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L. Asnin <sup>a</sup> & S. W. Park <sup>a</sup>

<sup>a</sup> Department of Molecular Biotechnology , Konkuk University , Seoul , South Korea

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## Isolation and analysis of bioactive compounds in *Capsicum* peppers

L. Asnin, and S. W. Park

Department of Molecular Biotechnology, Konkuk University, Seoul, South Korea

*An overview of the state-of-the-art in the extraction, isolation, and analytical determination of bioactive compounds in peppers of the genus Capsicum is presented. The review is structured by classes of phytochemicals. Both major and minor constituents of peppers are considered. Modern trends in analytical chemistry of nutrients in regard to pepper analysis with particular focus on chromatographic and related methods are discussed. Attention was paid to controversial questions of pepper analysis, including but not limited to problems of sample degradation and the completeness of extraction of target analytes. The rationale for choosing an optimal strategy of analysis is given.*

**Keywords** pepper, Capsicum, plant analysis, nutraceuticals, natural bioactive compounds

Address correspondence to S. W. Park, Konkuk University, Dept. of Molecular Biotechnology, 1 Hwayang-dong, Gwangjin-Gu, Seoul 143-701, South Korea

## INTRODUCTION

Pepper is an annual herbaceous plant of the *Capsicum* genus belonging to the *Solanaceae* family cultivated in regions of temperate and warm climate and valued for its characteristic pungency, aroma, and color appeal. Various authors ascribe 25 species to the genus, the five major domesticated species are: *Capsicum annuum* L., *Capsicum frutescens* L., *Capsicum baccatum* L., *Capsicum chinense* Jacq., and *Capsicum pubescens* Ruiz and Pav. (Basu and De, 2003). (To mention it specifically, *Capsicum* pepper is not related to the *Piper* genus of the family *Piperaceae*, which contains *Piper nigrum* L., the source of black and white pepper). The fruit is important economically due to vast quantities used (Thampi, 2003). It is sold as

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fresh, dried, or pickled pepper, ground powders (paprika), and in the form of processed products like purees, sauces, and oleoresins. The latter are pepper extracts, which, depending on type of pepper and an extraction method, can be either color-rich, used in foodstuff and cosmetics, or pungent-rich, serving as a source of pungent component capsaicin for pharmaceutical products (Pruthi, 2003; Zachariah and Gobinath, 2008) or self-defense weaponry (Reilly et al., 2001). The current trend to a healthier food and the continuing quest for new drugs explain the attention given to pepper as a source of bioactive compounds that can serve as drug candidates. Indeed, researches have proven antioxidant (Choi et al., 2006), anticancer (Maoka et al., 2001; Laviada, 2006), anti-inflammatory (Sancho et al., 2002; Szolcsányi, 2003), and antiulcer (Kang et al., 1995) properties of pepper extracts. A pepper-rich diet is supposed to be helpful against obesity (Yoshioka et el., 1995; Ohnuki et al., 2001) and promises other health benefits (Ahuja and Ball, 2006; Voutilainen et al., 2006; Loizzo et al., 2008). Revealing substances responsible for the biological activity of pepper, their isolation from the pepper matrix and development of new *Capsicum* varieties enriched in desirable phytochemicals are the objectives that heavily depend on a detailed and accurate qualitative and quantitative analysis.

There are several groups of valuable phytochemicals in *Capsicums*, including carbohydrates, which constitute approximately 85 percents of the dry matter, polyphenols (~ 0.5% dw), and minor, yet important, bioactives capsaicinoids, carotenoids, vitamins, *etc.* Methods of analysis for each group were developed, but respective information is scattered throughout the literature. Few attempts were made to critically review advances in the analysis of peppers. So, Daood (2009) shortly considered determination of carotenoids and vitamins. Manurakiza et al. (2003) discussed the analysis of capsaicinoids.

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Analytical methods for the assessment of color and pungency of *Capsicums* were also reviewed by Wall and Bosland (1998). A description of standard protocols for quality control of peppers was given by Pruthi (2003a). A general literature on the analysis of foods and plants is more abundant, including such an important compendium as the *Handbook of Food Analytical Chemistry* (Wiley). References to appropriate chapters of this compilation will be given below. These sources, however, rarely consider (if consider) specific problems of pepper analysis. In this context, the purpose of this review is to collect together information about analytical and preparative methods applied in pepper analysis. Works published during the last decade were mostly considered. It was done not only for the sake of brevity. The progress in instrumentation and analytical techniques during this period was so significant that some protocols and approaches that were in use by 2000 are out-of-date now.

## SAMPLE COLLECTION AND PRE-ANALYSIS STORAGE

Fresh fruits at different stages of maturity, fruits after storage and dry fruits, ground pepper or foodstuff become an object of analysis depending on a purpose of a research. An accurate representation of the composition of a fruit/product is only possible if no changes in the sample occur prior to the analysis. It must be understood that metabolic and/or chemical processes never stop in samples unless these are subjected to deep freezing in liquid nitrogen. Therefore, the storage period after sampling must be minimized, and storage conditions should be controlled. As mentioned, the deep freezing is the best conservation technique, although expensive and sometimes unavailable. A cheaper alternative for a short-term storage (several

days to a week) is stationary or portable (for transportation) refrigerators for -1–4°C. It is recommended to collect fresh fruits from growers immediately after harvest or purchase them at big retailers, which use industrial equipment for storage and transportation and can provide detail and trustworthy information concerning origination of samples and storage conditions.

For long-term storage, only temperatures lower than -20°C are allowed as a gradual change of phytochemical composition occurs at higher temperatures. Many researchers freeze-dry or lyophilize samples. Dehydration stops many intracellular metabolic processes; yet, spontaneous chemical reactions, especially oxidation by atmospheric oxygen, still proceed although with a slower rate. Therefore, the grinding of dry samples for storage is not recommended. Increase of the surface area of samples would result in a faster oxidation of pepper antioxidants.

## ISOLATION AND ANALYSIS OF BIOACTIVE COMPOUNDS

### *Volatiles*

Researchers study the composition of pepper volatiles in quest of bioactive (antioxidant) components (Jang et al., 2008), for monitoring the process of ripening (Pino et al., 2006; Forero et al., 2009; Liu et al., 2009), trying to understand the effect of processing conditions on quality and safety of foodstuff (Rios et al., 2008), or driven by mere curiosity (Naef et al., 2008). Kim et al. (2007) reported on the difference in the flavor of healthy and diseased peppers. The emission of volatile compounds by *Capsicum* plants as a defensive mechanism against insect herbivores was discussed by da Costa et al. (2011). Significant

efforts were made to identify the key odorants of different cultivars of pepper. Since Buttery et al. (1969) indicated that 2-methoxy-3-isobutylpyrazine, (*E,Z*)-2,6-nonadienal, and (*E,E*)-2,4-decadienal are important aroma compounds, many other substances were proven to play a role in the forming of a peculiar pepper flavor (Luning et al., 1994; Zimmermann and Schieberle, 2000) that, yet, depends on the variety of the fruit (Rodríguez-Burrueto et al., 2010). There were more than 300 components identified in the headspace of pepper samples (Cardeal et al., 2006). Considering a wide range of concentrations of volatiles and the fact that some of them presenting in trace amounts nonetheless define specific tones of pepper flavor, the complexity of pepper aroma analysis is becoming obvious.

#### *Sample preparation and extraction*

A variety of sample preparation methods is employed for the analysis of the volatile fraction of peppers, including manual or mechanical dry grinding (Cardeal et al., 2006; Sousa et al., 2006; Naef et al., 2008; Liu et al., 2009; Rodríguez-Burrueto et al., 2010), blendering with a solvent (Pino et al., 2006), or homogenization with liquid nitrogen (Jang et al., 2008). The choice of a sample preparation protocol is not always explained. Frequently, it seems to be directed by convenience or equipment available. At the same time, Luning et al. (1994) ascertained that sample preparation can influence the profile of volatiles. Authors who homogenized samples at low temperatures (Jang et al., 2008) obviously intended to prevent the loss of volatiles. Jiang and Kubota (2004) added methanol to the sample during homogenization to suppress enzymatic activity.

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For extraction of essential oil, different distillation methods are used. Note that a simple hydrodistillation in a Clevenger type apparatus (European Pharmacopea, 1983) is not commonly accepted in the analysis of *Capsicum* fruits. The procedure lasting for several hours exposes a sample to the risk of thermal degradation. Distillation under reduced pressure was proposed as an alternative (Jang et al., 2008). Pino et al. (2006), Naef et al. (2008), Liu et al. (2009), and Pino et al. (2011) used the technique of simultaneous steam distillation and solvent extraction in a Likens-Nickerson apparatus (Nickerson and Likens, 1966). The method allowed reducing the time of distillation to 0.5-1 h. Schieberle with coauthors (Engel et al., 1999) developed a method of the isolation of aroma compounds, called solvent assisted flavor evaporation, operating at temperatures < 40°C. Volatile compounds must be separated from the non-volatile material beforehand by means of extraction with a low-boiling solvent, such as diethyl ether or dichloromethane. The method was applied in the analysis of the volatile fraction of different varieties of sweet bell pepper (Zimmermann and Schieberle, 2000). Extracts obtained by distillation have to be dried with an appropriate dehydrator, usually, anhydrous Na<sub>2</sub>SO<sub>4</sub>. To detect minor constituents of aroma, a sample may need to be concentrated. Distillation with a Vigreux column, microdistillation, or concentration with nitrogen gas are commonly chosen methods for this.

Distillation techniques were criticized for being time-consuming, expensive, and possibly introducing artifacts at distillation and solvent interaction steps (Mazida et al., 2005; Rodríguez-Burrueto et al., 2010). Solid phase extraction (SPE) with bulk adsorbent and solid phase microextraction (SPME) with adsorbing fiber were suggested to overcome the mentioned problems (Mazida et al., 2005; Rodríguez-Burrueto et al., 2010). It must be noted, however, that the desorption step in the SPE

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and SPME methods is carried out at temperatures 220–250°C. Even if the time of the procedure usually does not exceed 5 min, it can cause a certain degradation of a sample due to high temperature. The elution of the trapped compounds with a proper solvent is an alternative (da Costa et al., 2011). However, the full recovery of the sample in this case must be proven.

The adsorbent employed for SPE is Tenax TA (Luning et al., 1994; van Ruth et al., 1995), selected for its high thermostability and adsorption capacity. In SPME, fibers coated with a complex absorbing liquid composing of divinylbenzene (DVB), carboxen, and polydimethylsiloxane (PDMS) are most popular (Mazida et al., 2005; Rios et al., 2008; Ziino et al., 2009; Rodríguez-Burrueto et al., 2010; Kollmannsberger et al., 2011), although other compositions, such as PDMS/DVB (Cardeal et al., 2006) or PDMS (Sousa et al., 2006), are occasionally used. Cardeal et al. (2006) have tested a pool of fiber materials including PDMS/DVB, PDMS, DVB/Carboxen/PDMS, polyacrilate, and Carbowax/DVB. They found that the first fiber is preferable to extract pepper VOCs.

Other approaches to the extraction of essential oil are found in the literature. A method of solvent-free solid injection (SFSI) based on the extraction of volatiles by carrier gas directly in an injector device of a gas chromatograph was proposed by Jing and Amirav (1997). Shim et al. (2003) have developed this technique using the Keele injector and adopted it to the analysis of peppers (Kim et al., 2007). The authors identified about 100 volatile compounds in samples of *C. annuum* using GC-MS detection. This number is comparable with the data of other researchers (van Ruth et al., 1995; Zimmermann and Schieberle, 2000; Liu et al., 2009), demonstrating the applicability of the method for a qualitative assay. The method has not been tested for quantitative analysis yet. Examples of typical chromatograms given in (Kim et al., 2007) suggest a rather

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pessimistic conclusion concerning quantitative determination of minor principles of aroma without preliminary concentration. Another drawback of the SFSI method is a relatively long exposure of samples to high temperatures (200-250 °C) in an injector. Kim et al. (2007) noted that the signals of some compounds (for example, 1*H*-pyrrole and 1*H*-indole) were affected by injection conditions.

Extraction with supercritical fluids (SCF) is also considered to be an option in the analysis of volatiles (Pourmortazavi and Hajimirsadeghi, 2007). Some non-volatile (under normal conditions) compounds, such as capsaicinoids and carotenoids will be also extracted due to their high solubility in supercritical fluids (del Valle et al., 2003; Uquiche et al., 2004). It was reported that the co-extraction of high boiling compounds can be reduced if the process is performed in a narrow temperature range of 40-50°C, below pressure of 15 MPa (Díaz-Reinoso et al., 2006). However, there were no evidences given so far that a selective SCF separation gives accurate representation of essential oil composition. Therefore, SCF extraction cannot be recommended as a method of choice for routine study of volatiles at present, for it could give biased information about flavor composition.

## *Separation, identification, and quantification*

The qualitative and quantitative analysis of VOCs is accomplished with classical GC or GC-MS techniques. Identification is performed using retention indices and/or mass spectra. Commercially available libraries of Wiley (McLafferty, 2009) and of NIST (2011) are to be used for mass-spectral identification. Useful databases of retention indices of pepper

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volatiles are abundantly published in the literature. The following references can be recommended (Luning et al., 1994; Zimmermann and Schieberle, 2000). Frequently, researchers benefit from the combination of GC-MS and GC methods (Zimmermann and Schieberle, 2000; Pino et al., 2006; Kim et al., 2007; Jang et al., 2008). As flame ionization detector (FID) is known to be superior to MS detector in terms of sensitivity and linear dynamic range, one can use GC/MS for identification and GC/FID for quantification of VOCs.

Both polar (FFAP, nitroterephthalic acid modified polyethylene glycol; Carbowax, polyethylene glicol 20M) and non-polar ((5%-phenyl)-methylpolysiloxane) capillary columns are used to separate aroma compounds. These two groups of chromatographic stationary phases exhibit ability to different types of intermolecular interactions that results in different chromatograms of the same mixture. Retention on non-polar columns correlates with the boiling temperature of analytes. Elution on polar stationary phases is influenced by specific intermolecular interactions (dipole-dipole, inductive interaction or hydrogen bonding). As a result, elution order heavily depends on the chemical structure of solutes, especially on their polar functionalities. A parallel use of both types of columns allows avoiding misidentification of compounds with similar chromatographic behavior as components co-eluted on one column are likely to be resolved on the other one.

Recently, the method of two-dimensional gas chromatography was applied to the analysis of aroma compounds of pepper (Cardeal et al., 2006). In this high-resolution chromatographic technique, two chromatographic columns of different polarity are serially coupled through a suitable interface. The authors used a cryofocusing system as a coupling device. Small fractions of eluate collected with a preset frequency at the outlet of the first column were transferred via the cryofocusing

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device to the second column. Thus, separation achieved on the first column was enhanced on the second one, allowing an unprecedented degree of resolution. Having compared a quadruple mass spectrometer (MS) and a time of flight MS as a detector for a GCxGC installation, the authors concluded an advantage of the ToFMS technique for its higher acquisition rate, sensitivity, and deconvolution capability.

Among other methods that can be used to solve specific tasks of the analysis of aroma compositions, the new emerging technique of E-nose (electronic nose) should be mentioned. E-nose is an instrument that comprises an array of electronic chemical sensors and an appropriate pattern recognition system capable of recognizing simple and complex odors. Mamatha et al. (2008) described the E-nose technique as not much informative but a fast and easy method for testing the quality of spices. The method was used for the study of peppers by Korel et al. (2002).

A combination of gas chromatography and olfactometry (GC/O) became a useful approach in the analysis of aroma over the last decades (van Ruth, 2001). Olfactometry is a method of detecting odor active compounds, usually by a group of trained assessors. In GC/O, the flow of effluent after a column is split for a detector and a special device, sniffting port (Linssen et al., 1993) that delivers the effluent to an assessor, who detects and describes flavor. Applications of the method to the study of pepper samples are exemplified in works (Luning et al., 1994; van Ruth et al., 1995; Zimmermann and Schieberle, 2000; Rodríguez-Burrueto et al., 2010).

## *Pungent principles*

Pungency is an important quality of peppers mostly defining the popularity of the fruit. Not surprising that a qualitative and quantitative evaluation of pungency was a topic of significant interest for a long time. The first approach was proposed by Scoville (1912), who developed a simple organoleptic test to determine pepper pungency. The method is still in use in food industry for trade classification of peppers according to Scoville heat units (SHU). However, the Scoville test is not informative as it does not reveal the composition of the pungent matter. At the present time, it is known that capsaicinoids are the compounds responsible for the hot-testing, spicy flavor imparted by many peppers. Those are amide derivatives of vanillylamine and carboxylic acids with eight to eleven carbon atoms (Figure 1). More than 20 capsaicinoids, differing only in fatty acid structure, have been described. The two major capsaicinoids are capsaicin and dihydrocapsaicin, which comprise over 80-90% of the total present in the fruit (Reilly et al., 2001; Manirakiza et al., 2003; Zachariah and Gobinath, 2008), except few varieties of *C. pubescens*, where their percentage can be as low as 26 % (Zewdie and Bosland, 2001). Due to the presence of a double bond in the alkyl chain, capsaicin can exist in the form of two configuration isomers. Only the (*E*)-isomer occurs naturally. Capsaicin and dihydrocapsaicin account for the strong pungency of *Capsicums*, being the most pungent compounds according to the Scoville test. Their SHU value is equal to  $16 \times 10^6$  as compared to SHU of  $9.1 \times 10^6$  or  $8.6 \times 10^6$  for nordihydrocapsaicin and homocapsaicin, respectively (Ravishankar et al., 2003).

Capsaicinoids are synthesized in the placenta of the fruits by an enzymatic condensation of vanillylamine and medium chain length fatty acids (Blum et al., 2003; Thiele et al., 2008). They are also found in a smaller but considerable amount (up to

24% of the total capsaicinoid content) in seeds and pericarp (Sukrasno and Yeoman, 1993; Kozukue et al., 2005; Monforte-González et al., 2010). Kozukue et al. (2005) reported that the distribution of capsaicinoids over the pericarp of Hansuwi red pepper (*C. annuum* L.) is not uniform, the smallest concentration found in the middle part of the pod and a higher content of pungent compounds found in the top and base parts of the pod. Pungency depends on maturity of fruit. It first appears within 10-20 days after flowering, increases in next 10-30 days, and evolves according to different trends (remains constant, decreases, or fluctuates) for different cultivars at the last stage of ripening (Sukrasno and Yeoman, 1993; Contreras-Padilla and Yahia, 1998; Kirschbaum-Titze et al., 2002; Manirakiza et al., 2003). The content of capsaicinoids in ripe fruits depends on a cultivar and on growth conditions to a lesser degree. According to this parameter, peppers of the genus *Capsicum* range as following (Maillard et al., 1997) (capsaicinoid content is given in brackets): *C. annuum* L. var. *annuum* (0.01–0.70%) < *C. baccatum* L. var. *pendulum* (0.11–0.25%) ≈ *C. pubescens* (0.12–0.36%) < *C. frutescens* L. (0.26–1.21%).

#### *Sample preparation and extraction*

The choice of a sample preparation protocol depends on the purpose of the following procedure. For preparative isolation of target substances, sample preparation is more complicated than for analysis. It consists, as a rule, of several steps including chromatographic purification. For analytical purposes, the thorough purification of a sample from non-target compounds is not necessary. The presence of concomitants that do not interfere with the quantification of target analytes is allowed in this case. Table 1 summarizes recent works on the analysis of pungent principles of peppers. It can be seen that both fresh fruits and

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dried samples are subjected to analysis. The drying step is reasonable and sometimes is inevitable if the composition of commercial products that are sold in dry form is of interest. Most often such samples come to a laboratory already dry. Otherwise, it must be kept in mind that enzymatic activity during the sample preparation step can affect the content of pungent compounds significantly (Pomar et al., 1997). Milling under cold conditions followed by an immediate extraction with acetone or ethanol cooled to temperature -20°C are methods to minimize sample degradation resulted from metabolic processes and from the contact of dissected tissues with air.

Different approaches to the extraction of pungent compounds from pepper tissues are in use (Table 1). Experiments with spiked analytes show that an intense treatment is not necessary for the full extraction of capsaicinoids. Recovery around 100%, at least, for major capsaicinoids was observed under mild conditions of extraction like stirring or sonicating at room temperature (r.t.) (Kurian and Starks, 2002; Kozukue et al., 2005; Chin-Chen et al., 2010; Liu et al., 2010). Thus, harsh extraction procedures involving microwave treatment or pressurized fluids at elevated temperatures developed by Barbero et al. (2006; 2008) are rather for industrial than laboratory use.

Polar organic solvents are commonly used as extractants, acetone, methanol, and ethanol being the most popular. The solubility of capsaicinoids in pure water is poor even under harsh conditions (Barbero et al., 2006; Barbero et al., 2008). Therefore, the latter is not applied for the quantitative evaluation of pungency. However, water extracts can be used for bioactivity tests (Antonious et al., 2007). Extracts are optionally concentrated by rotary vacuum evaporation. This step is desirable for samples with low content of capsaicinoids. An extract can be further subjected to an additional cleanup by means

of re-extraction, microcolumn chromatography, or TLC, but in most analytical publications it was proven to be unnecessary (see Table 1). The SPME method was also examined for the analysis of capsaicin and dihydrocapsaicin in ground pepper and pepper sauces; however, recovery achieved was poor, fluctuating between 20 and 88% depending on sample and analyte (Peña-Alvarez et al., 2009).

*Separation, identification, and quantification*

An analytical determination of pungent principles usually relies on HPLC, GC, LC/MS, or GC/MS methods. The application of TLC (Monforte-González et al., 2007; Monforte-González et al., 2010), NMR (Catchpole et al., 2003), and UV-Vis spectrometry (Davis et al., 2007) has also been reported. Since the latter methods give only the total capsaicinoid content, they are of limited interest nowadays.

