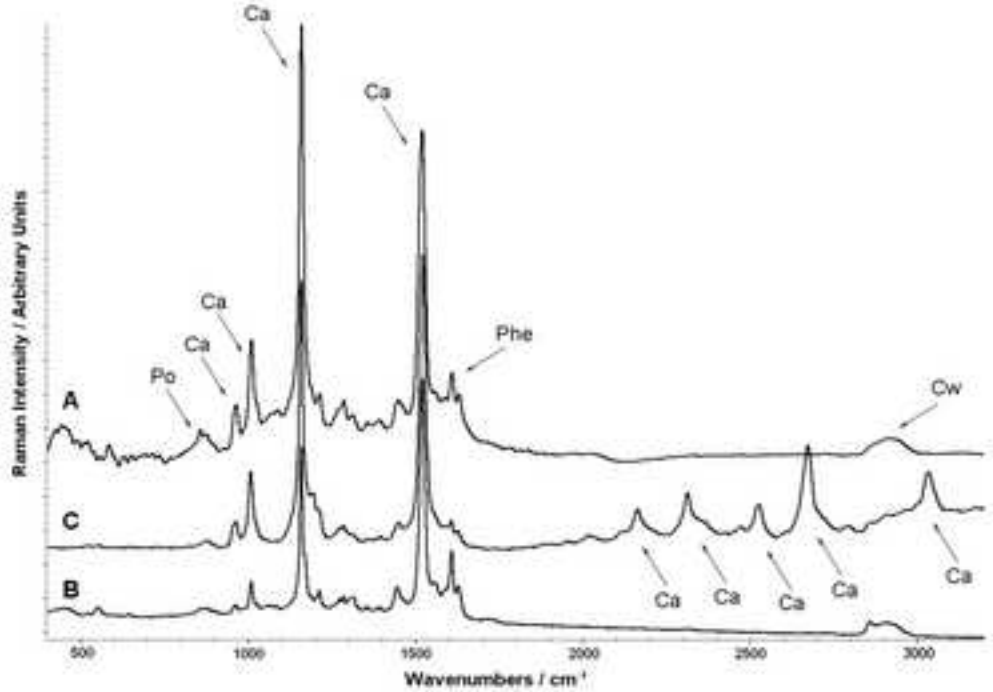
Vibrations: ν, stretching; δ, deformation (bending/scissoring); ω, wagging; τ, twisting; s, symmetric; as, asymmetric; ip, in-plane

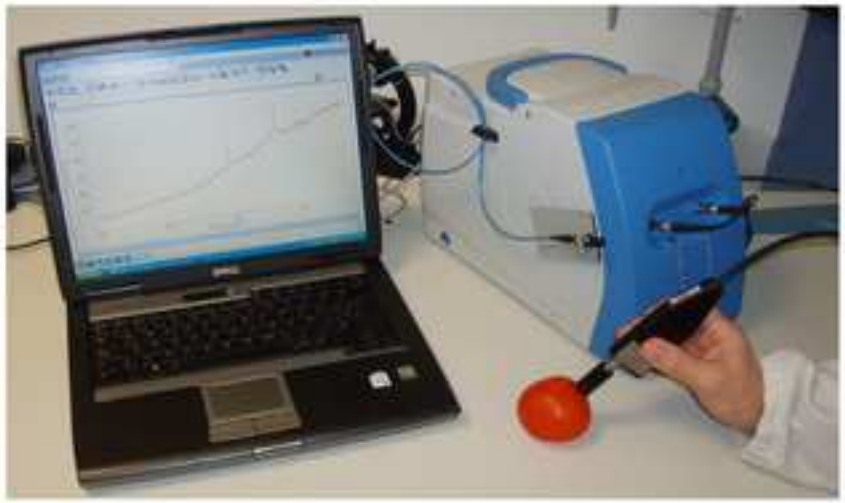
Table 2. Assignments of vibrational modes identified in the spectra of red tomato (ripe) obtained with both instruments and both laser excitation wavelengths.