For gas-chromatographic analysis, both polar and non-polar capillary columns are used. Temperature regime is commonly a complex program including a few increasing and constant temperature steps, with maximum temperature 250-280°C. In the past, most of GC methods needed derivatization and tedious clean-up steps to increase sample volatility and/or protect a column from degradation (Manirakiza et al., 2003). At present, the use of selective detectors (Thomas et al., 1998), efficient and stable columns together with the use of guard fused silica capillaries permits overcoming these obstacles. The resolution ability of capillary GC is sufficient to determine all alkaloids of interest, statistical indicators of quantitative analysis

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being satisfactory (Thomas et al., 1998; Antonious et al., 2007). All available protocols of the HPLC analysis of *Capsicum* alkaloids for the last decade are based on the use of C8 or C18 stationary phases of different brands (Table 1), although other sorts of brush-type adsorbents have been considered too (Thompson et al., 2005). Methanol or acetonitrile–water mixtures slightly acidified by the addition of acetic or formic acid are common mobile phases in these analyses. At the same time, a good separation of alkaloids with an unsaturated alkyl chain from their saturated analogues can be achieved by modifying the eluent with silver ion (Maillard et al., 1997; Thompson et al., 2005; Jin et al., 2009). Silver ion and alkene ligand form a strong complex; therefore retention of the capsaicins is selectively reduced comparing to dihydrocapsaicins. One more analytical separation technique for pepper alkaloids should be mentioned, capillary electrophoresis (CE) (Liu et al., 2010). This method, even if being of limited use in the area of consideration at the present time, is of interest due to low cost of analysis.

Qualitative analysis of pungent compounds by GC and GC/MS is performed based on the retention times and/or mass spectra using mass spectra libraries described above. Mass spectral identification in LC/MS faces several difficulties. There are no conventionally accepted comprehensive LC/MS or LC/MS/MS databases because soft ionization techniques used in LC/MS are highly dependent upon instrument design and operational parameters. Besides, the quality of spectral information is compromised by interferences from mobile phase and sample matrix (Sultan and Gabryelski, 2006). These complications can be overcome in the case of major capsaicinoids through collecting the mass spectra of commercially available standards on a given instrument under required conditions. A sophisticated identification scheme has been proposed for the determination of non-target components by Thurman et al. (2005), based on a combination of LC/MS information with searching of empirical

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formulas generated through accurate masses in the ChemIndex and/or Merck Index databases. Progress in LC/MS instrumentation further facilitates qualitative analysis (Picó et al., 2006). Problems of non-target compound identification using up-to-date equipment are discussed in (Picó et al., 2006; Soler and Picó, 2007). In the recognition of newly discovered substances, researchers rely on conventional methods of structure analysis (NMR, COSY, and so on) following the isolation of unknown compounds in pure form by means of preparative chromatography.

Quantification of pungent compounds is performed both by external and internal standard methods. Vanillin (Monforte-González, et al., 2007), bisphenol A (Korel et al., 2002), decanoyl- and octanoyl-vanillamides (Lu and Cwik, 1997; Reilly et al., 2001) have been proposed as internal standards (ISs) for the HPLC determination of capsaicinoids while codeine, tetracosane, and squalene have been used in GC analysis (Manirakiza et al., 2003). Since a list of commercially available pungent compounds is limited, detector calibration for a group of related analytes is frequently made using only one compound that is a representative of this group. Concentrations of other compounds of the group are derived from this calibration curve. For example, the contents of saturated and unsaturated capsaicinoids have been determined based on the calibration curves for capsaicin and dihydrocapsaicin, respectively (Kozukue et al., 2005; Barbero et al., 2006).

## *Capsinoids and capsiconinoids*

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Capsinoids are non-pungent analogues of capsaicin first found in *C. Annuum* cv. CH-19 Sweet (Yazawa et al., 1989; Kobata et al., 1998). They consist of a vanillyl moiety and a fatty acid chain conjugated with an ester bond (Figure 2). It was shown that capsinoid biosynthesis was caused by the functional loss of the putative-aminotransferase gene that is thought to catalyze the formation of vanillylamine from vanillin in the capsaicinoid biosynthetic pathway. Vanillyl alcohol is produced instead, which in turn leads to the production of capsinoids (Tanaka et al., 2010). Capsiconinoids (Figure 2) are another non-pungent capsaicin analogues found in the fruits of *C. baccatum* var. *praetermissum* (Kobata et al., 2008). Both groups of vanillyl derived esters exhibit biological activities similar to capsaicin except their considerably lower pungency (Kobata et al., 2008; Tanaka et al., 2009; Sasahara et al., 2010). They are also attractive targets for pharmaceutical studies because of their potency in apoptosis induction, antioxidant, anticancer, and immunosuppressive properties (Kobata et al., 2008).

The content of capsaicin-like substances strongly varies among pepper cultivars. If detected, it can range between 0.05 to 5.6 mg/g dw (Tanaka et al., 2009). In general, neither capsinoids nor capsiconinoids are detected when capsaicin amount in the fruit is low. Capsiconinoids first appear on the fiftieth day after flowering along with capsaicins in *C. baccatum* var. *praetermissum*. The concentration of capsiconinoids and capsaicins evolves correlatively, reaching a maximum within 25-35 days after flowering, and decreasing afterwards (Tanaka et al., 2009). A similar pattern was observed for capsinoids in the fruits of CH-19 Sweet pepper (Yazawa et al., 1989).

*Extraction, separation, and analysis*

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These compounds are extracted from the pepper matrix using aprotic solvents (Table 2), because capsinoids quickly degrade in protic liquids. The half-life period of capsiate in methanol is ~ 50 h at 25°C (Sutoh et al., 2001). Few examples of the preparative isolation of non-pungent capsaicin analogues capsiate and dihydrocapsiate (Kobata et al., 1998), capsiconate and dihydrocapsiconate (Kobata et al., 2008), and nordihydrocapsiate (Kobata et al., 1999) have been reported. Two chromatographic purification steps have been involved in all the cases, at least one step being performed on a reversed phase column using water-methanol mixtures as eluents. Because of the above-mentioned lability of vanillyl esters in such solvents, elution process must be performed quickly, followed by immediate isolation of target principles from the mobile phase.

The analytical separation of bioactives of interest can be easily carried out by means of reversed phase HPLC (Table 2). Singh et al. (2009) recommended the use of monolithic C18 columns. Sutoh et al. (2006) separated a complex mixture containing major capsinoids along with their metabolic precursors using a fluorinated stationary phase Fluofix IEW 425 (Wako Pure Chemicals, Japan). Standards of capsinoids and capsiconinoids for quantitative determination are not commercially available, but protocols for the synthesis of capsiate and dihydrocapsiate (Singh et al., 2009), and capsiconate and dihydrocapsiconate (Kobata et al., 2008) have been published.

## *Pigments*

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Chlorophylls and carotenoids constitute another group of valuable pepper nutrients responsible for its color. Chlorophylls are cyclic tetrapyrroles carrying a characteristic cyclopentenone ring (Figure 3), and carotenoids are C<sub>40</sub> isoprenoids containing 9 conjugated double bonds in the central polyenic chain, with different end groups. Despite an essential difference in chemical structure, these two classes of organic substances can be extracted and analyzed together that justifies their joint consideration in this section.

Historically, the analytical chemistry of plant pigments was developing driven by interest to plant physiology. In fact, photosynthesis studies gave rise to chromatography invented to separate chlorophylls from leave extracts (Tswett, 1906). Gradually, the focus of investigations has been shifting to dietary and pharmaceutical issues. Nowadays, researchers are interested in elucidating metabolic processes in peppers (Deli et al., 2001; Simkin et al., 2003; Roca et al., 2006; Ha et al., 2007), developing new varieties with improved properties (Hornero-Méndez and Minguez-Mosquera, 2000; Minguez-Mosquera et al., 2000), and in investigating the impact of agricultural practices on the enrichment of pigments in fruits (Russo and Howard, 2002; Pérez-López et al., 2007; Kim et al., 2008; Flores et al., 2009). There have been a number of publications in the last decade devoted to the evaluation of pepper quality depending on the carotenoid content (Breithaupt and Bamedi, 2001; Guil-Guerrero et al., 2006; Matsufuji et al., 2007; Topuz and Ozdemir, 2007; Marín et al., 2008; de Azevedo-Meleiro and Rodriguez-Amaya, 2009; Aruna and Baskaran, 2010). Interest in the fate of nutrients during postharvest treatment and food processing is another source of methods for the quantitation of carotenoids and chlorophylls in pepper products (Topuz and Ozdemir, 2003; Kim et al., 2004; Kidmose et al., 2006; Raffo et al., 2008; Gallardo-Guerrero et al., 2010).

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Nearly 100 chlorophylls have been described in the literature (Sheer, 2006), only chlorophylls *a* and *b*, their epimers (chlorophylls *a'* and *b'*), and products of their degradation being found in *Capsicum* fruits so far. The biosynthesis of chlorophylls is believed to start from the formation of 5-aminolevulinic acid (ALA) followed by conversion of eight molecules of ALA into chlorophyll precursors (Rüdiger and Grimm, 2006). Chlorophyll catabolism consists of three main enzymatic steps. In the hydrolysis reaction catalyzed by the enzyme chlorophyllase, a chlorophyll molecule loses the phytol fragment and converts into chlorophyllide. Mg-dechelatase promotes the removal of the Mg<sup>2+</sup>-ion, a process resulting in metal-free derivatives. Those are pheophytins derived from chlorophylls, and pheophorbides derived from chlorophyllides. Interesting that chlorophyll *b* does not appear to degrade directly but first reduces to chlorophyll *a* that explains the absence of chlorophyll *b*-type catabolites in plants (Hornero-Méndez and Mínguez-Mosquera, 2002; Kräutler and Hörtenersteiner, 2006). The enzyme pheophorbide *a* oxygenase is activated by the ripening and senescence processes, oxidizing pheophorbide *a* to colorless products (Hornero-Méndez and Mínguez-Mosquera, 2002). The content of chlorophylls steadily decreases during the ripening period until the total disappearance for most peppers at the developed color stage (Hornero-Méndez and Mínguez-Mosquera, 2002; Flores, et al., 2009a). However, chlorophylls persist in the ripe stage for so-called “stay-green” cultivars (for instance, *C. annuum* var. Negral), giving the fruit a characteristic chocolate color (Roca et al., 2006; Hornero-Méndez and Mínguez-Mosquera, 2002). The disappearance of chlorophylls is accompanied by a *de novo* biosynthesis of carotenoid pigments. The accumulation of carotenoids accounts for the development of new color of maturing fruits that can range from yellow to red depending on pigment composition. There are at least 34 carotenoids found in *Capsicum* peppers (Deli et al., 2001). Figure 4

shows the major carotenoids of red pepper. The figure also demonstrates metabolic links between them. It is seen that the red pigments, capsanthin and capsorubin (unique to the *Capsicum* genus), are at the end of the biosynthesis pathway. Therefore they can only accumulate in red peppers. The chromoplast pigments resulting from  $\beta$ -carotene are also accumulating. On contrary, the chloroplast xanthophylls lutein and neoxanthin gradually disappear as maturation proceeds (Hornero-Méndez and Minguez-Mosquera, 2000; Marín et al., 2004; Roca et al., 2006). The total content of carotenoids in the fully ripe fruits varies from 0.02 to 0.1% (fw) and the content of chlorophylls at the maximum ranges within 0.001-0.08% (fw) depending on variety and growth conditions. In immature green fruits, carotenoids present in non-esterified form. During ripening, selective carotenoid esterification with fatty acids increases with a gradual decrease of free pigments (Breithaupt and Schwack, 2000; Hornero-Méndez and Minguez-Mosquera, 2000a).

#### *Sample preparation and extraction*

Pepper pigments are fairly stable in their natural environment but become labile to degradation, oxidation, and isomerization caused by heat, light, air oxygen, or chemicals being separated from pepper tissues after trituration and extraction. Therefore, care must be taken during all stages of analysis to avoid pigment decomposition. General recommendations are to make homogenization, extraction, and subsequent procedures, such as filtration and centrifugation, as rapidly as possible at temperatures below 4°C in the dark or at least at subdued light. The use of amber glassware is desirable. Low temperature homogenization and extraction may seem unreasonable since following procedures (saponification,

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concentration in a rotary evaporator) are carried out at ambient or somewhat elevated temperatures. Nonetheless this measure is advocated to reduce the enzymatic activity of pepper oxidases released from the intracellular space (Schweiggert et al., 2007). The danger of enzymatic destruction is eliminated once the extract is separated from the pepper matrix given the enzymes are not transferred into the liquid phase. Storage of samples during experimental work on ice is also a reasonable precaution, and samples must be kept in a freezer for a long-term storage. Some researchers protect samples from oxidation by modifying extractants with antioxidants, such as ascorbic acid (2 g/100 ml) and butylated hydroxytoluene (BHT) (0.1-1 g/100 ml). On the other hand, Fraser et al. (2000) reported that the presence of BHT had no observable effect on the stability of carotenoids during extraction or storage at -20°C. Gokmen et al. (2002) homogenized samples in the presence of CaCO<sub>3</sub> to protect pigments from tissue acids.

Acetone is the most popular solvent for extraction. A typical extraction protocol is given in (Mínguez-Mosquera and Hornero-Méndez, 1993). After extraction, the liquid phase is mixed with 10% NaCl, and target analytes are transferred to diethyl ether. Other extractants like hexane (Guzman et al., 2010), ethyl acetate (Flores et al., 2009a), or methanol-ethyl acetate-petroleum ether (Breithaupt and Bamedi, 2001) have also been used. Weissenberg et al., (1997) and Deli et al. (2001) applied two solvents in series, methanol and diethyl ether. Kidmose et al. (2006) homogenized sweet bell pepper samples with methanol-water and then extracted carotenoids with an acetone-hexane mixture. Burns et al. (2003) extracted carotenoids and chlorophylls with methanol followed by the addition of Tris-HCl buffer (pH 7.5) and re-extraction with chloroform. It must be mentioned, however, that methanol-based extractants should be avoided if chlorophylls are to be analyzed, because chemically

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modified products of chlorophylls are formed easily in methanol solutions (Shioi, 2006). Extraction is carried out until complete decoloration of tissue. Three – four subsequent extractions are usually sufficient.

Because of the presence of esterified compounds in ripe peppers, an additional stage of saponification is recommended to improve appearance of chromatograms and to facilitate quantitation of carotenoids. Publications of Burns et al. (2003) and Guzman et al. (2010) give examples of challenges that researchers face when avoid saponification. For this procedure, an extract dissolved in an alcohol-miscible solvent and dried of water is mixed with 5-30% (w/v) KOH solution in MeOH or EtOH and left in a dark place for 0.5 to 24 h at r.t. A typical protocol can be found in (Mínguez-Mosquera and Hornero-Méndez, 1993). Occasionally, thermostating at moderate temperatures (below 50°C) is used (Matsufuji et al., 2007; Kim et al., 2008). Howard with coauthors (Howard et al., 2000; Russo and Howard, 2002) analyzing pepper fruits and Simkin et al. (2003) analyzing pepper leaves carried out saponification directly in the homogenized sample for 20-30 min at 40°C and 60°C, respectively. However, it was proven by Kimura et al. (1990) that the hydrolysis of carotenol esters is complete and the loss of analytes is minor in a room temperature saponification. Thus, an elevated temperature treatment is not only unnecessary but not recommended due to possible thermodegradation of analytes. Saponification destroys chlorophylls. Therefore, a part of the extract must be reserved and proceeded without saponification if the determination of chlorophylls is necessary.

*Separation, identification, and quantification*

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HPLC and TLC are heavily used techniques for the analysis of pepper pigments. Spectrophotometry, being popular in the past, is rarely used for research purposes at present, but it is still a “workhorse” method for the determination of the total chlorophyll and total carotenoid content in routine laboratories. Modern spectrophotometric assays allow differentiation between chlorophylls *a* and *b* (Porra, 2006). In the case of carotenoids, a separate quantification of the sums of the red and yellow pigments is possible (Hornero-Méndez and Mínguez-Mosquera, 2001).

TLC is a convenient method for a preliminary assay of pepper extracts. It rapidly gives information concerning qualitative composition of a sample in terms of major constituents. Typical eluents for silica gel TLC of pepper pigments are shown in Table 3. Chlorophylls can be easily identified based on their characteristic color. Groups of yellow and red pigments are also obvious to discover. Neoxanthin and  $\beta$ -carotene are identified based on their low and high  $R_f$  value, respectively. Recognition of the other yellow pigments is complicated because of similar retention.

HPLC delivers detail information about qualitative and quantitative composition of pepper extracts. Garrido and Zapata (2006) discussed works on the chromatography of chlorophylls made before 2001. Typical conditions for HPLC analysis applied during the last decade are summarized in Table 4. Complex gradient programs are usually used, analysis time ranging from 15 min to 1 h. Guzman et al. (2010) applied an UPLC technique to separate carotenoids of orange peppers. They did not gain any significant improvement as compared to regular HPLC in terms of analysis time. The use of monolithic columns promises a faster analysis (Garrido et al., 2003; Seppanen et al., 2003); however, those have not yet been tested on extracts of pepper pigments. Modification of mobile phases with minor additions of triethylamine or ammonium acetate was considered in

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1990th as an efficient way to reduce the on-column degradation of carotenoids (Hart and Scott, 1995, and refs. therein). On the other hand, review of modern HPLC methods shows a limited use of protective modifiers. Apparently, state-of-the-art stationary phases based on extra-pure silica with diminished activity of residual silanols due to endcapping expose no essential treat of catalytic transformations to analytes. Stationary phases with a long alkyl radical (C30) gaining popularity in the last decade (Guil-Guerrero et al., 2006; Ha et al., 2007; Pérez-López et al., 2007; Raffo et al., 2008) still more reduce the probability of contact between analytes and the silica support surface.

Chlorophylls and carotenoids can be analyzed jointly (Burns et al., 2004; Guzman et al., 2010), but if the stage of saponification is required, the chlorophyll and carotenoid fractions are chromatographed separately under similar or close conditions. The use of a DAD detector allows recording of UV-Vis spectra of eluted peaks. This information in combination with retention times is frequently sufficient for the qualitative analysis of pigments. Indeed, the absorption spectra of chlorophylls have peculiarities that allow an unambiguous distinguishing between chlorophyll *a*, chlorophyll *b* and corresponding pheophytins (Table 3; Kobayashi et al., 2006). Chlorophylls *a* and *b* and their *a'* and *b'* epimers can be distinguished by their different chromatographic behavior despite the adsorption spectra for the two epimeric forms are the same. The *a'* form is always less retained than the other one in reversed-phase systems (Gokmen et al., 2002; Suzuki and Shioi, 2003). Roca et al. (2006) demonstrated the identification of chlorophylls *a* and *b*, their 13<sup>2</sup>-OH-derivatives, chlorophyllide *a*, and pheophorbide *a* in “stay-green” mutants of *C. annuum* using combination of HPLC, TLC and UV-Vis data.

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Adsorption spectra are useful in the detection of carotenoids too, but an unambiguous recognition is not always possible. In such cases, the marker method or LC/MS are necessary. Reference data for UV-Vis and mass-spectral identification of carotenoids have been published by Mínguez-Mosquera and Hornero-Méndez (1993) (UV-Vis) and by de Azevedo-Meleiro and Rodriguez-Amaya (2009) (UV-Vis, MS), and excerpts are summarized in Table 3. Data for identification of carotenoid esters have been published by Breithaupt and Schwack (2000) and Schweiggert et al. (2005).

Quantification of chlorophylls and their derivatives is carried out by the external standard method. Chromatograms can be recorded at the wavelength of 660 nm, but wavelengths within a range of 430-450 nm are also appropriate. The latter setting is useful if chlorophylls and carotenoids are determined simultaneously with a fixed-wavelength UV detector. Standard samples of chlorophylls *a* and *b* are commercially available but because of their high cost are not frequently used. Researchers isolate the chlorophylls from plants according to protocols published by Perkins and Roberts (1962), Hornero-Méndez and Mínguez-Mosquera (2002), and Shioi (2006) or obtain it by means of semi-preparative HPLC from their own samples. Chlorophyllides are synthesized by enzymatic deesterification of chlorophylls (Mínguez-Mosquera et al., 1993). Pheophytins and pheophorbides are prepared by acidic treatment of respective chlorophylls and chlorophyllides (Perkins and Roberts, 1962; Mínguez-Mosquera et al., 1993; Seppanen et al., 2003). Carotenoids are quantified by means of both external and internal standard methods,  $\beta$ -apo-8'-carotenal serving as an IS. A recommended wavelength for UV monitoring is 450 nm. Quantification of carotenoid esters is problematic due to the lack of standards. Breithaupt and Bamedi (2001) used lutein dimyristate as an equivalent standard for all the ester analytes as a forced choice.

*Polyphenols and related antioxidants*

Substances containing a phenolic or polyphenolic fragment known as phenolics are important nutraceutical constituents of peppers. They contribute to the taste and flavor of the fruit to a degree (Serrano et al., 2010), yet are most valued for their health-beneficial effect. Phenolics exhibit antioxidant and antiradical properties (Liu, 2004), but anti-inflammatory, antimicrobial, antiallergenic effects have also been reported (Harborne and Williams, 2000; Liu, 2004; Shetty, 2004). The main body of researches on pepper involving the determination of phenolics is devoted to the effect that agricultural practices (Ren et al., 2001; Chassy et al., 2006; Pérez-López et al., 2007a; Flores et al., 2009; Serrano et al., 2010), storage conditions (Kevers et al., 2007; Raffo et al., 2007; Raffo et al., 2008; Matsufuji et al., 2009), and maturity (Howard et al., 2000; Marín et al., 2004; Navarro et al., 2006; Conforti et al., 2007; Oboh and Rocha, 2007; Oboh and Rocha, 2007a; Marín et al., 2008; Menichini et al., 2009) have on antioxidant activity of fruits. Comparative studies of the nutrient value of peppers are also found in the literature (Arabbi et al., 2004; Lee et al., 2005; Sun et al., 2007; Sim and Sil, 2008; Gorinstein et al., 2009; Nazzaro et al., 2009; Galvez Ranilla et al., 2010). De Jesus Ornelas-Paz et al. (2010) considered the effect of cooking on the phenolic content of Mexican peppers. Saha et al. (2010) used the total concentration of phenols as an indicator of fruit quality in pepper-breeding

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experiments. There are a few publications devoted to phenolic metabolism in peppers (Estrada et al., 2000; Pegard et al., 2005; Chmielowska et al., 2010).

Phenolic acids (Figure 5) and flavonoids (Figure 6) are major phenolic phytochemicals found in peppers. They are synthesized by plants as a result of adaptation to biotic and abiotic stress (Shetty, 2004). Similarly to capsaicinoids, phenolic antioxidants are derived from the phenylpropanoid pathway (Diaz et al., 1998; Davies and Schwinn, 2006), although more complicated mechanisms have been hypothesized (Shetty, 2004). During initial steps of this pathway, phenylalanine transforms to cinnamic acids. Flavonoids are products of the condensation of cinnamic acids with three malonyl-CoA groups and further can undergo enzymatic esterification with plant sugars. Considering a variety of those (glucose, rhamnose, galactose, xylose, arabinose, mannose, apiose and their multiple disaccharide and trisaccharide combinations) one can suggest a wide spectrum of flavonoid glycosides present in plants. So far, products of glycosylation at C6, C8, and O7 positions of flavons and at O3 and O7 positions of flavonols have been reported in peppers (Sukrasno and Yeoman, 1993; Materska et al., 2003; Marín et al., 2004; Materska and Perucka, 2005; Marín et al., 2008). Phenolic acids can undergo glycosylation too and respective derivatives are found in pepper extracts (Sukrasno and Yeoman, 1993; Materska et al., 2003; Marín et al., 2004; Materska and Perucka, 2005; Marín et al., 2008). Further metabolism of flavonoids would result in the formation of oligomeric compounds proanthocyanidins in many fruits and vegetables (Kennedy, 2005). Assays carried out by Hervert-Hernández et al. (2010) and Bahorun et al. (2004) did not reveal measurable amounts of proanthocyanidins in *Capsicum* peppers. At the same

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time, Gorinstein et al. (2009) determined tannins (condensed polyphenolic substances) in both hydrolyzed and non-hydrolyzed methanolic extracts of green pepper using an UV-Vis technique.

Plant phenolics are subdivided into 2 classes, so called free and bound phenolics. The first group of phytochemicals is extractable with hydro-organic solutions. This group consists of the aglycons and glycosides of monomeric phenolic acids and flavonoids. The fraction of bound phenolics includes polymerized phenols and compounds covalently attached to cell-wall macromolecules. Data on the distribution of the free and bound phenolics in peppers are controversial. Hervert-Hernández et al. (2010) and Gorinstein et al. (2009) reported a larger or comparable content of the bound compared to the free phenolics in *C. annuum* fruits. The fraction of bound phenolics comprises less than 10% of the total phenol content in red pepper according to Chu et al. (2002). On contrary, Oboh and Rocha (2007; 2007a) found that the free phenolics prevail over the bound fraction by 10-50% in *C. pubescens* and *C. annuum* peppers, both in unripe (green) and in ripe (red) fruits. This wide range of opinions can be resulted from different extraction protocols applied. So, Chu et al., and Oboh and Rocha used an alkali hydrolysis whereas Gorinstein with coworkers relied on the acidic hydrolysis method. A difference in genotypes, growing and harvesting conditions of the samples could also contribute to the above-mentioned inconsistencies.

Phenolic antioxidants are found both in pericarp, placenta, and seeds of peppers (Howard et al., 2000; Oboh and Rocha, 2007), with components majoring in the pericarp being minor in the seeds and *vice versa* (Sukrasno and Yeoman, 1993). The total content of phenolics ranges between 0.05 to 0.3% (fw) depending on variety, maturity stage, and growing conditions. The total flavonoid level, in general, decreases during maturation (Marín et al., 2004; Materska and Perucka, 2005; Marín et al.,

2008). The content of hydroxycinnamic acids changes insignificantly (Marín et al., 2008) or decreases (Estrada et al., 2000; Marín et al., 2004), although Materska and Peruchka (2005) reported an increase in glycosides of ferulic and sinapic acids when fruits pass from the green to red stage. A tendency to accumulation of free ferulic acid during ripening was also observed by Estrada et al. (2000). When the overall concentration of phenolic compounds was evaluated, both increasing (Howard et al., 2000; Pérez-López et al., 2007a; Flores et al., 2009; Saha et al., 2010) and decreasing (Howard et al., 2000; Navarro et al., 2006; Oboh and Rocha, 2007; Marín et al., 2008) trends were revealed. It is worth to note that the sign of trend can depend on an analytical technique used, spectrophotometric assay or HPLC (Howard et al., 2000). In the latter case, the total content is estimated by summation over all the peaks of phenolics found in a chromatogram. After harvesting, the content of the phenolic fraction does not undergo principal changes during a middle-term cold storage (Raffo et al., 2007; Raffo et al., 2008).

#### *Sample preparation and extraction*

Fresh, freeze-dried, air- or oven-dried (40-70°C) fruit samples are used for analysis. The presence of constitutional water does not interfere with the extraction of polyphenols, but dried samples are more convenient to handle. Fresh or dried plant material can be homogenized before extraction (homogenization in liquid nitrogen is a convenient procedure for fresh pepper); however, maceration with solvent is a more frequently used technique. Polyphenols are often better soluble in solvents less polar than water (Kim and Lee, 2005). Therefore, methanol (Kurian and Starks, 2002; Pegard et al., 2005; Conforti et al., 2007;

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Raffo et al., 2007; Sun et al., 2007; Nazzaro et al., 2009), ethanol (Menichini et al., 2009), or their aqueous solutions are used for extraction. In the case of methanol-water mixtures, the percentage of the organic part ranges from 50 to 90% (Estrada et al., 2000; Howard et al., 2000; Miean and Mohamed, 2001; Sakakibara et al., 2003; Arabbi et al., 2004; Baranowski et al., 2004; Marín et al., 2004; Chassy et al., 2006; Helmja et al., 2007; de Azevedo-Meleiro and Rodriguez-Amaya, 2009; Gorinstein et al., 2009; Matsufuji et al., 2009; Chmielowska et al., 2010; de Jesús Ornelas-Paz et al., 2010; Serrano et al., 2010). Hydro-ethanolic solvents contain 70 (Sim et al., 2008) or 80% (Lee et al., 2005; Materska and Peruchka, 2005; Saha et al., 2010) of alcohol. An acetone (80%)-water mixture has also been used (Chassy et al., 2006; Oboh and Rocha, 2007; Oboh and Rocha, 2007a; He et al., 2008). In order to ensure the complete extraction of soluble polyphenols, Hervert-Hernández et al., (2010) treated plant samples sequentially with methanol-water (50:50) and acetone-water (70:30). Modification of hydro-organic extractants with addition of acetic acid (2 to 5%, v/v) results in transferring anthocyanins to the liquid phase along with free phenolics (Howard et al., 2000; Arabbi et al., 2004; Kevers et al., 2007; Gorinstein et al., 2009).

The classical approach to the extraction of free phenolics consists in a prolonged (0.5-4 h) treatment of homogenized plant material with an extracting solvent with stirring, periodical shacking, or under reflux conditions. The temperature of treatment ranges between 0 and 80°C. A low temperature extraction is intended to prevent polyphenols from oxidation. It is usually used when the profile of phenolic phytochemicals is of interest (Arabbi et al., 2004; Marín et al., 2004). Extraction at elevated temperatures ensures a more complete transfer of phenolic substances into the liquid phase, although some of these compounds may be transferred in the oxidized form. Using sodium bisulfite (de Jesús Ornelas-Paz et al., 2010) or *tert*-

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butylhydroquinone (Miean and Mohamed, 2001; Raffo et al., 2007; Raffo et al., 2008) will prevent oxidative degradation of samples. Alternatively, a solvent can be modified with NaF in order to inactivate polyphenol oxidase activity (Marín et al., 2004; Serrano et al., 2010). The application of ultrasound was recommended to reduce extraction time and to enhance the recovery yield of polyphenols (Kim and Lee, 2005). In pepper studies, this technique was used both alone (Sakakibara et al., 2003; de Jesús Ornelas-Paz et al., 2010) and in combination with incubation of samples with extractants preceding the sonication (Helmja et al., 2007; Saha et al., 2010). The isolation of bound phenolics requires the use of hydrolyzing agents in combination with temperatures above 80°C. Different hydrolysis conditions would result in release of different fractions of the non-extractable matter. It partly explains inconsistencies in phenolic content frequently found in the literature. On the other hand, this circumstance can be used for the fractional extraction of phenolics (Figure 7).

Isolates *per se* or after hydrolysis can be directly subjected to analysis or can be further purified to remove interfering concomitants. The purification step is advisable in colorimetric assays of total phenolics since in this case concomitants cannot be monitored like in chromatography. Unfortunately, this recommendation is rarely followed, the works of Conforti et al. (2007) and Oboh and Rocha (2007) being rare exceptions. The liquid-liquid extraction (Conforti et al., 2007; Oboh and Rocha 2007) and SPE (Materska et al., 2003; Arabbi et al., 2004) techniques were used for the purification of phenolic isolates. In the former method, phenolics are transferred from aqueous or water-methanol solutions into ethyl acetate (Oboh and Rocha 2007) or chloroform (Conforti et al., 2007), respectively, remaining unwanted hydrophilic impurities in the water layer. The re-extraction procedure can be preceded by the partition of acidified water-methanol extracts against hexane to remove

hydrophobic admixtures (Conforti et al., 2007). In the SPE method, the matter of isolates is concentrated on a short column packed with a polyamide (Arabbi et al., 2004) or C18-type (Materska et al., 2003) adsorbent and washed with pure water. Then the phenolic fraction is eluted with methanol or a water-methanol mixture.

*Separation, identification, and quantification*

The measurement of the total phenolic content relies on a set of colorimetric assays. Applying different colorimetric reagents, a group analysis of phenolic antioxidants becomes possible as described in Table 5. A larger differentiation can be achieved by using different extraction protocols (Figure 7). The Folin-Ciocalteau (FC) reagent (Singleton and Rossi, 1965) is the main tool for the determination of total phenolics in plants that is in use for decades. Despite being considered to be a standard method, it delivers biased results in the presence of reducing hydroxyl-containing organics, such as ascorbic acid and sugars. A procedure for the correction of the results of the FC assay for the influence of those interferences in pepper analysis was described by Asami et al. (2003). Polyphenol glycosides that bear unesterified phenolic hydroxyls respond to the FC reagent. It does not hold true for totally esterified compounds. Review of the literature (see Table 5) suggests the borohydride-chloranil assay (He et al., 2008) to be a suitable method for the determination of total flavonoids. It must be mentioned, however, that the method has not yet been verified by other authors. Another circumstance that may have an effect on the results of colorimetric tests is the choice of standard. Gallic acid is a frequently used one for the quantification of total phenolics, although it is not a representative phytochemical for peppers. Chlorogenic and *p*-coumaric acids, catechol, and

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quercetin have also been used for standardization in the FC method. Quercetin as well as catechin is a common standard for the colorimetric quantification of flavonoids.

Detail information about composition of the phenolic fraction is obtained with the help of separating methods, HPLC being the most commonly used one. A summary of HPLC conditions for analysis of pepper extracts is given in Table 6. Helmja et al. (2007) developed a CE method for resolving several hydroxycinnamic acids, flavonols, and flavones and tested the method on a sample of chili pepper. Usually, the analysis of phenolics is carried out after hydrolysis in order to quantify these substances in the form of aglycons (Howard et al., 2000; Miean and Mohamed, 2001; Lee et al., 2005; Chassy et al., 2006; Kevers et al., 2007). There are few publications devoted to the chromatographic analysis of phenolic glycosides. This is because the identification of substituted polyphenols is a task of the utmost difficulty due to the lack of standards and reference data. Marín et al. (2004) were able to detect 5 hydroxycinnamic acid derivatives and 23 flavonoid glycosides (derivatives of apigenin, quercetin, luteolin and chrysoeriol) in bell sweet pepper using combination of HPLC-DAD and LC/MS/MS data. Materska et al. (2003) and Materska and Perucka (2005) found 9 glycosylated derivatives of sinapic acid, ferulic acid, quercetin, luteolin and apigenin in an extract of the pericarp of hot pepper. Raffo et al. (2007) recognized 2 derivatives of hydroxycinnamic acid in red bell pepper based on the mentioned data of Marín with coauthors, but 2 major and a few minor flavonoids remained unidentified. Baranowski et al. (2004) have reported a simultaneous determination of glycosides and aglycons in paprika samples, 16 compounds being determined in total. A comprehensive database of polyphenols in fruits and vegetables including peppers was collected by Sakakibara et al. (2003). They performed identification based on retention times

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and UV-Vis spectra recorded by a DAD detector. In addition to the above-mentioned papers containing information about UV-Vis and mass-spectra of phenolic glycosides from peppers, a general literature describing identification techniques for flavonoids (Harborne and Mabry, 1982; Frison and Sporns, 2005) can be useful.

A direct quantification of phenolic derivatives is rarely optional due to the lack of commercially available standards. If synthesis or preparative isolation of target analytes is not possible, available representatives of groups of phytochemicals to be measured can be used for standardization. Then results will be expressed in equivalent quantities with respect to standards taken. So, hydroxycinnamic acid derivatives were quantified as equivalents of chlorogenic acid, and flavonoids as equivalents of quercetin 3-rutinoside (Raffo et al., 2008) or quercetin (Arabbi et al., 2004). Summing the above discussion, one can conclude that the qualitative and quantitative analysis of aglycons does not appear to be problematic, while it is so in the case of phenolic derivatives.

Preparative separation of complex phenolics from pepper extract was reported by Materska et al. (2003) and Materska and Perucka (2005), who adopted an earlier method (Stochmal et al., 2001) developed for the isolation of flavonoid glycosides from alfalfa. The method is based on the fractional elution of a pepper extract on a (40 x 3 cm) LiChroprep RP-18 column using stepwise gradient of methanol in water from 0 to 100% with a 5% increment. Fractions containing more than one compound were further purified on a (25 x 0.8 cm) Eurospher 100 RP-18 column with the mobile phase acetonitrile-H<sub>3</sub>PO<sub>4</sub> (99:1). Sukrasno and Yeoman (1993) isolated 3-*O*-rhamnosylquercetin and 7-*O*-glucosylluteolin by column chromatography on Sephadex LH-20 with 80% EtOH aq. followed by preparative TLC on pre-coated silica gel K5 with the solvent system

EtOAc-EtOH-HOAc-H<sub>2</sub>O (15:3:1:5). Iorizzi et al., (2001) separated the *n*-butanol soluble fraction of the methanol extract from *C. annuum* var. *acuminatum* using droplet counter current chromatography. *n*-BuOH-Me<sub>2</sub>CO-H<sub>2</sub>O (3:1:5) was the stationary phase. Earlier eluted fractions contained *p*-coumaric acid 4-*O*-β-glucoside, trans-sinapoyl and vanillyl β-glucosides, and 3-*O*-rhamnosylquercetin. These fractions were subjected to preparative reversed-phase HPLC to isolate each compound in the pure form.

#### ***Carbohydrates: Monosaccharides***

Three sources contribute to the carbohydrate content of peppers: free sugars, polysaccharides, and carbohydrate residues of different glycoconjugates. The latter are rarely considered as carbohydrate nutrients. Rather, they are attributed to a class of parent compounds (flavonoids, lipids, capsaicinoids and so on). They can contribute to the total carbohydrate content, depending on a method of analysis used. This section concerns the analysis of free sugars, while the next section discusses polysaccharides.

Free sugars play an important role in taste characteristics of fruits, thus affecting their consumer attractiveness. They are also known to be major nutrients which support pollen development (Shaked et al., 2004) and influence the tolerance of seeds to a desiccation stress (Demir et al., 2008). The analysis of free sugars in peppers is exemplified by studies devoted to the effect of agricultural practices (Moreno et al., 2000; Flores et al., 2009), a post-harvest treatment (Faustino et al., 2007; Flores

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et al., 2007; Cagno et al., 2009; Sgroppo and Pereyra, 2009; Gallardo-Guerrero et al., 2010), and storage (Srinivasa et al., 2006; Raffo et al., 2007; Raffo et al., 2008) on the quality of the fruits. Sugars were considered in comparative studies of different pepper varieties (Antonious et al., 2006; Matsufuji et al., 2007) and in investigations aimed at the understanding of ripening process (Navarro et al., 2006; Bernardo et al., 2008; Flores et al., 2009a). The enlightening of physiological phenomena in pepper plant was an objective of the work of Martínez-Ballesta et al., (2004). An interesting investigation was undertaken by Kamilova et al. (2006), who analyzed exudates of plant organs to understand disease resistance mechanisms.

From a physiological point of view, sugars are assimilates that participate in the photosynthetic fixation of carbon in plants (Nielsen et al., 1991). Due to this universal role, sugars are found in all parts of the pepper plant in high quantities. In general, sugar content in a mature fruit ranges between 0.5 to 7% (fw), depending on a genotype, geographical location, and growth conditions. Among hot peppers, *C. frutescens* has, on average, the lowest level of reducing sugars, followed by (in an ascending order) *C. annuum*, *C. chinense*, and *C. baccatum* (Antonious et al., 2006). The content of sugars in sweet varieties of *C. annuum* species is still higher. The major sugars are glucose, fructose, and sucrose. Kamilova et al. (2006) reported measurable concentration of maltose in pepper exudates, although only trace amounts of this disaccharide in plant tissues were documented (Raffo et al., 2007). Nonstructural sugars are accumulated in the fruit pericarp. Their total concentration in this tissue (expressed on a dry weight base) is roughly tenfold larger than in seeds or in leaves.

During the ripening process, the concentration of glucose and fructose increases (Nielsen et al., 1991; Matsufuji et al., 2007; Bernardo et al., 2008), whereas that of sucrose follows different trajectories depending on a cultivar. Some authors

observed a decrease in the sucrose content as ripening progressed (Navarro et al., 2006; Flores et al., 2009; Flores et al., 2009a). In the works of Nielsen et al. (1991) (*C. annuum* L. cv. Trophy), Luning et al. (1994) (*C. annuum* L. cv. Mazurka and cv. Evident), and Kim et al. (2002) (*C. annuum* L. cv. Nockwang) the sucrose content went through the maximum, reducing significantly upon achieving the ripe stage. In two varieties of Spanish pepper, sucrose somewhat increased from the green to the red stage (Bernardo et al., 2008). Finally, the sucrose level remained unaffected by maturity in a *C. annuum* L. cv. Signal sweet pepper (Matsufuji et al., 2007).

After harvesting, the sugar content changes due to enzyme activity. It undergoes only limited changes during a short-term (less than 2 weeks) storage at a temperature of 4-8°C (Raffo et al., 2007; Raffo et al., 2008; Sgroppo and Pereyra, 2009). One week storage at a temperature of 10°C resulted in a 7 to 30% loss in total sugars, depending on a pre-storage treatment (Sgroppo and Pereyra, 2009).

#### *Sample preparation and extraction*

Sugars are stable at ambient conditions therefore no special protection of samples from environmental factors is required. They are also relatively stable in contact with pepper enzymes. Nielsen et al. (1991) used a radiolabeled internal standard to show that sucrose undergoes no cleavage throughout the extraction and preparation for HPLC analysis.

A standard extractant for free carbohydrates is 80% ethanol (Moreno et al., 2000; Shaked et al., 2004; Antonious et al., 2006; Matsufuji et al., 2007; Bernardo et al., 2008; Demir et al., 2008; Sgroppo and Pereyra, 2009). Extraction is usually

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carried out under reflux (80°C) for 1 h. Pure water, both hot (Gallardo-Guerrero et al., 2010) and at r.t., (Flores et al., 2009a) was also used. Water was applied to collect exudates of pepper plants from stonewool media (Kamilova et al., 2006). Aqueous neutral buffers (pH 7.0) can be used instead of water (Kim et al., 2002). It is convenient if enzymatic methods will be used for quantification. Raffo et al. (2007; 2008) relied on a room temperature extraction with a (1:1) acetonitrile-water mixture. Frequently, several groups of phytochemicals need to be analyzed. Then more universal solvents should be used. So, sugars and chlorophylls can be extracted simultaneously with 80% acetone (Flores et al., 2007).

Coloured pigments are common concomitants in sugar isolates when organic extractants are used. Many researchers prefer to remove them before proceeding to the analysis. The most popular technique is the filtration through a C18 cartridge following the evaporation of the organic solvent and the reconstitution of the sample in water (Navarro et al., 2006; Flores et al., 2007; Matsufuji et al., 2007). Martínez-Ballesta et al. (2004), who analyzed pepper leaves, directly filtered leaf sap through a C18 Sep-pak filter. Priya Sethu with coworkers (1996) purified ethanol extracts by passage through Dowex 1x8 ( $H^+$ ) and Dowex 50W ( $OH^-$ ) resins.

### *Separation, identification, and quantification*

There are the three common approaches to analyze carbohydrate isolates: colorimetry, chromatography, and enzymatic assays. Colorimetry has been recognized for a long time as a simple, cheap, and fast technique for the quantification of the sum of soluble saccharides. The phenol-sulfuric acid method (Dubois et al., 1956) and the anthrone method (Hewitt, 1958) are used for the measurement of the overall concentration of carbohydrate species in solutions, including those conjugated to other

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molecules, such as flavonoids or proteins. For the assessment of reducing sugars, the use of dinitrosalicylic acid has been recommended (Miller, 1959).

Determination of individual sugars is possible with the help of chromatographic methods, which are summarized in Table 7. HPLC with a refractive index detector is the standard method of choice. When a higher sensitivity is required, GC of trimethylsilanized samples (Brobst and Lott, 1966) or HPLC with a UV-detector and post-column derivatization of solutes (Kamilova, et al., 2006) can be recommended. Due to simple and predictable composition of the free sugar fraction, the identification of analytes by retention times is sufficient. Quantification is most frequently carried out by means of external standardization. The internal standard approach was occasionally used, with sorbitol (Gallardo-Guerrero et al., 2010) or lactose (Bernardo et al., 2008) serving as ISs.

During the last two decades, commercially available sucrose/D-glucose/D-fructose enzymatic assays appeared. The method is simple, fast, and sensitive enough to determine sugars in pepper pods (Kim et al., 2002; Cagno et al., 2009). Its applicability for the analysis of other plant organs with lower carbohydrate content has not been tested yet. The method is based on the spectrometric measurement of NADPH produced in the enzymatic oxidation of D-glucose in the presence of NADP<sup>+</sup>. D-fructose and sucrose are determined after enzymatic conversion (cleavage in the case of sucrose) to D-glucose (Raugel, 1999). Despite mentioned advantages, some drawbacks are associated with the enzymatic assay. It is sensitive to an interfering influence of organics able to reduce NADP<sup>+</sup>. Therefore, the co-extraction of pepper antioxidants can compromise the results of analysis unless measures are undertaken to remove such compounds from the sample. Secondly, as the

quantification of fructose and sucrose is a multistep procedure, an experimental error, being cumulative, is knowingly higher than for HPLC analysis.