785 nm Portable	785 nm Confocal	514 nm Confocal	Vibrational assignments	Molecular assignments
445 w, br	443 w, br			
		457 m, br		
517 vw	517 vw	517 vw		lycopene
548 m	548 w	546 m		
642 w	641 w	642 w		
853 w	853 vw		ν_{as}(C-O-C)	polysaccharide
				carotene
872 vw	870 vw	873 w		carotene
	889 vw, br			carotene
896 vw		893 w		
960 w	958 w	958 m, br	$\rho_{ip}(CH_3)$ (ν_4)	carotenoid
	964 vw		$\rho_{ip}(CH_3)$ (ν_4)	carotenoid
981 w		980 vw		cuticular wax
1007 m	1007 m	1004 m	ν(C-CH_3) [ν_3)	carotene
1050 vw, br	1053 vw	1045 vw		cuticular wax
1063 vw, br	1062 vw	1066 vw, br	ν(C-C)	cuticular wax
1078 vw,br	1079 vw	1078 vw, br		cuticular wax
1157 vs	1156 s	1156 s	ν(C-C) (ν_2)	carotene
1166 sh	1167 m	1167 s	ring δ_{ip}(CH)	cuticular wax
	1184 vw			carotene
1209 m	1209 m	1209 w, br		carotene
1251 vw	1253 vw, sh	1253 vw, br		β-carotene
1269 w	1269 w		ν_4	β-carotene
			δ_{ip}(=C-H)	lipids
1281 w	1281 vw			carotene
		1293 m		cuticular wax
1310 m	1310 m, br	1311 m		β-carotene
1351 vw,br	1351 w			carotene
1388 w,br	1390 vw, br			carotene
1441 m	1441 m, br	1443 vw, br	$\delta(CH_2)$	cuticular wax
			$\delta(CH_3)$	carotene
1520 s	1518 vs	1516 s	ν(C=C) (ν_1)	carotene
1554 m	1553 w, br	1556 s		phytoene/phytofluene
1584 w	1585 w	1584 w	aromatic ν(C-C)	phenolic compounds
1605 m	1606 m	1604 vs	aromatic ring	phenolic compounds
1624 w	1623 m, br	1623 s		phenolic compounds
	1720 vw, br		ν(C=O)	cuticular wax
		2161 w	$\nu_2+\nu_3$	carotene ct
	2311 vw	2312 w	$2\nu_2$	carotene ov
		2464 vw		lycopene ov or ct
		2521 w	$\nu_1+\nu_3$	carotene ct
		2665 w	$\nu_1+\nu_2$	carotene ct
		2723 sh	ν(CH) aliphatic + overtones δ(CH)	lipids
2858 w	2854 m	2855 m	$\nu_s(CH_2)$	cuticular wax
		2898 m	ν(CH) + $\nu(CH_2)$	cellulose
2901 w, br	2906 m, br	2910 m	$\nu_s(CH_3)$	cuticular wax
2925 w, br		2924 m	$\nu_{as}(CH_2)$	cuticular wax
		2963 vw, sh	$\nu_{as}(CH_3)$	lipids
		3023 w	$2\nu_1$	carotene ov

	3320 vw	$2\nu_2+\nu_3$	carotene ov or ct
	3460 vw, br		carotene ov or ct
	3529 vw, br		carotene ov or ct
	3619 vw, br		lycopene ov or ct
	3670 vw	$\nu_1+\nu_2+\nu_3$	carotene ct
	3815 w		carotene ov or ct
	3974 vw		carotene ov or ct

Vibrations: ν, stretching; δ, deformation (bending/scissoring); ω, wagging; τ, twisting; ρ, rocking; s, symmetric; as, asymmetric; ip, in-plane; ov, overtone; ct, combination-tone





The portable and confocal Raman instruments using 514 and 785 nm laser excitation wavelengths provide different and complementary spectroscopic information about tomato fruits composition.

The major compounds detected using Raman spectroscopy in unripe tomatoes are cutin and cuticular waxes and in ripe tomatoes are carotenes, polyphenols and polysaccharides.

It is possible to differentiate spectroscopically lycopene from beta-carotene in ripe tomatoes.

Phytoene/phytofluene was spectroscopically detected in tomatoes, which was not already included in works based on Raman spectroscopy application on fruit and vegetables.

New generation of Raman portable instruments can offer reliable results for on-site analysis in cultivars and foodstuffs.