### ***Carbohydrates: Polysaccharides***

In food chemistry, plant polycarbohydrates are divided into the two large groups: starches and non-starch polysaccharides (NSP) or dietary fiber (Englyst and Hudson, 1996). These groups are distinctive in physico-chemical properties due to different structure of polymeric chains, nature of constituent sugars, and type(s) of glycosidic linkages present. Starch is a mixture of  $\alpha$ -glucan polysaccharides amylose and amilopectin in proportions that depend on the botanical origin. Amylose is a homopolymer of D-glucose linked through  $\alpha$ -D-(1-4) linkages. It has an essentially linear structure. On average, it consists of 500 to 6000 glucose units that can be distributed among 1 to 20 chains. Each chain has an average degree of polymerization DP = 500. Amilopectin is a branched polymer composed of  $\alpha$ -D-(1-4)-linked glucose segments containing short (DP = 20-25) glucose chains in  $\alpha$ -D-(1-6) branches. Its molecular weight ranges between  $10^6$  and  $10^7$  g/mol (Bello-Perez et al., 2010). In plants, amylose and amilopectin are aggregated in discrete granular water-insoluble starch particles, generally ranging in size between 1 and 100  $\mu\text{m}$  (Bertolini, 2010). Besides the polysaccharides, starch granules contain lipids and can be surrounded by proteins, lipids,  $\beta$ -glucans (Biliaderis, 1992; Vasanthan et al., 2005).

# ACCEPTED MANUSCRIPT

NSP are mostly originated from cell wall material. Those are cellulose, pectins, and hemicelluloses or matrix glycans (xyloglucans, arabinoxylans, glucomannans), whose function is to non-covalently cross-link the cellulose microfibrils (Carpita and Gibeaut, 1993). Pectins are another class of substances that held together cellulose fibers in the cellular walls (Conforti and Zinck, 2002). They are polymers of D-galacturonic acid. Neutral sugar units, which include arabinose, galactose or rhamnose, may be present as side chains (Bemiller and Whistler, 1996). The carboxyl groups of polygalacturonic acid can be fully or partially methoxylated resulting in a wide range of polymer esters with different solubility in water and aqueous alkali solutions (Howard and Buescher, 1990). Popov et al. (2011) reported a small fraction of pectic galacturonan acetylated at the O3 position in sweet bell pepper. Not fully methylated polygalacturonic acid molecules can be cross-linked by  $\text{Ca}^{2+}$  cations in the cell wall. This fraction is water-insoluble, yet, can be transferred into a solution with the help of chelating agents extracting the  $\text{Ca}^{2+}$  ions (Melton and Smith, 2005). Besides carbohydrates, the insoluble part of the cell wall fibers may contain lignin, a three-dimensional phenolic heteropolymer that is covalently associated with polysaccharides in plant cell walls (López-Hernández et al., 1996; Pomar et al., 2004).

Studies of polysaccharide content were mostly induced by interest in the dietary value of peppers (Villanueva-Suárez et al., 2003; Martínez et al., 2007; Bernardo et al., 2008) and in techniques to protect the dietary value and textural quality during storage (Gu et al., 1999; Conforti and Zinck, 2002). Helyes et al. (2005) have studied the effect of climatic changes on pepper fruits. Estrada et al. (2000) and Harpster et al. (2002) have considered the metabolism of pepper ripening. Elucidating the dehydration process was an objective of the work of Gallardo-Guerrero et al. (2010). Several research groups investigated the

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effect of different factors on pollen germination (Alonia et al., 2001; Shaked et al., 2004) and characteristics of pepper plants (Flores et al., 2007). Popov et al. (2011) have explored the anti-inflammatory activity of pectic polysaccharides isolated from sweet pepper.

*Capsicum* peppers contain roughly comparable quantities of starch and NSP in the edible part. For example, 0.8, 2.2, and 0.7% (fw) of starch, fiber (cellulose and hemicellulose), and pectin were found in Padrón peppers (*C. annuum* var. Longum) (López-Hernández et al., 1996). In spite of this fact, pepper is advertised as a source of only dietary fiber. Therefore, starch is rarely quantified in pepper fruits since its content is of low interest for consumers and breeders. On the other hand, researchers determine starch in pollen, leaves and roots of pepper plants as an indicator of plant vigor (Alonia et al., 2001; Shaked et al., 2004; Helyes et al., 2005; Flores et al., 2007). There is a large variation in published values of starch content in leaves. Helyes et al. (2005) have reported a figure of 113 mg glucose/g dw, whereas Flores et al. (2007) have found it to be less than 15 mg glucose/g dw. Different groups published comparable data on the concentration of starch in pollen that ranges between 70 and 250 mg glucose/g dw depending on the phase of plant development (Alonia et al., 2001; Shaked et al., 2004).

The review of data on fiber content in pepper fruits is complicated because of different extraction protocols employed and different terminology used. It especially concerns the term *fiber*. Sometimes this term (as dietary fiber) means the total NSP according to the recommendations of Englyst and Hudson (1996), sometimes it stands for the cellulose fraction or the sum of cellulose and hemicelluloses, and sometimes it includes lignin that is not a carbohydrate at all. In general, there is a reasonable agreement between different laboratories in the overall NSP in the pepper pericarp. It ranges between 1.6 and 3%

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(fw) (López-Hernández et al., 1996; Howard et al., 1997; Villanueva-Suárez et al., 2003; Martínez et al., 2007; Zachariah and Gobinath, 2008; Bernardo et al., 2008). Apparently, genotype does not affect significantly the total level of polysaccharides at least for *C. annum* species (Bernardo et al., 2008). The effect of the ripening process on the overall content of fibrous polymers is also not pronounced (Martínez et al., 2007; Bernardo et al., 2008), although maturation results in the redistribution of the matter between relatively long chain and relatively short chain glycans (Harpster et al., 2002). On contrast, a significant fall in the concentrations of all groups of the alcohol-insoluble carbohydrates has been observed during a post-harvest storage (Priya Sethu et al., 1996) unless special protective measures were applied (Howard et al., 1997; Gu et al., 1999; Conforti and Zinck, 2002).

Neutral sugars constituting cell wall polysaccharides are glucose, galactose, xylose, arabinose, mannose, and rhamnose (Priya Sethu et al., 1996; Villanueva-Suárez et al., 2003; Popov et al., 2011). For the total dietary fiber in green pepper, the following series was observed (Villanueva-Suárez et al., 2003): glucose (6-7% dw.) > galactose (1.9) > xylose (0.8-1.1) ≈ arabinose (0.6-1.0) > mannose (0.5) > rhamnose (0.1). Priya Sethu et al. (1996) and Popov et al. (2011) have shown that monosaccharide profile depends on a fraction of NSP. In some fractions, galactose and arabinose or xylose can be predominant sugars, glucose being a minor one.

## *Starch analysis*

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Starch is quantified in the form of glucose after hydrolysis. Sample preparation includes homogenization using any convenient method followed by the extraction of soluble sugars with room temperature 80% acetone (Flores et al., 2007), hot ethanol (Shaked et al., 2004), or 80% ethanol under reflux (López-Hernández et al., 1996). Extraction time is 30 min to 1 h. The procedure is repeated twice to ensure complete removal of free sugars. The alcohol (or acetone) insoluble solids (AIS) are recovered by centrifugation and subjected to hydrolysis. Chemical hydrolysis with acidic solutions (boiling 3% HCl (Flores et al., 2007) or 35% HClO<sub>4</sub> (Helyes et al., 2005)) is still used in pepper analysis, although the method was criticized for its low specificity and sensitivity (Vasantha et al., 2005). Nowadays, enzymatic digestion is a method of choice (López-Hernández et al., 1996; Alonia et al., 2001). The procedure includes gelatinization in refluxing water or in hot (100°C) 0.2 M KOH for 0.5-2 h followed by the adjustment of pH to a value of 4.6-5.0 with acetic acid or a citrate/phosphate buffer. Then enzyme amyloglucosidase is added and the mixture is incubated at 55°C for several hours (not less than 1 h). Released glucose is determined by one of the methods described in the previous section.

## *Non-starch polysaccharides: Fractionation and analysis*

Sample preparation may follow the same initial steps to obtain AIS as for starch analysis described above. Sometimes additional precautions are taken to suppress activity of pepper enzymes and to remove cell wall proteins, lipids, and lignins. Pepper enzymes released into the intercellular space after disruption of the cell walls catalyze compositional changes and the depolymerization of fibrous materials thus compromising the results of the following fractionation of NSP. Priya Sethu et al.

(1996) stored a sliced pepper under 80% ethanol at 0°C for a month prior to analysis to inactivate the enzymes. Howard et al. (1997) boiled samples in ethanol for 10 min for the same purpose. Conforti et al. (2002) homogenized pepper in cold (-20°C) acetone followed by washing with 80% then 100% acetone. The acetone-insoluble residue was suspended in a mixture of phenol, acetic acid, and water (2:1:1) at 4°C for 5 min, then was diluted with acetone, filtered, and dried. Thus prepared AIS were believed to contain no active enzymes. A disadvantage of this method is that phenol alters pectin solubility by removing calcium from the cell walls (Vasantha et al., 2005).

Purification of AIS from non-carbohydrate contaminants is achieved by triple 2 h extraction with hexane-CH<sub>2</sub>Cl<sub>2</sub> at 80°C (Priya Sethu et al., 1996). A more comprehensive purification procedure was used by Harpster et al. (2002). The ethanol-insoluble residue was subjected to sequential extraction with Tris-buffered phenol (to remove proteins), 95% ethanol, chloroform-methanol (1:1), and acetone. Obtained AIS were incubated overnight at r.t. in 20 mM HEPES (pH 7) containing α-amylase to remove starch.

A common approach to AIS analysis consists in a sequential extraction of pectins (usually divided into three subfractions) and matrix glycans. The remaining insoluble fiber (IF) residue represents mainly the cellulose fraction. Several protocols have been proposed for NSP fractionation, summarized in Table 8. In this table, the residue left after extraction on a higher fractionation step is applied to a lower step. Each extraction is repeated 2-6 times. The water or alkali extraction steps can be optionally omitted that would result in the loss of certain information regarding the composition of fractions. It must be remembered that if starch is not separated from NSP prior to the analysis, it will contaminate the IF fraction. Starch will not

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interfere with analysis of pectins if those are quantified in the form of galacturonic acid. However, if the neutral sugars of pectic substances are of interest, the breakthrough of starch and products of its hydrolysis into the pectin fractions must be avoided.

Simpler extraction protocols are occasionally used in studies of pepper polysaccharides. Being faster, these methods are less informative. So, Villanueva-Suárez et al. (2003) separated starch and NSP applying termamyl ( $\alpha$ -amylase) at pH 5.2 and temperature 100°C for 10 min followed by treatment with a mixture of pancreatin and pullulanase (amylopectin debranching enzyme) at pH 7 and temperature 50°C for 30 min. This procedure gives the total dietary fiber. The official AOAC method 991.43 (AOAC, 1998) as well as its modified version (McCleary et al., 2010) allows differentiation between the water-soluble and insoluble parts of dietary fiber. The later fraction comprises a few chemically distinctive groups of carbohydrates; therefore an explanatory value of such information is limited. So, Martínez et al., (2007) have applied this method to find no noticeable effect of ripening on the cell wall composition of pepper. In a more detail study, Harpster et al. (2002) using size-exclusion chromatography have demonstrated that some changes in a cell wall polysaccharide profile do happen during maturation. One more simplified fractionation protocol have been proposed by Popov et al. (2011), who isolated pectic substances by digesting a pepper homogenize with simulated gastric juice, followed by centrifugation and ultrafiltration. Two fractions were collected with molecular mass 100-300 kDa and over 300 kDa. Both the fractions were contaminated with proteins.

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The solid matter of the liquid fractions obtained after fractional extraction can be evaluated gravimetrically after recovering by precipitation with ethanol or by evaporation of the solvent. However, methods of analysis based on the hydrolysis of the fractions and the measurement of the constituent sugars are more informative. Protocols of hydrolysis found in the literature summarized in Table 8. As seen, some protocols are universal, suitable for several groups of polysaccharides; other protocols are selective, allowing the hydrolysis of specific molecules. Pectins are quantified in the form of galacturonic acid through the colorimetric reaction with *m*-hydroxydiphenyl (Kintner and Van Buren, 1982; Filisetti-Cozzi and Carpita, 1991). The carbazole method used for the uronic acid assay in the past is not recommended because of interference from neutral sugars. López-Hernández et al. (1996) have assessed the quantity of released galacturonic acid by HPLC with a polar column Spherisorb NH<sub>2</sub> (250 x 4.6 mm, 5 µm) using 0.1 M sodium acetate (pH 4.6) as the mobile phase. Neutral sugars in the hydrolysates of the pectin and hemicellulose fractions are measured by GC after derivatization to alditol acetates (Villanueva-Suárez et al., 2003; Popov et al., 2011). β-D-allose (Villanueva-Suárez et al., 2003) and *myo*-inositol (Popov et al., 2011) were suggested as ISs. Neutral monosaccharides can be also analyzed by HPLC as described above. Villanueva-Suárez et al. (2003) have compared GC and HPLC methods. They concluded that both methodologies are in good agreement. Determination of neutral sugars and galacturonic acid in one sample was reported by Paik et al. (2003). They reduced the saccharides with NaBH<sub>4</sub> to aldитols and aldonic acid, respectively, followed by their separation on an anion-exchange resin Dowex-1 (CH<sub>3</sub>COO<sup>-</sup>-form). The neutral and acidic fractions were converted to alditol acetates according to Jones and Albersheim (1972) and measured by the GC method as mentioned above.

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The IF is usually determined by the gravimetric method or as the difference between the total polysaccharides and the sum of the soluble fractions. More accurate and detailed data on the composition of IF is of low interest as this material is not digestible by humans. In the former approach, a dried sample of the IF fraction is refluxed with water containing a neutral detergent for 1 h. The residue is dried, weighed, incinerated at 550°C and then reweighed. The amount of IF is calculated as the difference in mass (López-Hernández et al., 1996). The second approach is exemplified by works of Howard with coauthors (Howard et al., 1997; Gu et al., 1999).

Semi-automated extractors for the fractionation of dietary fiber, for example, Dosi-fiber (JS Selecta, Spain) and Fibertec (Foss, Denmark), are manufactured. This equipment is designed for analysis according to official (AOAC, AACC, ISO) methods; therefore, the choice of solvents, regimes, and sample load is restricted.

Finally, consider the application of preparative chromatography for analysis and deeper purification of the solutions obtained by extraction. Anion-exchange columns with DEAE-cellulose or DEAE-agarose have been used for the fractionation of pectic polysaccharides isolated with simulated gastric juice (Popov et al., 2011) or hot water (Paik et al., 2003), respectively. In both cases, a stepwise elution with NaCl was applied. The main fractions were collected with 0.2-0.3 M NaCl. Paik with coauthors (2003) have additionally purified dialyzed eluates after ion-exchange chromatography on a size-exclusion Sephadex G-100 column. Size-exclusion chromatography has also been used for the characterization of the molecular mass distribution of different polysaccharide fractions in work by Harpster et al. (2002). For pectic extracts, a Sepharose CL-2B column (95 x

1.5 cm) was used, eluted with 0.2 M CH<sub>3</sub>COONH<sub>4</sub> at pH 5.0. For matrix glycans, a Sepharose CL-6B column of the same dimensions was used, eluted with 0.1 M NaOH.

### **Vitamins**

The term “vitamins” describes a group of nutrients that are required for normal functioning of the human body. They serve (i) as coenzymes or their precursors; (ii) as components of the antioxidative defense systems; (iii) as factors of genetic regulation; and (iv) in specialized functions, such as a photoreceptive cofactor in vision (Gregory, 1996; Combs, 2008). Vitamins comprise natural compounds of diverse chemical structure, classified together based on their physiological activity. Thirteen substances or group of substances are generally recognized as vitamins (Combs, 2008), which may be categorized into two groups according to their solubility. The fat-soluble vitamins are represented by vitamins A, D, E, and K. The water-soluble vitamins include vitamin C and the members of the vitamin B, niacin, and folic acid groups.

The main body of studies on vitamins in pepper during the last decade was focused on the nutritional value of the fruits (Burns et al., 2003; Perucka and Materska, 2003; Antonious et al., 2006; Phillips et al., 2006; Matsufuji et al., 2007; Topuz and Ozdemir, 2007; Bernardo et al., 2008; Marín et al., 2008; Gorinstein et al., 2009; Nazzaro et al., 2009; Isabelle et al., 2010; Wahyuni et al., 2011). Researchers also considered the influence of post-harvest treatment on the antioxidant content (Daood et al., 2006; Kim et al., 2008a; Sgroppo and Pereyra, 2009; Matsufuji et al., 2009) and the evolution of the phytochemical

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composition of peppers during storage (Marín et al., 2004; Kevers et al., 2007; Raffo et al., 2008). Effects of fertilization, agricultural practices, and environmental conditions were studied in (Navarro et al., 2006; Pérez-López et al., 2007a; Flores et al., 2009; Flores et al., 2009a, Ghoname et al., 2009; Lizarazo et al., 2010; Serrano et al., 2010). One publication was devoted to the biosynthesis and metabolism of vitamins in *Capsicums* (Koch et al., 2002). Antimutagenic properties of peppers in connection with their vitamin C content have been discussed by Ramirez-Victoria et al. (2001). Bernhardt and Schlich (2006) discussed the impact of different cooking methods on the vitamin content.

*Capsicum* peppers are considered to be an excellent source of vitamins C, E, and precursors of vitamin A, so called provitamin A (Horward and Wildman, 2007), although other vitamins have also been found (Phillips et al., 2006; Helmja et al., 2007). The provitamin A compounds,  $\alpha$ - and  $\beta$ -caroten, and  $\beta$ -cryptoxanthin, have been discussed above and are not considered here. Vitamin C (L-ascorbic acid) and vitamin E (tocopherols) are known for their antioxidant properties (Horward and Wildman, 2007; Combs, 2008) and believed to be synthesized by plants as protectants against reactive oxygen species, which are generated in photosynthetic and respiratory processes (Smirnoff, 1996; Combs, 2008; Lizarazo et al., 2010). Because of different solubility of these substances, their distribution and, consequently, targets in plant tissues are somewhat different. While ascorbate is found in all subcellular compartments, where it neutralizes reactive forms of oxygen (Smirnoff, 1996, Munné-Bosch and Alegre, 2002), tocopherols function primarily in thylakoid membranes, inhibiting lipid oxidation by scavenging various free radicals (Smirnoff et al., 2001; Combs, 2008; Lizarazo et al., 2010).

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Vitamin C exists in two forms, L-ascorbic acid (AA) and a product of its oxidation L-dehydroascorbic acid (DHAA) (Figure 8). Some authors do not consider DHAA as a vitamer (Daood et al., 1996; Matsufuji et al., 2009), whereas other authors do (Horward and Wildman, 2007; Marín et al., 2008) because it can be converted metabolically to AA (Combs, 2008). Usually, DHAA accounts for only a small fraction of the total vitamin C in *Capsicums* (Márkus et al., 1999; Marín et al., 2004; Matsufuji et al., 2009), rarely achieving the value of 20% (Márkus et al., 1999) and does not contribute to the antioxidant capacity of the vitamin (Matsufuji et al., 2009). In plants, AA is synthesized according to the mannose-galactose-galactonolactone pathway (Smirnoff, 2000; Smirnoff et al., 2001). Alternative pathways have also been hypothesized (Smirnoff et al., 2001). Expectedly, the AA content in peppers correlates to the level of sugar precursors (Horward and Wildman, 2007). It varies depending on genotype, geographical origin of a fruit (Daood et al., 1996; Antonious et al., 2006; Horward and Wildman, 2007; Wahyuni et al., 2011), and on weather conditions (Márkus et al., 1999). Fertilization conditions affect this value within ca.  $\pm$  20% (Pérez-López et al., 2007a; Flores et al., 2009; Ghoname et al., 2009; Serrano et al., 2010). AA increases with ripening from 0.05-0.2 mg/g fw at the immature green stage to 0.8-2.6 mg/g fw for fully ripe fruits (Horward and Wildman, 2007; Pérez-López et al., 2007a; Serrano et al., 2010).

AA concentration in harvested fruits is controlled by relationship between the consumption and biosynthesis processes. The magnitude of changes depends on the ripening stage at the harvest time and on post-harvest treatment. So, Raffo et al. (2008) and Kevers et al. (2007) reported minor changes in the AA concentration in red and yellow peppers stored at 4-8°C for 9 and 34 days, respectively. At the same time, chopped green pepper stored at 4°C demonstrated a decrease in the AA content

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from ca. 0.5 to 0.3 mg/g fw followed by an increase to 0.4 mg/g fw in a course of a 15-day experiment. Vitamin C monotonically decreased by 30% when the storage temperature was elevated to 10°C (Sgroppi and Pereyra, 2009). Its content decreased by 90% during 4 months in a dry powdered pepper, in which biosynthesis processes were suppressed, whereas the chemical oxidation of the vitamin took place freely (Daood et al., 1996).

Vitamin E is a collective name for several isoprenoid vitamers, tocotrienols and tocopherols (Figure 8).  $\alpha$ -tocopherol is the most active one. Tocopherols are synthesized in the isoprenoid pathway that starts from isopentenyl pyrophosphate (PP) and proceeds through the formation of intermediate phytol-PP (Arango and Heise, 1998; Fraser et al., 2000). Tocopherol molecules contain three chiral centers at C2, C4', and C8' positions, making possible eight stereoisomers. Only the (*RRR*)-form is naturally occurring. All four tocopherols ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) are found in peppers.  $\alpha$ -tocopherol is the predominant vitamer in pericarp tissues, whereas  $\gamma$ -tocopherol is one in seeds (Horward and Wildman, 2007; Daood, 2009). There were a few reports about minor amounts of tocotrienols in red *Capsicum* (Daood, 2009, Isabelle et al., 2010). Their presence could account for less than 1% of the total vitamin E content (Isabelle et al., 2010). The tocopherol concentration in pepper fruits strongly depends on cultivar and environmental conditions (Horward and Wildman, 2007; Wahyuni et al., 2011) and is positively influenced by light irradiation (Lizarazo et al., 2010). The level of tocopherols changes as ripening progresses. In general, the  $\alpha$ -tocopherol content in pericarp tissue and the  $\gamma$ -tocopherol content in seeds increases until the red succulent stage and then can decline (Márkus et al., 1999; Koch et al., 2002; Horward and Wildman, 2007; Daood, 2009). Usually, the researcher expects to find the concentration of  $\alpha$ -tocopherol in pericarp and  $\gamma$ -tocopherol in seeds of matured red peppers within the range

of 0.1-3 and 0.3-0.5 mg/g fw, respectively. The content of other tocopherols in pods is considerably lower and depends on many factors. The ratio of  $\alpha/\beta$  tocopherols rarely achieves the value of 2 (Koch et al., 2002; Wahyuni et al., 2011). The percentage of  $\delta$ -tocopherol found in red pepper sold in Singapore composed less than 1% of the total vitamin E (Isabelle et al., 2010). The dehydration of fruits results in a decrease of the tocopherol fraction, the more pronounced the higher the drying temperature (Daood et al., 2006). It is worth to note that although powdered dry red pepper is considered to be a fair source of vitamin E (Horward and Wildman, 2007), the latter is completely destroyed during 3 months of ambient storage (Daood et al., 1996).

Folate is another vitamin reported in peppers (Vahteristo et al., 1997; Phillips et al., 2006). This general name stands for a group of derivatives of folic (pteroylmonoglutamic) acid (Figure 8). The folates participate in several important physiologic processes, such as single-carbon metabolism, methionine synthesis, and histidine catabolism and are considered to be important in preventing fetal neural tube defects (Combs, 2008). Sweet peppers studied by Phillips et al. (2006) contained 5-methyltetrahydrofolate (5-MTHF) and 5-formyltetrahydrofolate (5-FTHF) in comparable amounts and a trace level of 10-formylfolate. Vahteristo et al. (1997) reported a tenfold exceeding of 5-MTHF over 5-FTHF and tetrahydrofolate. The total folate content varied in these works between 0.2 and 0.7  $\mu\text{g/g}$  fw. Actually, a significant portion of the folates exist in plants in the form of polyglutamates (Combs, 2008). Those are enzymatically hydrolyzed to monoglutamate entities before analysis as will be discussed below.

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## *Sample preparation, extraction, and analysis*

Vitamins are determined both in fresh fruits and in dry powdered products. Fresh peppers may be freeze-dried or lyophilized before disintegration. Soft dehydration is even advisable if samples have to be stored for a long time. Usually, the pericarp of fruits is subjected to analysis unless the composition of seeds and placenta is of special interest. In studies of the dietary intake of vitamins, it is reasonable to analyze the whole edible part. In analysis of tocopherols and folates, samples should be prepared under reduced light to avoid photodegradation. When analyzing folates, some researchers additionally protect samples from degradation by keeping them in an ice box (Vahteristo et al., 1997).

Disintegration of pericarp tissues is performed by cutting into pieces, blending, or crashing in a mortar with liquid N<sub>2</sub>. Daood with co-authors macerated fresh pepper in a mortar with quartz sand and removed the moisture with methanol (Daood et al., 1996; Márkus et al., 1999). Dried pepper is convenient to grind in a mortar, a laboratory mill, or a coffee blender. A few drops of phosphoric acid added to the sample at the beginning of disintegration prevent the enzymatic conversion of AA to DHAA (Márkus et al., 1999). The following extraction and analysis procedures depend on solubility of analytes. Respective analytical protocols for water-soluble and fat-soluble vitamins are summarized in Tables 9 and 10, necessary explanations are given below for each vitamin considered.

**Vitamin C.** Water-soluble vitamins are extracted with aqueous solutions. In the case of vitamin C, it is usually 2-6% solutions of metaphosphoric acid (Daood et al., 2006; Bernardo et al., 2008; Kim et al., 2008a; Ghoname et al., 2009; Matsufuji et al., 2009; Nazzaro et al., 2009; Sgroppo and Pereyra, 2009; Gallardo-Guerrero et al., 2010; Isabelle et al., 2010;

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Saha et al., 2010), sometimes modified with minor amounts of complexing agents, EDTA (Topuz and Ozdemir, 2007) or diethylenetriaminepentaacetic acid (Wahyuni et al., 2011). Organic acids can also be used (Antonious et al., 2006; Marín et al., 2008). Other solvents can be recommended when AA is extracted along with other water-soluble phytochemicals. So, AA, sugars, and organic acids were co-extracted with a neutral ( $\text{pH} \approx 7$ ) phosphate buffer (Serrano et al., 2010). A water-ethyl acetate system was applied to extract both lipophilic and hydrophilic organics. AA, sugars and phenolic compounds were then quantified in the water layer (Flores et al., 2009a). A mixture of acetone, water, and acetic acid (70:28:2) was used to co-extract AA and flavonoids (Kevers et al., 2007).

Both AA and DHAA are not bound strongly to the sample matrix as they are mainly located in the cytoplasm. Therefore, they can be isolated under mild conditions. A usual extraction protocol consists in fast homogenization (< 2 min) with or without solvent, followed by shaking the slurry with the solvent during 10-15 min at ambient temperature. If vitamin C is analyzed along with hardly extractable compounds, the procedure is performed under harsh conditions ensuring complete transfer of these compounds to the liquid phase.

Many researchers purify vitamin C extracts by SPE with C18 cartridges (Marín et al., 2004; Navarro et al., 2006; Topuz and Ozdemir, 2007, Kim et al., 2008a; Marín et al., 2008). Partition against ethyl acetate (Gorinstein et al., 2009) is a cost-effective alternative to SPE purification. On the other hand, current HPLC methods allow a good separation of AA and DHAA from each other and from interferences (Flores et al., 2009, Daood et al., 1996); therefore, the clean-up of extracts is mainly recommended for non-chromatographic quantification methods. Those are titration with 2,6-dichlorophenolindophenol (2,6-

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DCPIP) (Ramirez-Victoria et al., 2001, Ghoname et al., 2009) or spectrophotometric methods. In the last case, 2,6-DCPIP (Antonious et al., 2006; Kevers et al., 2007), 2,4-dinitrophenylhydrazine (Matsufuji et al., 2009), Cu(II)-neocuproine (Gorinstein et al., 2009), or  $\alpha,\alpha$ -dipiridyl in the presence of FeCl<sub>3</sub> (Perucka and Materska, 2003) are used as coloring agents. A highly specific UV-enzymatic assay, in which AA is oxidized by guaiacol peroxidase, was adopted for the analysis of pepper extracts by Bernardo et al. (2008). Two HPLC techniques, ion-pair and reversed phase chromatography, are popular for the determination of the reduced and oxidized forms of vitamin C along with over organic acids (citric, malic, oxalic, and succinic). Details are given in Table 9. In contrast to AA, DHAA has a weak UV absorption. Márkus et al. (1999) have increased the sensitivity of the HPLC assay with respect to DHAA by using a post-column derivatization with *o*-phenylenediamine followed by detection with a fluorescent detector (FD). Marín et al. (2008) applied the same reagent for a pre-injection derivatization of samples. Chromatograms were recorded with an UV-detector set at 261 nm for AA and 348 nm for the DHAA-derivate. The possibilities of micellar electro kinetic chromatography in the analysis of AA were demonstrated by Helmja et al. (2007).

Main concomitants of organic acids in aqueous pepper extracts are sugars and polyphenolics. These compounds are UV-transparent at wavelengths applied to monitor AA (220-250 nm) and/or are not eluted along with the vitamers of interest, hence, do not interfere with analysis either. However, if a simultaneous determination of AA and sugars is desirable, they can be separated on a Supelcogel C-610H column (sulfonated polystyrene/divinylbenzene) (Serrano et al., 2010). The

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identification and quantification of AA and DHAA do not present difficulties in view of relatively simple chromatograms and availability of standards.

**Folates.** In few works on the folates in pepper, those were extracted with neutral (pH 6) potassium hydrophosphate buffers modified with 10 mM ascorbic acid and 10 mM 2-mercaptoethanol (Vahteristo et al., 1997; Phillips et al., 2006). The presence of AA was necessary to prevent the oxidation of the analytes during extraction. The isolate was further degassed with argon and incubated at 37°C and pH 6 sequentially with  $\alpha$ -amilaze (1 h), a protease (3h), and rat plasma deconjugase (14 h) (Phillips et al., 2006) or hog kidney deconjugase (3 h) (Vahteristo et al., 1997). With the last enzyme, pH must be adjusted to 4.9. The first two enzymes destroy starch and proteins. Either deconjugase converts folate polyglutamates to monoglutamates. Then the sample was cleaned-up by means of anion-exchange SFE with a SAX cartridge eluted with 1 M NaCl in 0.1 M phosphate buffer (pH 6) containing 25% of acetonitrile. The analysis of the samples was made by HPLC (Table 9) with MS (Phillips et al., 2006) or FD (Vahteristo et al., 1997) detection. The method of external standardization was used for quantification.

**Vitamin E.** Tocopherols and tocotrienols, being reductants, are slowly oxidized by atmospheric oxygen to quinons; the process is catalyzed by light, heat, and alkalinity. They are also photolabile compounds, especially sensitive to radiation in the UV region (Ball, 2006). Therefore, sample preparation procedures must be performed under reduced light. It is convenient to equip a laboratory with fluorescent lightening fitted with appropriate UV filters for this purpose. The extraction of vitamin E is performed either at ambient temperature (Koch et al., 2002; Perucka and Materska, 2003; Daood et al., 2006, Phillips et al.,

2006; Isabelle et al., 2010; Lizarazo et al., 2010) or on ice (Burns et al., 2003; Matsufuji et al., 2007; Wahyuni et al., 2011) by shaking for 10-15 min or by quick homogenization with a solvent repeated until the complete exhaustion of color. The cold extraction is used when tocopherols are analyzed together with carotenoids and is necessary to prevent the degradation of carotenoids. Solvents for the isolation of tocopherols must combine lipophilic properties with a certain polarity that is important for the solvation of polar functionalities of tocopherols and breaking bonds between matrix proteins and the analytes (Ball, 2006). Mixed solvents containing a chlorohydrocarbon ( $\text{CHCl}_3$ ,  $\text{CCl}_4$ , 1,2-dichloroethane) and methanol are most popular (Márkus et al., 1999; Daood et al., 2006; Wahyuni et al., 2011), although acetone is also used (Matsufuji et al., 2007; Lizarazo et al., 2010). Koch et al. (2002) have applied *i*-octane and hexane-*i*-propanol (3:2, v/v), referring to older protocols. Nowadays, the use of pure hydrocarbons for direct solvent extraction of vitamin E from foodstuff ceases in favor of mixtures with a polar co-solvent (Lee et al., 1999). The recovery yield is supposed to be higher if the sample is sequentially treated with different solvents. One such method has been adopted in pepper analysis (Isabelle et al., 2010). Another method gaining popularity is based on the use of immiscible liquids (Burns et al., 2003; Wahyuni et al., 2011). A typical protocol consists in the extraction of the sample with a polar organic solvent followed by the addition of an aqueous buffer. Finally, a hydrophobic solvent (chlorophorm or ethyl acetate) is added to accumulate the liposoluble analytes. This approach allows purification of the extracts from UV interferences (Fraser et al., 2000). The direct extraction of fat-soluble vitamins can be achieved with supercritical fluids as reviewed by Ball (2006) and Blake (2007). Neither this nor another emerging technique – matrix solid phase dispersion (Ball, 2006; Blake, 2007) – have been tested with pepper samples.

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In the past, the saponification of extracts or homogenizates was strongly recommended, aiming to destroy lipids and pigments, which can interfere with analysis, and to assure the release of tocopherols from the sample matrix (Ball, 2006; Blake, 2007). Nowadays, this procedure is avoided by many researchers as unnecessary since the above-mentioned extraction systems allow a high recovery yield (Lee et al., 1999; Vilasoa-Martínez et al., 2008) in combination with sufficient sample purification. When applied, the saponification is made by refluxing the extract for 15-30 min with 30-35% KOH dissolved in methanol or water-ethanol modified with pyrogallol, ascorbic acid, or BHT (Márkus et al., 1999; Daood et al., 2006; Matsufuji et al., 2007). The vitamin E fraction is re-extracted from the saponification mixture with hexane or petroleum ether. The extraction must be repeated three times (Blake, 2007). The sample solution may need to be concentrated depending on the sensitivity of an analytical method employed. This is made by evaporation under reduced pressure or with a stream of N<sub>2</sub>.

Both NP and RP HPLC can be used for the separation and quantification of vitamers E (Table 10). The NP mode provides a benefit of compatibility with hydrophobic liquids used for the extraction of fat-soluble vitamins. To inject the sample into a RP system, it must be transferred to a water miscible solvent. Although NP chromatography is an object of a well known criticism (catalytic activity of the stationary phase, sensitivity to residual moisture of the mobile phase, *etc.*), examples found in the literature demonstrate an excellent separation of tocopherols and tocotrienols on silica with binary eluents in the isocratic regime. On contrary, the application of RP HPLC requires the use of complex ternary or quaternary mixtures under gradient elution conditions (Table 10). Note, however, that such systems allow simultaneous separation of tocopherols and carotenoids, which cannot be achieved on silica. Lee, Su, and Ong (2004) described a two-column switching system able to

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separate capsaicinoids, carotenoids, chlorophylls, and tocopherols in one run, which they used to analyze *Capsicum* fruits (Isabelle et al., 2010).

Since tocopherols are fluorescent compounds, a fluorescent detector can be used for recording chromatograms, providing high selectivity and sensitivity (Koch et al., 2002; Daood et al., 2006; Matsufuji et al., 2007, Isabelle et al., 2010; Lizarazo et al., 2010; Wahyuni et al., 2011). The extinction and emission wavelengths are usually set at 295 and 320-340 nm, respectively. FD-chromatograms of the lipophilic fraction of pepper extracts contain a limited number of interfering peaks, as a rule, ubiquinons (Daood et al., 1996; Daood, 2009); therefore, the identification of the vitamers E is easy and can be made based on the retention times of standards, which are commercially available for all four tocopherols. UV-chromatograms are more complex due to co-elution of carotenoids. In this case, detection at a wavelength of 290 nm is recommended to minimize the influence of concomitants (Burns et al., 2003; Fraser et al., 2000). Quantification of tocopherols is frequently carried out by comparison with external calibration curves. For internal standardization,  $\alpha$ -tocopheryl acetate is an appropriate IS as it is not found in peppers. It can, however, be found in fortified foodstuff (Lee et al., 1999). It is important to note that this IS must be added to samples after saponification. Otherwise it will be converted to  $\alpha$ -tocopherol.

The preparative isolation of vitamins for analytical or biological assays is not of high interest. As these compounds are commercially available and their structures are known, there is no necessity in purified samples. There was only one preparative protocol published in the last decade. Perucka and Materska (2003) separated tocopherols from the lipophilic fraction of a pepper extract by TLC on silica gel with petroleum ether-diethyl ether (9:1).

## CONCLUSION

When in 1932 Svent-Györgyi isolated ascorbic acid from pepper (Combs, 2008), it was considered to be a great scientific achievement. It took almost a year to elucidate the structure of the vitamin. Today a similar analysis is a routine procedure in laboratories all over the world and takes no more than one day. Current development of analytical instrumentation and methodology has achieved such a state that the determination of many groups of phytochemicals, at least all main nutrients, has become possible, is relatively fast and not exceptionally expensive. Complete profiling of bioactive principles of pepper can be accomplished by a team of a few researchers for several days. Required equipment includes GC/MS and HPLC. LC/MS is desirable but not mandatory. Instead, a combination of preparative chromatography and conventional techniques of the identification of organic substances can be used.

Despite the recent achievements, some aspects of the phytochemical analysis of pepper still require improvement. One of such problems is the quantification of conjugated phytochemicals (polyphenol glycosides, carotenoid esters, *etc.*) that is currently neither accurate nor convenient due to the lack of standards, published reference data, and reliable and commercially available peak identification algorithms. Another issue lacking attention of researchers is the enantiomeric composition of pepper phytochemicals. Many of those are chiral molecules and can exist in plants in different enantiomeric forms, as the pepper aroma compound linalool (Jiang and Kubota, 2004), or occur naturally in one enantiomeric form but experience

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racemization during post-harvest treatment (Friedman, 1999; Polak, 2011). Actual distribution of optical isomers in pepper tissues is not known for majority of such compounds. Therefore, the application of chiral analytical methods may give new interesting information about chemical composition and biochemistry of the genus *Capsicum*.

Finally, it is worth to mention that advanced analytical techniques such as CE, capillary liquid chromatography, and micellar electrokinetic chromatography proven to be useful in plant analysis (Tolstikov et al., 2003; Sun et al., 2005; Ganzena, 2008; Elder et al., 2010) still did not find their way into the studies of *Capsicums*. There have been just few reports published on CE analysis of bioactives extracted from peppers (Helmja et al., 2007; Liu et al., 2010). Much more work needs to be done on the study of matrix effects of pepper extracts in a new analytical environment and on development and validation of new analytical protocols until we enjoy advantages of these methods.

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## REFERENCES

# ACCEPTED MANUSCRIPT

- Ahuja, K. D. K., and Ball, M. J. (2006). Effects of daily ingestion of chilli on serum lipoprotein oxidation in adult men and women. *Brit. J. Nutr.* **96**: 239–242.
- Alonia, B., Peet, M., Pharr, M., and Karni, L. (2001). The effect of high temperature and high atmospheric CO<sub>2</sub> on carbohydrate changes in bell pepper (*Capsicum annuum*) pollen in relation to its germination. *Physiol. Plant.* **112**: 505–512.
- Antonious, G. F., Kochhar, T. S., Jarret, R. L., and Snyder, J. C. (2006). Antioxidants in hot pepper: variation among accessions. *J. Environ. Sci. Health.* **B41**: 1237–1243.
- Antonious, G. F., Meyer, J. E., Rogers, J. A., and Hu, Y.-H. (2007). Growing hot pepper for cabbage looper, *Trichoplusia ni* (Hübner) and spider mite, *Tetranychus urticae* (Koch) control. *J. Environ. Sci. Health.* **B42**: 559–567.
- AOAC Int. (1998). Total, soluble, and insoluble dietary fiber in foods. In: Official methods of analysis. 16th ed., 4th revision. Method 991.43. AOAC Int., Gaithersburg, MD.
- Arabbi, P. R., Genovese, M. I., and Lajolo, F. M. (2004). Flavonoids in vegetable foods commonly consumed in Brazil and estimated ingestion by the Brazilian population. *J. Agric. Food Chem.* **52**: 1124–1131.
- Arango, Y., and Heise, K.-P. (1998). Tocopherol synthesis from homogentisate in *Capsicum annuum* L. (yellow pepper) chromoplast membranes: evidence for tocopherol cyclase. *Biochem. J.* **336**: 531–533.

# ACCEPTED MANUSCRIPT

- Aruna, G., and Baskaran, V. (2010). Comparative study on the levels of carotenoids lutein, zeaxanthin and b-carotene in Indian spices of nutritional and medicinal importance. *Food Chem.* **123**: 404–409.
- Asami, D. K., Hong, Y.-J., Barrett, D. M., and Mitchell, A. E. (2003). Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. *J. Agric. Food Chem.* **51**: 1237–1241.
- Bahorun, T., Luximon-Ramma, A., Crozier, A., and Aruoma, O. I. (2004). Total phenol, flavonoid, proanthocyanidin and vitamin C levels and antioxidant activities of Mauritian vegetables. *J. Sci. Food Agric.* **84**: 1553–1561.
- Ball, G. F. M. (2006). Vitamins in foods. Analysis, bioavailability, and stability. CRC Press, Boca Raton, FL.
- Baranowski, R., Kabut, J., and Baranowska, I. (2004). Analysis of mixture of catechins, flavones, flavanones, flavonols, and anthocyanidins by RP-HPLC. *Anal. Lett.* **37**: 157–165.
- Barbero, G. F., Palma, M., and Barroso, C. G. (2006). Determination of capsaicinoids in peppers by microwave-assisted extraction–high-performance liquid chromatography with fluorescence detection. *Anal. Chim. Acta.* **578**: 227–233.
- Barbero, G. F., Palma, M., and Barroso, C. G. (2006a). Pressurized liquid extraction of capsaicinoids from peppers. *J. Agric. Food Chem.*, **54**: 3231–3236.

# ACCEPTED MANUSCRIPT

- Barbero, G. F., Liazid, A., Palma, M., and Barroso, C. G. (2008). Fast determination of capsaicinoids from peppers by high-performance liquid chromatography using a reversed phase monolithic column. *Food Chem.* **107**: 1276–1282.
- Basu, S. K., and De, A. K. (2003). *Capsicum*: historical and botanical perspectives. In: *Capsicum: the genus Capsicum*, pp. 1–15. De, A. K., Ed., Taylor & Francis, London.
- Bello-Perez, L. A., Rodriguez-Ambriz, S. L., Sanchez-Rivera, M. M., and Agama-Acevedo, E. (2010). Starch macromolecular structure. In: *Starches: Characterization, properties, and applications*, pp. 33–58. Bertolini, A. C., Ed., CRC Press, Boca Raton, FL.
- Bemiller, J. N., and Whistler, R. L. (1996). Carbohydrates. In: *Food chemistry*. 3rd ed., pp.157–223. Fennema, O. R., Ed., Marcel Dekker, New York.
- Bernardo, A., Martínez, S., Álvarez, M., Fernández, A., and López, M. (2008). The composition of two spanish pepper varieties (Fresno de la Vega and Benavente-Los Valles) in different ripening stages. *J. Food Quality*. **31**: 701–716.
- Bernhardt, S., and Schlich, E. (2006). Impact of different cooking methods on food quality: Retention of lipophilic vitamins in fresh and frozen vegetables. *J. Food Eng.* **77**: 327–333.
- Bertolini, A. C. (2010). Trends in starch applications. In: *Starches: characterization, properties, and applications*, pp. 1–19. Bertolini, A. C., Ed., CRC Press, Boca Raton, FL.

# ACCEPTED MANUSCRIPT

- Biliaderis, C. G. (1992). Structure and phase transitions of starch in food systems. *Food Technol.* **46**: 98–109.
- Blake, Ch. J. (2007). Status of methodology for the determination of fat-soluble vitamins in foods, dietary supplements, and vitamin premixes. *J. AOAC Int.* **90**: 897–910.
- Blum, E., Mazourek, M., O'Connell, M., Curry, J., Thorup, T., Liu, K., Jahn, M., and Paran, I. (2003). Molecular mapping of capsaicinoid biosynthesis genes and quantitative trait loci analysis for capsaicinoid content in *Capsicum*. *Theor. Appl. Genet.* **108**: 79–86.
- Breithaupt, D. E., and Schwack, W. (2000). Determination of free and bound carotenoids in paprika (*Capsicum annuum* L.) by LC/MS. *Eur. Food Res. Technol.* **211**: 52–55.
- Breithaupt, D. E., and Bamedi, A. (2001). Carotenoid esters in vegetables and fruits: a screening with emphasis on  $\beta$ -cryptoxanthin esters. *J. Agric. Food Chem.* **49**: 2064–2070.
- Brobst, K. M., and Lott, C. E. (1966). Determination of some components in corn sirup by gas-liquid chromatography of the trimethylsilyl derivatives. *Cereal Chem.* **43**: 35–43.
- Burns, J., Fraser, P. D., and Bramley, P. M. (2003). Identification and quantification of carotenoids, tocopherols and chlorophylls in commonly consumed fruits and vegetables. *Phytochemistry*. **62**: 939–947.

# ACCEPTED MANUSCRIPT

- Buttery, R. G., Seifert, R. M., Guadagni, D. G., and Ling, L. C. (1969). Characterization of some volatile constituents of bell peppers. *J. Agric. Food Chem.* **17**: 1322–1327.
- Cagno, R. D., Surico, R. F., Minervini, G., Angelis, M. D., Rizzello, C. G., and Gobbetti, M. (2009). Use of autochthonous starters to ferment red and yellow peppers (*Capsicum annum* L.) to be stored at room temperature. *Int. J. Food Microbiol.* **130**: 108–116.
- Cardeal, Z. L., Gomes da Silva, M. D. R., and Marriott, P. J. (2006). Comprehensive two-dimensional gas chromatography/mass spectrometric analysis of pepper volatiles. *Rapid Commun. Mass Spectrom.* **20**: 2823–2836.
- Carpita, N. C., and Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**: 1–30.
- Catchpole, O. J., Grey, J. B., Perry, N. B., Burgess, E. J., Redmond, W. A., and Porter, N. G. (2003). Extraction of chili, black pepper, and ginger with near-critical CO<sub>2</sub>, propane, and dimethyl ether: analysis of the extracts by quantitative nuclear magnetic resonance. *J. Agric. Food Chem.* **51**: 4853–4860.
- Chang, C.-C., Yang, M.-H., Wen, H.-M., and Chern, J.-C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **10**: 178–182.

# ACCEPTED MANUSCRIPT

- Chassy, A. W., Bui, L., Renaud, E. N. C., Van Horn, M., and Mitchell, A. E. (2006). Three-year comparison of the content of antioxidant microconstituents and several quality characteristics in organic and conventionally managed tomatoes and bell peppers. *J. Agric. Food Chem.* **54**: 8244–8252.
- Chin-Chen, M.-L., Carda-Broch, S., Bose, D., and Esteve-Romero, J. (2010). Direct injection and determination of the active principles of spices using micellar liquid chromatography. *Food Chem.* **120**: 915–920.
- Chmielowska, J., Veloso, J., Gutiérrez, J., Silvar, C., and Díaz, J. (2010). Cross-protection of pepper plants stressed by copper against a vascular pathogen is accompanied by the induction of a defence response. *Plant Sci.* **178**: 176–182.
- Choi, S.-H., Suh, B.-S., Kozukue, E., Kozukue, N., Levin, C. E., and Friedman, M. (2006). Analysis of the contents of pungent compounds in fresh Korean red peppers and in pepper-containing foods. *J. Agric. Food Chem.* **54**: 9024–9031.
- Chu, Y.-F., Sun, J., Wu, X., and Liu, R. H. (2002). Antioxidant and antiproliferative activities of common vegetables. *J. Agric. Food Chem.* **50**: 6910–6916.
- Combs, G. F. (2008). The Vitamins: Fundamental aspects in nutrition and health; 3rd ed., Elsevier, Amsterdam.
- Conforti, F. D., and Zinck, J. B. (2002). Hydrocolloid-lipid coating affect on weight loss, pectin content, and textural quality of green bell peppers. *J. Food Sci.* **67**: 1360–1363.

# ACCEPTED MANUSCRIPT

- Conforti, F., Statti, G. A., and Menichini, F. (2007). Chemical and biological variability of hot pepper fruits (*Capsicum annuum* var. *acuminatum* L.) in relation to maturity stage. *Food Chem.* **102**: 1096–1104.
- Contreras-Padilla, M., and Yahia, E. M. (1998). Changes in capsaicinoids during development, maturation, and senescence of chile peppers and relation with peroxidase activity. *J. Agric. Food Chem.* **46**: 2075–2079.
- da Costa, J. G., Pires, E. V., Riffel, A., Birkett, M. A., Bleicher, E., and Sant'Ana, A. E. G. (2011). Differential preference of *Capsicum* spp. cultivars by *Aphis gossypii* is conferred by variation in volatile semiochemistry. *Euphytica*. **177**: 299–307.
- Daood, H. G. (2009). Analytical and technological aspects on bioactive compounds in spice red pepper. *Acta Alimentaria*. **38(Suppl.)**: 87–97.
- Daood, H. G., Vinkler, M., Mirkus, F., Hebshi, E. A., and Biacs, P. A. (1996). Antioxidant vitamin content of spice red pepper (paprika) as affected by technological and varietal factors. *Food Chem.* **55**: 365–312.
- Daood, H. G., Kapitány, J., Biacs, P., and Albrecht, K. (2006). Drying temperature, endogenous antioxidants and capsaicinoids affect carotenoid stability in paprika (red pepper spice). *J. Sci. Food Agric.* **86**: 2450–2457.
- Davies, K. M., and Schwinn, K. E. (2006). Molecular biology and biotechnology of flavonoid biosynthesis. In: Flavonoids, pp. 143–218. Andersen, O. M., and Markham, K. R., Eds., CRC Press, Boca Raton, FL.

# ACCEPTED MANUSCRIPT

- Davis, Ch. B., Markey, C. E., Busch, M. A., and Busch, K. W. (2007). Determination of capsaicinoids in habanero peppers by chemometric analysis of UV spectral data. *J. Agric. Food Chem.* **55**: 5925–5933.
- de Azevedo-Meleiro, C. H., and Rodriguez-Amaya, D. B. (2009). Qualitative and quantitative differences in the carotenoid composition of yellow and red peppers determined by HPLC-DAD-MS. *J. Sep. Sci.* **32**: 3652–3658.
- de Jesús Ornelas-Paz, J., Martínez-Burrola, J. M., Ruiz-Cruz, S., Santana-Rodríguez, V., Ibarra-Junquera, V., Olivas, G. I., and Pérez-Martínez, J. D. (2010). Effect of cooking on the capsaicinoids and phenolics contents of Mexican peppers. *Food Chem.* **119**: 1619–1625.
- del Valle, J. M., Jiménez, M., Napolitano, P., Zetzl, C., and Brunner, G. (2003). Supercritical carbon dioxide extraction of pelletized Jalapeño peppers. *J. Sci. Food Agric.* **83**: 550-556.
- Deli, J., Molnár, P., Matus, Z., and Tóth, G. (2001). Carotenoid composition in the fruits of red paprika (*Capsicum annuum* var. *Lycopersiciforme rubrum*) during ripening; biosynthesis of carotenoids in red paprika. *J. Agric. Food Chem.* **49**: 1517–1523.
- Demir, İ., Tekin, A., Ökmen, Z. A., Okcu, G., and Kenanoğlu, B. B. (2008) Seed quality, and fatty acid and sugar contents of pepper seeds (*Capsicum annuum* L.) in relation to seed development and drying temperatures. *Turk. J. Agric. Forest.* **32**: 529–536.

# ACCEPTED MANUSCRIPT

- Diaz, J., Bernal, A., Merino, F., and Ros Barcelo, A. (1998). Phenolic metabolism in *Capsicum annuum* L. *Recent Res. Dev. Phytochem.* **2**: 155–169.
- Díaz-Reinoso, B., Moure, A. S., Domíngues, H., and Parajó, J. C. (2006). Supercritical CO<sub>2</sub> Extraction and Purification of Compounds with Antioxidant Activity. *J. Agric. Food Chem.* **54**: 2441–2469.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). Colourimetric methods for determination of sugars and related substances. *Anal. Chem.* **28**: 796–801.
- Elder, D. P., Snodin, D., and Teasdale, A. (2010). Analytical approaches for the detection of epoxides and hydroperoxides in active pharmaceutical ingredients, drug products and herbals. *J. Pharm. Biomed. Anal.* **51**: 1015–1023.
- Engel, W., Bahr, W., and Schieberle, P. (1999). Solvent assisted flavour evaporation - a new and versatile technique for the careful and direct isolation of aroma compounds from complex food matrices. *Eur. Food Res. Technol.* **209**: 237–241.
- Englyst, H. N., and Hudson, G. J. (1996). The classification and measurement of dietary carbohydrates. *Food Chem.* **57**: 15–21.
- Estrada, B., Bernal, M. A., Díaz, J., Pomar, F., and Merino, F. (2000). Fruit development in *Capsicum annuum*: Changes in capsaicin, lignin, free phenolics, and peroxidase patterns. *J. Agric. Food Chem.* **48**: 6234–6239.
- European Pharmacopea. Vol 1. (1983). Maissonue, Sainte Ruffine.

# ACCEPTED MANUSCRIPT

- Faustino, J. M. F., Barroca, M. J., and Guiné, R. P. F. (2007). Study of the drying kinetics of green bell pepper and chemical characterization. *Food Bioprod. Process.* **85**: 163–170.
- Filisetti-Cozzi, T. M. C. C., and Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Anal. Biochem.* **197**: 157–162.
- Flores, P., Castellar, I., Hellín, P., Fenoll, J., and Navarro, J. (2007). Response of pepper plants to different rates of mineral fertilizers after soil biofumigation and solarization. *J. Plant Nutr.* **30**: 367–379.
- Flores, P., Hellín, P., Lacasa, A., López, A., and Fenoll, J. (2009). Pepper antioxidant composition as affected by organic, low-input and soilless cultivation. *J. Sci. Food Agric.* **89**: 2267–2274.
- Flores, P., Hellín, P., and Fenoll, J. (2009a). Effect of manure and mineral fertilization on pepper nutritional quality. *J. Sci. Food Agric.* **89**: 1581–1586.
- Forero, M. D., Quijano, C. E., and Pino, J. A. (2009). Volatile compounds of chile pepper (*Capsicum annuum* L. var. *glabriusculum*) at two ripening stages. *Flavour Fragr. J.* **24**: 25–30.
- Fraser, P. D., Pinto, M. E. S., Holloway, D. E., and Bramley, P. M. (2000). Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *Plant J.* **24**: 551–558.
- Friedman, M. (1999). Chemistry, nutrition, and microbiology of D-amino acids. *J. Agric. Food Chem.* **47**: 3457–3479.

# ACCEPTED MANUSCRIPT

Frison, S., and Sporns, P. (2005). Identification of flavonol glycosides using MALDI-MS. In: Handbook of food analytical chemistry: pigments, colorants, flavors, texture, and bioactive food components, pp. 511–518. Wrolstad, R. E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, Ch. F., Smith, D., and Sporns, P., Eds., Wiley-Interscience, Hoboken.

Gallardo-Guerrero, L., Pérez-Gálvez, A., Aranda, E., Mínguez-Mosquera, M. I., and Hornero-Méndez, D. (2010). Physicochemical and microbiological characterization of the dehydration processing of red pepper fruits for paprika production. *LWT - Food Sci. Technol.* **43**: 1359–1367.

Galvez Ranilla, L., Kwon, Y.-I., Apostolidis, E., and Shetty, K. (2010). Phenolic compounds, antioxidant activity and in vitro inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. *Bioresource Technol.* **101**: 4676–4689.

Ganzena, M. (2008). Quality control of herbal medicines by capillary electrophoresis: potencial, requirements and applications. *Electrophoresis.* **29**: 3489–3503.

Garrido, J. L., Rodríguez, F., Campaña, E., and Zapata, M. (2003). Rapid separation of chlorophylls *a* and *b* and their demetallated and dephytylated derivatives using a monolithic silica C<sub>18</sub> column and a pyridine-containing mobile phase. *J. Chromatogr A.* **994**: 85–92.

# ACCEPTED MANUSCRIPT

- Garrido, G. L., and Zapata, M. (2006). Chlorophyll analysis by new high performance liquid chromatography methods. In: Chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications, pp. 109–121. Grimm, B., Porra, R. J., Rüdiger, W., and Scheer, H., Eds., Springer, Dordrecht.
- Ghoneim, A. A., Dawood, M. G., Riad, G. S., and El-Tohamy, W. A. (2009). Effect of nitrogen forms and biostimulants foliar application on the growth, yield and chemical composition of hot pepper grown under sandy soil conditions. *Res. J. Agric. Bio. Sci.* **5**: 840–852.
- Gokmen, V., Bahçeci, S., and Acar, J. (2002). Liquid chromatographic method for the determination of chlorophylls, carotenoids, and their derivatives in fresh and processed vegetables. *J. Liq. Chrom. Rel. Technol.* **25**: 1201–1213.
- Gorinstein, S., Park, Y.-S., Heo, B.-G., Namiesnik, J., Leontowicz, H., Leontowicz, M., Ham, K.-S., Cho, J.-Y., and Kang, S.-G. (2009). A comparative study of phenolic compounds and antioxidant and antiproliferative activities in frequently consumed raw vegetables. *Eur. Food Res. Technol.* **228**: 903–911.
- Gregory, J. F. (1996). Vitamins. In: Food Chemistry, 3rd ed., pp. 531–616. Fennema, O. R., Ed., Marcell Dekker, New York.
- Gu, Y. S., Howard, L. R., and Wagner, A. B. (1999). Firmness and cell wall characteristics of pasteurized Jalapeño pepper rings as affected by calcium chloride and rotary processing. *J. Food Sci.* **64**: 494–497.
- Guil-Guerrero, J. L., Martínez-Guirado, C., del Mar Rebolloso-Fuentes, M., and Carrique-Pérez, A. (2006). Nutrient composition and antioxidant activity of 10 pepper (*Capsicum annuum*) varieties. *Eur. Food Res. Technol.* **224**: 1–9.

# ACCEPTED MANUSCRIPT

- Guzman, I., Hamby, S., Romero, J., Bosland, P. W., and O'Connell, M. A. (2010). Variability of carotenoid biosynthesis in orange colored *Capsicum* spp. *Plant Sci.* **179**: 49–59.
- Ha, S.-H., Kim, J.-B., Park, J.-S., Lee, S.-W., and Cho, K.-J. (2007). A comparison of the carotenoid accumulation in *Capsicum* varieties that show different ripening colors: deletion of the capsanthin-capsorubin synthase gene is not a prerequisite for the formation of a yellow pepper. *J. Exper. Bot.* **58**: 3135–3144.
- Harborne, J. B., and Mabry, T. J. (1982). The flavonoids: advances in research. Vol. 1. 744 pp. Chapman and Hall, London–New York.
- Harborne, J. B., and Williams, Ch. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry*. **55**: 481–504.
- Harpster, M. H., Brummell, D. A., and Dunsmuir, P. (2002). Suppression of a ripening-related endo-1,4- $\beta$ -glucanase in transgenic pepper fruit does not prevent depolymerization of cell wall polysaccharides during ripening. *Plant Mol. Biol.* **50**: 345–355.
- Hart, D. J., and Scott, K. J. (1995). Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chem.* **54**: 101–111.
- He, X., Liu, D., and Liu, R. H. (2008). Sodium Borohydride/Chloranil-Based Assay for Quantifying Total Flavonoids. *J. Agric. Food Chem.* **56**: 9337–9344.

# ACCEPTED MANUSCRIPT

- Helmja, K., Vaher, M., Gorbatšova, J., and Kaljurand, M. (2007). Characterization of bioactive compounds contained in vegetables of the Solanaceae family by capillary electrophoresis. *Proc. Estonian Acad. Sci. Chem.* **56**: 172–186.
- Helyes, L., Tuba, Z., Balogh, J., and Réti, K. (2005). Production ecophysiology of hungarian green pepper under elevated air CO<sub>2</sub> concentration. *J. Crop Improv.* **13**: 333–344.
- Hervert-Hernández, D., Sáyago-Ayerdi, S. G., and Goñi, I. (2010). Bioactive compounds of four hot pepper varieties (*Capsicum annuum* L.), antioxidant capacity, and intestinal bioaccessibility. *J. Agric. Food Chem.* **58**: 3399–3406.
- Hewitt, B. R. (1958). Spectrophotometric determination of total carbohydrate. *Nature*. **182**: 246–247.
- Hornero-Méndez, D., and Minguez-Mosquera, M. I. (2000). Carotenoid biosynthesis changes in five red pepper (*Capsicum annuum* L.) cultivars during ripening. Cultivar selection for breeding. *J. Agric. Food Chem.* **48**: 1617–1622.
- Hornero-Méndez, D., and Minguez-Mosquera, M. I. (2000a). Xanthophyll esterification accompanying carotenoid overaccumulation in chromoplast of *capsicum annuum* ripening fruits is a constitutive process and useful for ripeness index. *J. Agric. Food Chem.* **48**: 3857–3864.
- Hornero-Méndez, D., and Mínguez-Mosquera, M. I. (2001). Rapid spectrophotometric determination of red and yellow isochromic carotenoid fractions in paprika and red pepper oleoresins. *J. Agric. Food Chem.* **49**: 3584–3588.

# ACCEPTED MANUSCRIPT

Hornero-Méndez, D., and Mínguez-Mosquera, M. I. (2002). Chlorophyll disappearance and chlorophyllase activity during ripening of *Capsicum annuum* L fruits. *J. Sci. Food Agric.* **82**: 1564–1570.

Hornero-Méndez, D., Costa-García, J., Mínguez-Mosquera, M. I. (2002a). Characterization of carotenoid high-producing *capsicum annuum* cultivars selected for paprika production. *J. Agric. Food Chem.* **50**: 5711–5716.

Howard, L. R., and Buescher, R. W. (1990). Cell wall characteristics and firmness of fresh pack cucumber pickles affected by pasteurization and calcium chloride. *J. Food Biochem.* **14**: 31–43.

Howard, L. R., Burma, P., and Wagner, A. B. (1997). Firmness and cell wall characteristics of pasteurized jalapeño pepper rings as affected by preheating and storage. *J. Food Sci.* **62**: 89–92.

Howard, L. R., Talcott, S. T., Brenes, C. H., and Villalon, B. (2000). Changes in phytochemical and antioxidant activity of selected pepper cultivars (*Capsicum* species) as influenced by maturity. *J. Agric. Food Chem.* **48**: 1713–1720.

Howard, L. R., and Wildman, R. E. C. (2007). Antioxidant vitamin and phytochemical content of fresh and processed pepper fruit (*Capsicum annuum*). In: Handbook of neutraceuticals and functional food. 2nd ed., pp.165–190. Wildman, R. E. C., Ed., CRC Press, Boca Raton, FL.

Iorizzi, M., Lanzotti, V., De Marino, S., Zollo, F., Blanco-Molina, M., Macho, A., and Muñoz, E. (2001). New glycosides from *Capsicum annuum* L. var. *acuminatum*. Isolation, structure determination, and biological activity. *J. Agric. Food Chem.* **49**: 2022–2029.

# ACCEPTED MANUSCRIPT

Isabelle, M., Lee, B. L., Lim, M. T., Koh, W.-P., Huang, D., and Ong, Ch. N. (2010). Antioxidant activity and profiles of common vegetables in Singapore. *Food Chem.* **120**: 993–1003.

Jang, H.-W., Ka, M.-H., and Lee, K.-G. (2008). Antioxidant activity and characterization of volatile extracts of *Capsicum annuum* L. and *Allium* spp. *Flavour Fragr. J.* **23**: 178–184.

Jiang, L., and Kubota, K. (2004). Differences in the volatile components and their odor characteristics of green and ripe fruits and dried pericarp of Japanese pepper (*Xanthoxylum Piperitum* DC.). *J. Agric. Food Chem.* **52**: 4197–4203.

Jin, R., Pan, J., Xie, H., Zhou, B., and Xia, X. (2009). Separation and quantitative analysis of capsaicinoids in chili peppers by reversed-phase. *Chromatographia*. **70**: 1011–1013.

Jing, H., and Amirav, A. (1997). Pesticide analysis with the pulsed-flame photometer detector and a direct sample introduction device. *Anal. Chem.* **69**: 1426–1435.

Jones, Y. M., and Albersheim, P. O. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharide. *Plant Physiol.* **49**: 926–936.

Kamilova, F., Kravchenko, L. V., Shaposhnikov, A. I., Azarova, T., Makarova, N., and Lugtenberg, B. (2006). Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. *Mol. Plant-Microbe Interact.* **19**: 250–256.

# ACCEPTED MANUSCRIPT

Kang, J. Y., Yeoh, K. G., Chia, H. P., Lee, H. P., Chia, Y. W., Guan, R. and Yap, I. (1995). Chili protective factor against peptic ulcer? *Dig. Dis. Sci.* **40**: 576–579.

Kennedy, J. A. (2005). Proanthcyanidins: extraction, purification, and determination of subunit composition by HPLC. In: *Handbook of food analytical chemistry: pigments, colorants, flavors, texture, and bioactive food components*, pp. 499–509. Wrolstad, R. E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, Ch. F., Smith, D., and Sporns, P., Eds., Wiley-Interscience, Hoboken.

Kevers, C., Falkowski, M., Tabart, J., Defraigne, J.-O., Dommes, J., and Pincemail, J. (2007). Evolution of antioxidant capacity during storage of selected fruits and vegetables. *J. Agric. Food Chem.* **55**: 8596–8603.

Kidmose, U., Yang, R.-Y., Thilsted, S. H., Christensen, L. P., and Brandt, K. (2006). Content of carotenoids in commonly consumed Asian vegetables and stability and extractability during frying. *J. Food Compos. Anal.* **19**: 562–571.

Kim, Y. S., Park, J. Y., Kim, K. S., Ko, M. K., Cheong, S. J., and Oh, B.-J. (2002). A thaumatin-like gene in nonclimacteric pepper fruits used as molecular marker in probing disease resistance, ripening, and sugar accumulation. *Plant Mol. Biol.* **49**: 125–135.

Kim, S., Park, J., and Hwang, I. K. (2004). Composition of main carotenoids in korean red pepper (*Capsicum annuum*, L.) and changes of pigment stability during the drying and storage process. *J. Food Sci.* **69**: FCT39–FCT44.

# ACCEPTED MANUSCRIPT

- Kim, D.-O., and Lee, Ch. Y. (2005). Extraction and isolation of polyphenolics. In: Handbook of food analytical chemistry: pigments, colorants, flavors, texture, and bioactive food components, pp. 471–482. Wrolstad, R. E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, Ch. F., Smith, D., and Sporns, P., Eds., Wiley-Interscience, Hoboken.
- Kim, I.-K., Abd El-Aty, A. M., Shin, H.-Ch., Lee, H. B., Kim, I.-S., and Shim, J.-H. (2007). Analysis of volatile compounds in fresh healthy and diseased peppers (*Capsicum annuum* L.) using solvent free solid injection coupled with gas chromatography-flame ionization detector and confirmation with mass spectrometry. *J. Pharm. Biomed. Anal.* **45**: 487–494.
- Kim, S., Ha, T. Y., and Park, J. (2008). Characteristics of pigment composition and color value by the difference of harvesting times in Korean red pepper varieties (*Capsicum annuum*, L.). *Int. J. Food Sci. Technol.* **43**: 915–920.
- Kim, K. W., Bae, R. N., and Lee, S. K. (2008a). Disinfestations of the Oriental tobacco budworm in green hot pepper by ultra high carbon dioxide: Implications for postharvest fruit quality. *J. Plant Biol.* **51**: 180–185.
- Kimura, M., Rodriguez-Amaya, D. B., and Godoy, H. T. (1990). Assessment of the saponification step in the quantitative determination of carotenoids and provitamins A. *Food Chem.* **35**: 187–195.
- Kintner, D. K., and Van Buren, J. P. (1982). Carbohydrate interference and its correction in pectin analysis using the *m*-hydroxydiphenyl method. *J. Food Sci.* **47**: 756–759.

# ACCEPTED MANUSCRIPT

- Kirschbaum-Titze, P., Hiepler, C., Mueller-Seitz, E., and Petz, M. (2002). Pungency in paprika (*Capsicum annuum*). 1. Decrease of capsaicinoid content following cellular disruption. *J. Agric. Food Chem.* **50**: 1260–1263.
- Kirschbaum-Titze, P., Hiepler, C., Mueller-Seitz, E., and Petz, M. (2002a). Pungency in paprika (*Capsicum annuum*). 2. Heterogeneity of capsaicinoid content in individual fruits from one plant. *J. Agric. Food Chem.* **50**: 1264–1266.
- Kobata, K., Todo, T., Yazawa, S., Iwai, K., and Watanabe, T. (1998). Novel capsaicinoid-like substances, capsiate and dihydrocapsiate, from the fruits of a nonpungent cultivar, CH-19 Sweet, of pepper (*Capsicum annuum* L.). *J. Agric. Food Chem.* **46**: 1695–1697.
- Kobata, K., Sutoh, K., Todo, T., Yazawa, S., Iwai, K., and Watanabe, T. (1999). Nordihydrocapsiate, a new capsinoid from the fruits of a nonpungent pepper, *Capsicum annuum*. *J. Nat. Prod.* **62**: 335–336.
- Kobata, K., Tate, H., Iwasaki, Yu., Tanaka, Y., Ohtsu, K., Yazawa, S., and Watanabe, T. (2008). Isolation of coniferyl esters from *Capsicum baccatum* L., and their enzymatic preparation and agonist activity for TRPV1. *Phytochemistry*. **69**: 1179–1184.
- Kobayashi, M., Akiyama, M., Kano, H., and Kise, H. (2006). Spectroscopy and structure determination. In: Chlorophylls and bacteriochlorophylls: Biochemistry, biophysics, functions and applications, pp. 79–94. Grimm, B., Porra, R. J., Rüdiger, W., and Scheer, H., Eds., Springer, Dordrecht.

# ACCEPTED MANUSCRIPT

- Koch, M., Arango, Y., Mock, H.-P., and Heise, K.-P. (2002). Factors influencing  $\alpha$ -tocopherol synthesis in pepper fruits. *J. Plant Physiol.* **159**: 1015–1019.
- Kollmannsberger, H., Rodríguez-Burrueto, A., Nizza, S., and Nuez, F. (2011). Volatile and capsaicinoid composition of ají (*Capsicum baccatum*) and rocoto (*Capsicum pubescens*), two Andean species of chile peppers. *J. Sci. Food Agric.* **91**: 1598–1611.
- Korel, F., Bağdatlioğlu, N., Balaban, M. Ö., and Hişil, Y. A. (2002). Ground red peppers: capsaicinoids content, scoville scores, and discrimination by an electronic nose. *J. Agric. Food Chem.* **50**: 3257–3261.
- Kozukue, N., Han, J.-S., Kozukue, E., Lee, S.-J., Kim, J.-A., Lee, K.-R., Levin, C. E., and Friedman, M. (2005). Analysis of eight capsaicinoids in peppers and pepper-containing foods by high-performance liquid chromatography and liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **53**: 9172–9181.
- Kräutler, B., and Hörtensteiner, S. (2006). Chlorophyll catabolites and the biochemistry of chlorophyll breakdown. In: Chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications, pp. 237–260. Grimm, B., Porra, R. J., Rüdiger, W., and Scheer, H., Eds., Springer, Dordrecht.
- Kurian, A. L., and Starks, A. N. (2002). HPLC analysis of capsaicinoids extracted from whole orange habañero chili peppers. *J. Food Sci.* **67**: 956–962.

# ACCEPTED MANUSCRIPT

Larsen, E., and Christensen, L. P. (2005). Simple saponification method for the quantitative determination of carotenoids in green vegetables. *J. Agric. Food Chem.* **53**: 6598–6602.

Laviada, I. (2006). Induction of apoptosis in prostate tumor PC-3 cells and inhibition of xenograft prostate tumor growth by the vanilloid capsaicin. *Apoptosis*. **11**: 89–99.

Lee, J., Suknark, K., Kluitse, Y., Phillips, R. D., and Eitenmiller, R. R. (1999). Rapid liquid chromatographic assay of vitamin E and retinyl palmitate in extruded weaning foods. *J. Food Sci.* **64**: 968–672.

Lee, B. L., Su, J., and Ong, C. N. (2004). Monomeric C18 chromatographic method for the liquid chromatographic determination of lipophilic antioxidants in plants. *J. Chromatogr. A*. **1048**: 263–267.

Lee, J. J., Crosby, K. M., Pike, L. M., Yoo, K. S., and Leskovar, D. I. (2005). Impact of genetic and environmental variation on development of flavonoids and carotenoids in pepper (*Capsicum* spp.). *Sci. Hortic.* **106**: 341–352.

Linssen, J. P. H., Janssens, J. L. G. M., Roozen, J. P. R., and Posthumus, M. A. (1993). Combined gas chromatography and sniffing port analysis of volatile compounds of mineral water packed in polyethylene laminated packages. *Food Chem.* **46**: 367–371.

Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.* **134**: 3479S–3485S.

# ACCEPTED MANUSCRIPT

- Liu, R., Xiong, K., Chao-Luo, Y., ze-Dai, X., min-Liu, Z., and tong-Xue, W. (2009). Changes in volatile compounds of a native Chinese chilli pepper (*Capsicum frutescens* var) during ripening. *Int. J. Food Sci. Technol.* **44**: 2470–2475.
- Liu, L., Chen, X., Liu, J., Deng, X., Duan, W., and Tan, S. (2010). Determination of capsaicin and dihydrocapsaicin in *Capsicum annuum* and related products by capillary electrophoresis with a mixed surfactant system. *Food Chem.* **119**: 1228–1232.
- Lizarazo, K., Fernández-Marín, B., Becerril, J. M., and García-Plazaola, J. I. (2010). Ageing and irradiance enhance vitamin E content in green edible tissues from crop plants. *J. Sci. Food. Agric.* **90**: 1994–1999.
- Loizzo, M. R., Tundis, R., Menichini, F., Statti, G. A., and Menichini, F. (2008). Influence of ripening stage on health benefits properties of *Capsicum annuum* Var. *acuminatum* L.: In vitro studies. *J. Med. Food.* **11**: 184–189.
- López-Hernández, J., Oruña-Concha, M. J., Simal-Lozano, J., Vázquez-Blanco, M. E., and González-Castro, M. J. (1996). Chemical composition of Padron peppers (*Capsicum annuum* L.) grown in Galicia (N.W. Spain). *Food Chem.* **51**: 557–559.
- Lu, J., and Cwik, M. (1997). Determination of capsaicin and zucapsaicin in human serum by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. B.* **701**: 135–139.

# ACCEPTED MANUSCRIPT

Luning, P. A., de Rijk, T., Wicher, H. J., and Roozen, J. P. (1994). Gas chromatography, mass spectrometry, and sniffing port analyses of volatile compounds of fresh bell peppers (*Capsicum annuum*) at different ripening stages. *J. Agric. Food Chem.* **42**: 977–983.

Maillard, M.-N., Giampaoli, P., and Richard, H. M. J. (1997). Analysis of eleven capsaicinoids by reversed-phase high performance liquid chromatography. *Flavour Fragr. J.* **12**: 409–413.

Mamatha, B. S., Prakash, M., Nagarajan, S., and Bhat, K. K. (2008). Evaluation of the flavor quality of pepper (*piper nigrum* L.) cultivars by gc-ms, electronic nose and sensory analysis techniques. *J. Sens. Stud.* **23**: 498–513.

Manirakiza, P., Covaci, A., and Schepens, P. (2003). Pungency principles in *Capsicum* - analytical determinations and toxicology. In: *Capsicum: the genus Capsicum*, pp. 71–86. De, A. K., Ed., Taylor & Francis, London.

Maoka, T., Mochida, K., Kozuka, M., Ito, Y., Fujiwara, Y., Hashimoto, K., Enjo, F., Ogata, M., Nobukuni, Y., Tokuda, H., and Nishino, H. (2001). Cancer chemopreventive activity of carotenoids in the fruits of red paprika *Capsicum annuum* L. *Cancer Lett.* **172**: 103–109.

Marín, A., Ferreres, F., Tomás-Barberá, F. A., and Gil, M. I. (2004). Characterization and quantitation of antioxidant constituents of sweet pepper (*Capsicum annuum* L.). *J. Agric. Food Chem.* **52**: 3861–3869.

Marín, A., Gil, M. I., Flores, P., Hellín, P., and Selma, M. V. (2008). Microbial Quality and Bioactive Constituents of Sweet Peppers from Sustainable Production Systems. *J. Agric. Food Chem.* **56**: 11334–11341.

# ACCEPTED MANUSCRIPT

Márkus, F., Daoood, H. G., Kapitány, J., and Biacs, P. A. (1999). Change in the carotenoid and antioxidant content of spice red pepper (paprika) as a function of ripening and some technological factors. *J. Agric. Food Chem.* **47**: 100–107.

Martínez, S., Curros, A., Bermúdez, J., Carballo, J., and Franco, I. (2007). The composition of Arnoia peppers (*Capsicum annuum* L.) at different stages of maturity. *Int. J. Food Sci. Nutr.* **58**: 150–161.

Martínez-Ballesta, M. C., Martínez, V., and Carvajal, M. (2004). Osmotic adjustment, water relations and gas exchange in pepper plants grown under NaCl or KCl. *Environ. Exper. Bot.* **52**: 161–174.

Materska, M., Piacente, S., Stochmal, A., Pizza, C., Oleszek, W., and Perucka, I. (2003). Isolation and structure elucidation of flavonoid and phenolic acid glycosides from pericarp of hot pepper fruit *Capsicum annuum* L. *Phytochemistry*. **63**: 893–898.

Materska, M., and Perucka, I. (2005). Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (*Capsicum annuum* L.). *J. Agric. Food Chem.* **53**: 1750–1756.

Matsufuji, H., Ishikawa, K., Nunomura, O., Chino, M., and Takeda, M. (2007). Anti-oxidant content of different colored sweet peppers, white, green, yellow, orange and red (*Capsicum annuum* L.). *Int. J. Food Sci. Technol.* **42**: 1482–1488.

Matsufuji, H., Furukawa, S., Teranishi, K., Kawaharada, K., Chino, M., Yamagata, K., Ogihara, H., and Yamasaki, M. (2009). Effects of nonthermal processes on the inactivation of microorganisms and antioxidants in minimally processed vegetables. *Food Sci. Technol. Res.* **15**: 153–162.

# ACCEPTED MANUSCRIPT

- Mazida, M. M., Salleh, M. M., and Osman. H. (2005). Analysis of volatile aroma compounds of fresh chilli (*Capsicum annuum*) during stages of maturity using solid phase microextraction (SPME). *J. Food Compos. Anal.* **18**: 427–437.
- McCleary, B. V., DeVries, J. W., Rader, J. I., Cohen, G., Prosky, L., Mugford, D. C., Champ, M., and Okuma, K. (2010). Determination of total dietary fiber (CODEX Definition) by enzymatic-gravimetric method and liquid chromatography: collaborative study. *J. AOAC Int.* **93**: 221–233.
- McLafferty, F. (2009). Wiley Registry of Mass Spectral Data, 9th Edition Upgrade. Wiley.
- Melton, L. D., and Smith, B. G. (2005). Cell wall polysaccharides. In: Handbook of Food Analytical Chemistry: water, proteins, enzymes, lipids, and carbohydrates, pp. 695–744. Wrolstad, R.E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, Ch. F., Smith, D., and Sporns, P., Eds., Wiley, Hoboken.
- Menichini, F., Tundis, R., Bonesi, M., Loizzo, M. R., Conforti, F., Statti, G., De Cindio, B., Houghton, P. J., and Menichini, F. (2009). The influence of fruit ripening on the phytochemical content and biological activity of *Capsicum chinense* Jacq. cv Habanero. *Food Chem.* **114**: 553–560.
- Miean, K. H., and Mohamed, S. (2001). Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *J. Agric. Food Chem.* **49**: 3106–3112.
- Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31**: 426–428.

# ACCEPTED MANUSCRIPT

Mínguez-Mosquera, M. I., and Hornero-Méndez, D. (1993). Peppers (*Capsicum annuum* L.), paprika, and oleoresin by reversed-phase HPLC. *J. Agric. Food Chem.* **41**: 1616–1620.

Mínguez-Mosquera, M. I., Gallardo-Guerrero, L., and Gandul-Rojas, B. (1993). Characterization and separation of oxidized derivatives of pheophorbide *a* and *b* by thin-layer and high-performance liquid chromatography. *J. Chromatogr.* **633**: 295–299.

Mínguez-Mosquera, M. I., Pérez-Gálvez, A., and Garrido-Fernández, J. (2000). Carotenoid content of the varieties *Jaranda* and *Jariza* (*Capsicum annuum* L.) and response during the industrial slow drying and grinding steps in paprika processing. *J. Agric. Food Chem.* **48**: 2972–2976.

Monforte-González, M., Medina-Lara, F., Gutiérrez-Carbajal, G., and Vázquez-Flota, F. (2007). Capsaicinoid quantitation by in situ densitometry of thin layer chromatography plates. *J. Liq. Chrom. Rel. Technol.* **30**: 1697–1704.

Monforte-González, M., Guzmán-Antonio, A., Uuh-Chim, F., and Vázquez-Flota, F. (2010). Capsaicin accumulation is related to nitrate content in placentas of habanero peppers (*Capsicum chinense* Jacq.). *J. Sci. Food Agric.* **90**: 764–768

Moreno, D. A., Víllora, G., Ruiz, J. M., Olivares, J., López-Lefebre, L., Hernández, J., and Romero, L. (2000). The Capsicum (*Capsicum annuum* L. cv. lamuyo) foliar content of carbohydrates and pigments: Response to NK dosage. *Commun. Soil Sci. Plant Anal.* **31**: 2335–2343.

# ACCEPTED MANUSCRIPT

- Munné-Bosch, S., and Alegre, L. (2002). Interplay between ascorbic acid and lipophilic antioxidant defences in chloroplasts of water-stressed *Arabidopsis* plants. *FEBS Lett.* **524**: 145–148.
- Naef, R., Velluz, A., and Jaquier, A. (2008). New volatile sulfur-containing constituents in a simultaneous distillation-extraction extract of red bell peppers (*Capsicum annuum*). *J. Agric. Food Chem.* **56**: 517–527.
- Navarro, J. M., Flores, P., Garrido, C., and Martinez, V. (2006). Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. *Food Chem.* **96**: 66–73.
- Nazzaro, F., Caliendo, G., Arnesi, G., Veronesi, A., Sarzi, P., and Fratianni, F. (2009). Comparative content of some bioactive compounds in two varieties of *Capsicum annuum* L. sweet pepper and evaluation of their antimicrobial and mutagenic activities. *J. Food Biochem.* **33**: 852–868.
- Nickerson, G. B., and Likens, S. T. (1966) Gas chromatographic evidence for the occurrence of hop oil components in beer. *J. Chromatogr.* **21**: 1–5.
- Nielsen, T. H., Skjærbæk, H. C., and Karlsen, P. (1991). Carbohydrate metabolism during fruit development in sweet pepper (*Capsicum annuum*) plants. *Phisiol. Plant.* **82**: 311–319.
- NIST (National Institute of Standards and Technology). (2011). NIST 11 Mass Spectral Library (NIST2011/EPA/NIH).

# ACCEPTED MANUSCRIPT

Oboh, G., and Rocha, J. B. T. (2007). Distribution and antioxidant activity of polyphenols in ripe and unripe tree pepper (*Capsicum pubescens*). *J. Food Biochem.* **31**: 456–473.

Oboh, G., and Rocha, J. B. T. (2007a). Polyphenols in red pepper [*Capsicum annuum* var. *aviculare* (Tepin)] and their protective effect on some pro-oxidants induced lipid peroxidation in brain and liver. *Eur. Food Res. Technol.* **225**: 239–247.

Ohnuki, K., Haramizu, S., Oki, K., Watanabe, T., Yazawa, S., and Fushiki, T. (2001). Administration of capsiate, a non-pungent capsaicin analog, promotes energy metabolism and suppresses body fat accumulation in mice. *Biosci. Biotechnol. Biochem.* **65**: 2735–2740.

Paik, S.-Y., Ra, K. S., Chang, I. S., Park, Y. C., Park, H. S., Baik, H. S., Yun, J. W., and Choi, J. W. (2003). Purification and characterization of complement-activating acidic polysaccharides from the fruits of *Capsicum annum*. *J. Biochem. Mol. Biol.* **36**: 230–236.

Pegard, A., Brizard, G., Fazari, A., Soucaze, O., Abad, P., and Djian-Caporalino, C. (2005). Histological characterization of resistance to different root-knot nematode species related to phenolics accumulation in *Capsicum annum*. *Phytopathol.* **95**: 158–165.

# ACCEPTED MANUSCRIPT

- Peña-Alvarez, A., Ramírez-Maya, E., and Alvarado-Suárez, L. A. (2009). Analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase microextraction–gas chromatography–mass spectrometry. *J. Chromatogr. A.* **1216**: 2843–2847.
- Pérez-López, A. J., López-Nicolas, J. M., Núñez-Delicado, E., Del Amor, F. M., and Carbonell-Barrachina, Á. A. (2007). Effects of Agricultural Practices on color, carotenoids composition, and minerals contents of sweet peppers, cv. Almuden. *J. Agric. Food Chem.* **55**: 8158–8164.
- Pérez-López, A. J., del Amor, F. M., Serrano-Martínez, A., Fortea, M. I., and Núñez-Delicado, E. (2007a). Influence of agricultural practices on the quality of sweet pepper fruits as affected by the maturity stage. *J. Sci. Food Agric.* **87**: 2075–2080.
- Perkins, H. J., and Roberts, D. W. A. (1962). Purification of chlorophylls, pheophytins, and pheophorbides for specific activity determinations. *Biochim. Biophys. Acta.* **58**: 486–498.
- Perucka, I., and Materska, M. (2003). Antioxidant activity and content of capsaicinoids isolated from paprika fruits. *Pol. J. Food Nutr. Sci.* **12/53**: 15–18.
- Perucka, I., and Oleszek, W. (2000). Extraction and determination of capsaicinoids in fruit of hot pepper *Capsicum annuum* L. by spectrophotometry and high-performance liquid chromatography. *Food Chem.* **71**: 287–291.

# ACCEPTED MANUSCRIPT

- Phillips, K. M., Ruggio, D. M., Ashraf-Khorassani, M., and Haytowitz, D. B. (2006). Difference in folate content of green and red sweet peppers (*Capsicum annuum*) Determined by liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **54**: 9998–10002.
- Picó, Y., Font, G., Ruiz, M. J., and Fernández, M. (2006). Control of pesticide residues by liquid chromatography-mass spectrometry to ensure food safety. *Mass Spectrom. Rev.* **25**: 917–960.
- Pino, J., Sauri-Duch, E., and Marbot, R. (2006). Changes in volatile compounds of Habanero chile pepper (*Capsicum chinense* Jack. cv. Habanero) at two ripening stages. *Food Chem.* **94**: 394–398.
- Pino, J., Fuentes, V., and Barrios O. (2011). Volatile constituents of Cachucha peppers (*Capsicum chinense* Jacq.) grown in Cuba. *Food Chem.* **125**: 860–864.
- Polak, B. (2011). Confirmation of chirality of some natural products by the HPLC method. In: High performance liquid chromatography in phytochemical analysis, pp. 373–396. Waksmanzka-Hajnos, M., Sherma, J., Eds., CRC Press, Boca Raton, FL.
- Pomar, F., Bernal, M. A., Díaz, J., and Merino, F. (1997). Purification, characterization and kinetic properties of pepper fruit acidic peroxidase. *Phytochemistry*. **46**: 1313–1317.

# ACCEPTED MANUSCRIPT

- Pomar, F., Novo, M., Bernal, M. A., Merino, F., and Ros Barceló, A. (2004). Changes in stem lignins (monomer composition and crosslinking) and peroxidase are related with the maintenance of leaf photosynthetic integrity during *Verticillium* wilt in *Capsicum annuum*. *New Phytol.* **163**: 111–123.
- Popov, S. V., Ovodova, R. G., Golovchenko, V. V., Popova, G. Yu., Viatyasev, F. V., Shashkov, A. S., and Ovodov, Yu. S. (2011). Chemical composition and anti-inflammatory activity of a pectic polysaccharide isolated from sweet pepper using a simulated gastric media. *Food Chem.* **124**: 309–315.
- Porra, R. J. (2006). Spectrometric assays for plant, algal and bacterial chlorophylls. In: Chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications, pp. 96–107. Grimm, B., Porra, R. J., Rüdiger, W., and Scheer, H., Eds., Springer, Dordrecht.
- Pourmortazavi, S. M., and Hajimirsadeghi, S. S. (2007). Supercritical fluid extraction in plant essential and volatile oil analysis. *J. Chromatogr. A.* **1163**: 2–24.
- Priya Sethu, K. M., Prabha, T. N., and Tharanath, R. N. (1996). Post-harvest biochemical changes associated with the softening phenomenon in *Capsicum annuum* fruits. *Phytochemistry*. **42**: 961–966.
- Pruthi, J. S. (2003). Advances in post-harvest processing technologies of *Capsicum*. In: Capsicum: the genus *Capsicum*, pp. 175–213. De, A. K., Eds., Taylor & Francis, London.

# ACCEPTED MANUSCRIPT

- Pruthi, J. S. (2003a). Chemistry and quality control of *Capsicums* and *Capsicum* products. In: *Capsicum: the genus Capsicum*, pp. 25–71. De, A. K., Ed., Taylor & Francis, London.
- Quach, H. T., Steeper, R. L., and Griffin, G. W. (2004). An improved method for the extraction and thin-layer chromatography of chlorophyll a and b from spinach. *J. Chem. Educ.* **81**: 385–387.
- Raffo, A., Baiamonte, I., Nardo, N., and Paoletti, F. (2007). Internal quality and antioxidants content of cold-stored red sweet peppers as affected by polyethylene bag packaging and hot water treatment. *Eur. Food Res. Technol.* **225**: 395–405.
- Raffo, A., Baiamonte, I., and Paoletti, F. (2008). Changes in antioxidants and taste-related compounds content during cold storage of fresh-cut red sweet peppers. *Eur. Food Res. Technol.* **226**: 1167–1174.
- Ramirez-Victoria, P., Guzman-Rincon, J., Espinosa-Aguirre, J. J., and Murillo-Romero, S. (2001). Antimutagenic effect of one variety of green pepper (*Capsicum* spp.) and its possible interference with the nitrosation process. *Mutation Res.* **496**: 39–45.
- Raugel, P.-J. (1999). Rapid food analysis and hygiene monitoring: kits, instruments, and systems. Springer, Berlin.
- Ravishankar, G.A., Suresh, B., Girindhar, P., Ramachandra Rao, S., and Sudhakar Johnson, T. (2003). Biotechnological studies on *Capsicum* for metabolite production and plant improvement. In: *Capsicum: the genus Capsicum*, pp. 96–128. De, A. K., Eds., Taylor & Francis, London.

# ACCEPTED MANUSCRIPT

- Reilly, Ch. A., Crouch, D. J., and Yost, G. S. (2001). Quantitative analysis of capsaicinoids in fresh peppers, oleoresincapsicum and pepper spray products. *J. Forensic Sci.* **46**: 502–509.
- Ren, H., Endo, H., and Hayashi, T. (2001). Antioxidative and antimutagenic activities and polyphenol content of pesticide-free and organically cultivated green vegetables using water-soluble chitosan as a soil modifier and leaf surface spray. *J. Sci. food Agric.* **81**: 1426–1432.
- Rios, J. J., Fernández-García, E., Mínguez-Mosquera, M. I., and Pérez-Gálvez, A. (2008) Description of volatile compounds generated by the degradation of carotenoids in paprika, tomato and marigold oleoresins. *Food Chem.* **106**: 1145–1153.
- Roca, M., Hornero-Méndez, D., Gandul-Rojas, B., and Mínguez-Mosquera, M. I. (2006). Stay-green phenotype slows the carotenogenic process in *Capsicum annuum* (L.) fruits. *J. Agric. Food Chem.* **54**: 8782–8787.
- Rodríguez-Burrueto, A., Kollmannsberger, H., González-Mas, M. C., Nitz, S., and Nuez, F. (2010). HS-SPME comparative analysis of genotypic diversity in the volatile fraction and aroma-contributing compounds of *Capsicum* fruits from the *annuum-chinense-frutescens* complex. *J. Agric. Food Chem.* **58**: 4388–4400.
- Rüdiger, W., and Grimm, B. (2006). Chlorophyll metabolism, an overview. In: Chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications, pp. 134–146. Grimm, B., Porra, R. J., Rüdiger, W., and Scheer, H., Eds., Springer, Dordrecht.

# ACCEPTED MANUSCRIPT

- Russo, V. M., and Howard, L. R. (2002). Carotenoids in pungent and non-pungent peppers at various developmental stages grown in the field and glasshouse. *J. Sci. Food Agric.* **82**: 615–624.
- Saha, S., Hedau, N. K., Kumar, S., Mahajan, V., and Gupta, H. S. (2010). Variability in hot pepper for phytochemicals offers promising tools in plant-breeding programmes. *Acta Agric. Scand. B.* **60**: 227–234.
- Sakakibara, H., Honda, Y., Nakagawa, S., Ashida, H., and Kanazawa, K. (2003). Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *J. Agric. Food Chem.* **51**: 571–581.
- Sancho, R., Lucena, C., Macho, A., Calzado, M.A., Blanco-Molina, M., Minassi, A., Appendino, G., and Muños, E. (2002). Immunosuppressive activity of capsaicinoids: capsiate derived from sweet peppers inhibits NF- $\kappa$ B activation and is a potent anti-inflammatory compound in vivo. *Eur. J. Immunol.* **32**: 1753–1763.
- Sasahara, I., Furunata, Y., Iwasaki, Y., Inoue, N., Sato, H., Watanabe, T., and Takanashi, M. (2010). Assessment of the biological similarity of three capsaicin analogs (capsinoids) found in non-pungent chili pepper (CH-19 sweet) fruits. *Biosci. Biotechnol. Biochem.* **74**: 274–278.
- Schweiggert, U., Kammerer, D. R., Carle, R., and Schieber, A. (2005). Characterization of carotenoids and carotenoid esters in red pepper pods (*Capsicum annuum* L.) by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**: 2617–2628.

# ACCEPTED MANUSCRIPT

Schweiggert, U., Kurz, C., Schieber, A., and Carle, R. (2007). Effects of processing and storage on the stability of free and esterified carotenoids of red peppers (*Capsicum annuum* L.) and hot chilli peppers (*Capsicum frutescens* L.). *Eur. Food Res. Technol.* **225**: 261–270.

Scoville, W. L. (1912). Note on capsicums. *J. Amer. Pharm. Assoc.* **1**: 453–454.

Seppanen, C. M., Rahmani, M., and Csallany, A. S. (2003). Simultaneous determination of chlorophylls, pheophytins,  $\beta$ -carotene, tocopherols, and tocotrienols in olive and soybean oils by high-performance liquid chromatography. *J. Food Sci.* **68**: 1644–1647.

Serrano, M., Zapata, P. J., Castillo, S., Guillén, F., Martínez-Romero, D., and Valero, D. (2010). Antioxidant and nutritive constituents during sweet pepper development and ripening are enhanced by nitrophenolate treatments. *Food Chem.* **118**: 497–503.

Sgroppi, S. C., and Pereyra, M. V. (2009). Using mild heat treatment to improve the bioactive related compounds on fresh-cut green bell peppers. *Int. J. Food Sci. Technol.* **44**: 1793–1801.

Shaked, R., Rosenfeld, K., and Pressman, E. (2004). The effect of low night temperatures on carbohydrates metabolism in developing pollen grains of pepper in relation to their number and functioning. *Sci. Hortic.* **102**: 29–36.

# ACCEPTED MANUSCRIPT

- Sheer, H. (2006). An overview of chlorophylls and bacteriochlorophylls: biochemistry, biophysics, Functions and applications. In: Chlorophylls and bacteriochlorophylls: biochemistry, biophysics, functions and applications, pp. 1–26. Grimm, B., Porra, R. J., Rüdiger, W., and Scheer, H., Eds., Springer, Dordrecht.
- Shetty, K. (2004). Role of proline-linked pentose phosphate pathway in biosynthesis of plant phenolics for functional food and environmental applications: a review. *Process Biochem.* **39**: 789–803.
- Shim, J. H., Lee, Y. S., Kim, M. R., Lee, C. J., and Kim, I. S. (2003). Use of the Keele injector for sample introduction for gas chromatographic analysis of vinclozolin in lettuces. *J. Chromatogr. A.* **1015**: 233–237.
- Shioi, Y. (2006). Large scale chlorophyll preparations using simple open-column chromatographic methods. In: Chlorophylls and bacteriochlorophylls: Biochemistry, biophysics, functions and applications, pp. 123–131. Grimm, B., Porra, R. J., Rüdiger, W., and Scheer, H., Eds., Springer, Dordrecht.
- Sim, K. H., and Sil, H. Y. (2008). Antioxidant activities of red pepper (*Capsicum annuum*) pericarp and seed extracts. *Int. J. Food Sci. Technol.* **43**: 1813–1823.
- Simkin, A. J., Zhu, Ch., Kuntz, M., and Sandmann, G. (2003). Light-dark regulation of carotenoid biosynthesis in pepper (*Capsicum annuum*) leaves. *J. Plant Physiol.* **160**: 439–443.
- Singh, S., Jarret, R., Russo, V., Majetich, G., Shimkus, J., Bushway, R., and Perkins, B. (2009). Determination of capsinoids by HPLC-DAD in *Capsicum* species. *J. Agric. Food Chem.* **57**: 3452–3457.

# ACCEPTED MANUSCRIPT

- Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolibdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **16**: 144–158.
- Smirnoff, N. (1996). The function and metabolism of ascorbic acid in plants. *Ann. Bot.* **78**: 661–669.
- Smirnoff, N. (2000). Ascorbic acid: metabolism and functions of a multi-faceted molecule. *Curr. Opin. Plant Biol.* **3**: 229–235.
- Smirnoff, N., Conklin, P. L., and Loewus, F. A. (2001). Biosynthesis of ascorbic acid in plants: A renaissance. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* **52**: 437–467.
- Soler, C., and Picó, Y. (2007). Recent trends in liquid chromatography-tandem mass spectrometry to determine pesticides and their metabolites in food. *Trends Anal. Chem.* **26**: 103–115.
- Sousa, E. T., de M. Rodrigues, F., Martins, C. C., de Oliveira, F. S., de P. Pereira, P. A., and de Andrade, J. B. (2006). Multivariate optimization and HS-SPME/GC-MS analysis of VOCs in red, yellow and purple varieties of *Capsicum chinense* sp. peppers. *Microchem. J.* **82**: 142–149.
- Srinivasa, P. C., Prashanth, K. V. H., Susheelamma, N. S., Ravi, R., and Tharanathan, R. N. (2006). Storage studies of tomato and bell pepper using eco-friendly films. *J. Sci. Food Agric.* **86**: 1216–1224.

# ACCEPTED MANUSCRIPT

- Stochmal, A., Piacente, S., Pizza, C., De Riccardis, F., Leitz, R., and Oleszek, W. (2001). Alfalfa (*Medicago sativa* L.) flavonoids. 1. Apigenin and luteolin glycosides from aerial parts. *J. Agric. Food Chem.* **49**: 753–758.
- Sukrasno, N., and Yeoman, M. M. (1993). Phenylpropanoid metabolism during growth and development of *Capsicum frutescens* fruits. *Phytochemistry*. **32**: 839–844.
- Sultan, J., and Gabryelski, W. (2006). Structural identification of highly polar nontarget contaminants in drinking water by ESI-FAIMS-Q-TOF-MS. *Anal. Chem.* **78**: 2905–2917.
- Sun, X., Yang, X., and Wang, E. (2005). Chromatographic and electrophoretic procedures for analyzing plant pigments of pharmacologically interests. *Anal. Chim. Acta*. **547**: 153–157.
- Sun, T., Xu, Z., Wu, C.-T., Janes, M., Prinyawiwatkul, W., and No, H. K. (2007). Antioxidant activities of different colored sweet bell peppers (*Capsicum annuum* L.). *J. Food Sci.* **72**: S98–S102.
- Sutoh, K., Kobata, K., and Watanabe, T. (2001). Stability of capsinoid in various solvents. *J. Agric. Food Chem.* **49**: 4026–4030.
- Sutoh, K., Kobata, K., Yazawa, S., and Watanabe, T. (2006). Capsinoid is biosynthesized from phenylalanine and valine in a non-pungent pepper, *Capsicum annuum* L. cv. CH-19 Sweet. *Biosci. Biotechnol. Biochem.* **70**: 1513–1516.

# ACCEPTED MANUSCRIPT

- Suzuki, Y., and Shioi, Y. (2003). Identification of chlorophylls and carotenoids in major teas by high-performance liquid chromatography with photodiode array detection. *J. Agric. Food Chem.* **51**: 5307–5314.
- Szolcsányi, J. (2003). Future perspectives of capsaicin research. In: *Capsicum: the genus capsicum*, pp. 248–269. De, A. K., Ed., Taylor & Francis, London.
- Tanaka, Y., Hosokawa, M., Otsu, K., Watanabe, T., and Yazawa, S. (2009). Assessment of capsinoid composition, nonpungent capsaicinoid analogues, in *Capsicum* cultivars. *J. Agric. Food Chem.* **57**: 5407–5412.
- Tanaka, Y., Hosokawa, M., Miwa, T., Watanabe, T., and Yazawa, S. (2010). Newly mutated putative-aminotransferase in nonpungent pepper (*Capsicum annuum*) results in biosynthesis of capsinoids, capsaicinoid analogues. *J. Agric. Food Chem.* **58**: 1761–1767.
- Thampi, P. S. S. (2003). A glimpse of the world trade in Capsicum. In: *Capsicum: the genus Capsicum*, pp. 16–24. De, A. K., Ed., Taylor & Francis, London.
- Thiele, R., Mueller-Seitz, E., and Petz, M. (2008). Chili pepper fruits: presumed precursors of fatty acids characteristic for capsaicinoids. *J. Agric. Food Chem.* **56**: 4219–4224.
- Thomas, B. V., Schreiber, A. A., and Weisskopf, C. P. (1998). Simple method for quantitation of capsaicinoids in peppers using capillary gas chromatography. *J. Agric. Food Chem.* **46**: 2655–2663.

# ACCEPTED MANUSCRIPT

- Thompson, R. Q., Phinney, K. W., Sander, L. C., and Welch, M. J. (2005). Reversed-phase liquid chromatography and argentation chromatography of the minor capsaicinoids. *Anal. Bioanal. Chem.* **381**: 1432–1440.
- Thurman, E. M., Ferrer, I., and Fernández-Alba, A. R. (2005). Matching unknown empirical formulas to chemical structure using LC/MS TOF accurate mass and database searching: example of unknown pesticides on tomato skins. *J. Chromatogr. A.* **1067**: 127–134.
- Tolstikov, V. V., Lommen, A., Nakanishi, K., Tanaka, N., and Fiehn, O. (2003). Monolithic silica-based capillary reversed-phase liquid chromatography/electrospray mass spectrometry for plant metabolomics. *Anal. Chem.* **75**: 6737–6740.
- Topuz, A., and Ozdemir, F. (2003). Influences of  $\gamma$ -irradiation and storage on the carotenoids of sun-dried and dehydrated paprika. *J. Agric. Food Chem.* **51**: 4972–4977.
- Topuz, A., and Ozdemir, F. (2007). Assessment of carotenoids, capsaicinoids and ascorbic acid composition of some selected pepper cultivars (*Capsicum annuum* L.) grown in Turkey. *J. Food Compos. Anal.* **20**: 596–602.
- Tsumura, F., Ohsako, Y., Haraguchi, Y., Kumagai, H., Sakurai, H., and Ishii, K. (1993). Rapid enzymatic assay for ascorbic acid in various foods using peroxidase. *J. Food Sci.* **58**: 619–622.
- Tswett, M. (1906). Adsorptionsanalyse und chromatographische Methode. Anwendung auf die Chemic des Chlorophylls. *Ber. Dtsch. Botan. Ges.* **24**: 384–393.

# ACCEPTED MANUSCRIPT

- Uquiche, E., del Valle, J. M., and Ortiz, J. (2004). Supercritical carbon dioxide extraction of red pepper (*Capsicum annuum* L.) oleoresin. *J. Food Eng.* **65**: 55–66.
- Vahteristo, L., Lehikoinen, K., Ollilainen, V., and Varo, P. (1997). Application of an HPLC assay for the determination of folate derivatives in some vegetables, fruits and berries consumed in Finland. *Food Chem.* **59**: 589–597.
- Valverde, J., This, H., and Vignolle, M. (2007). Quantitative determination of photosynthetic pigments in green beans using thin-layer chromatography and a flatbed scanner as densitometer. *J. Chem. Educ.* **84**: 1505–1507.
- van Ruth, S. M. (2001). Methods for gas chromatography-olfactometry: a review. *Biomol. Eng.* **17**: 121–128.
- van Ruth, S. M., Roozen, J. P., and Coijnsen, J. L. (1995). Volatile compounds of rehydrated French beans, bell peppers and leeks. Part 1. Flavour release in the mouth and in three mouth model systems. *Food Chem.* **53**: 15–22.
- Vasanthan, Th., Hoover, R., and Ratnayake, W. S. (2005). Starch and Starch Derivatives. In: Handbook of Food Analytical Chemistry: water, proteins, enzymes, lipids, and carbohydrates, pp. 671–693. Wrolstad, R.E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, Ch. F., Smith, D., and Sporns, P., Eds., Wiley, Hoboken.
- Vilasoa-Martínez, M., Calaza-Ramos, C., López-Hernández, J., Lage-Yusty, M. A., Losada, P. P., and Rodríguez-Bernaldo de Quirós, A. (2008). Determination of vitamin E and carotenoid pigments by high performance liquid chromatography in shell of *Chionoecetes opilio*. *Anal. Chim. Acta*. **617**: 225–229.

# ACCEPTED MANUSCRIPT

- Villanueva-Suárez, M. J., Redondo-Cuenca, A., Rodríguez-Sevilla, M. D., and De Las Heras Martínez, M. (2003). Characterization of nonstarch polysaccharides content from different edible organs of some vegetables, determined by GC and HPLC: comparative study. *J. Agric. Food Chem.* **51**: 5950–5955.
- von Roepenack-Lahaye, E., Degenkolb, T., Zerjeski, M., Franz, M., Roth, U., Wessjohann, L., Smidt, J., Scheel, D., and Clemens, S. (2004). Profiling of arabidopsis secondary metabolites by capillary liquid chromatography coupled to electrospray ionization quadrupole time-of-flight mass spectrometry. *Plant Physiol.* **134**: 548–559.
- Voutilainen, S., Nurmi, T., Mursu, J., and Rissanen, T. H. (2006). Carotenoids and cardiovascular health. *Am. J. Clin. Nutr.* **83**: 1265–1271.
- Wahyuni, Y., Ballester, A.-R., Sudarmonowati, E., Bino, R. J., and Bovy, A. G. (2011). Metabolite biodiversity in pepper (*Capsicum*) fruits of thirty-two diverse accessions: Variation in health-related compounds and implications for breeding. *Phytochemistry.* **72**: 1358–1370.
- Wall, M. M., and Bosland. P. W. (1998). Analytical methods for color and pungency of chiles (capsicums). In: Instrumental Methods in Food and Beverage Analysis, pp. 347–373. Wetzel D. L. B., and Charalambous, G., Eds., Elsevier, Amsterdam.

# ACCEPTED MANUSCRIPT

- Weissenberg, M., Schaeffler, I., Menagem, E., Barzilai, M., and Levy, A. (1997). Isocratic non-aqueous reversed-phase high-performance liquid chromatographic separation of capsanthin and capsorubin in red peppers (*Capsicum annuum* L.), paprika and oleoresin. *J. Chromatogr. A.* **757**: 89–95.
- Yazawa, S., Suetome, N., Okamoto, K., and Namiki, T. (1989). Content of capsaicinoids and capsaicinoid-like substances in fruit of pepper (*Capsicum annuum* L.) hybrids made with ‘CH-19 Sweet’ as a parent. *J. Jpn. Soc. Hortic. Sci.* **58**: 601–607.
- Yoshioka, M., Lim, K., Kikuzato, Sh., Kiyanaga, A., Tanaka, H., Shindo, M., and Suza, M. Effects of red-pepper diet on the energy metabolism in men. (1995). *J. Nutr. Sci. Vitaminol.* **41**: 647–656.
- Zachariah, T. J., and Gobinath P. (2008). Paprika and chili. In: Chemistry of spices, pp. 260–286. Parthasarathy, V. A., Chempakam, B., and Zachariah, T. J., Eds., CABI, Wallingford.
- Zewdie, Y., and Bosland, P. W. (2001). Capsaicinoid profiles are not good chemotaxonomic indicators for *Capsicum* species. *Biochem. Syst. Ecol.* **29**: 161–169.
- Ziino, M., Condurso, C., Romeo, V., Tripodi, G., and Verzera, A. (2009). Volatile compounds and capsaicinoid content of fresh hot peppers (*Capsicum annuum* L.) of different Calabrian varieties. *J. Sci. Food Agric.* **89**: 774–780.
- Zimmermann, M., and Schieberle, P. (2000). Important odorants of sweet bell pepper powder (*Capsicum annuum* cv. *annuum*): differences between samples of Hungarian and Moroccan origin. *Eur. Food Res. Technol.* **211**: 175–180.

**Table 1** Summary of analytical protocols for the determination of capsaicinoids.

Refs.	Matrix (Compounds determined*)	Sample preparation	Extraction	Analysis
a	Placenta; pericarp (frozen) (C, DHC, NDHC)	Blendered	Acetone, 3-5 min, r.t. <sup>**</sup>	GC: AT-1701 (14% cyanopropylphenyl-86% methylsiloxan) (30 m x 0.25 mm ID, 0.25 µm), 160-270°C
b	1. Ground pepper  2. Sauces (C, DHC)	1. Ground to a fine powder  2. Homogenized	SPME: PDMS/DVD fiber immersed in water for 30 min	GC/MS: ZB-5M (30 m x 0.32 mm ID, 0.25 µm), 40- 300°C

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c	Fresh mature fruits (C, DHC)	Oven dry, 80°C	55- 1. H <sub>2</sub> O (100 ml) + 10 drops of 2% sodium dioctylsulfosuccinat e, 1 h, r.t.  2. MeOH, 2 min (blending)	GC/MS: HP-1 (25 m x 0.2 mm ID, 0.33 µm), 250°C
d, e	Pericarp; placenta; seeds (TCC)	Freeze ground	dry, Acetone, overnight, r.t.	TLC: Silica gel 60; cyclohexane-CHCl <sub>3</sub> -acetic acid (70:20:10).  Quantification by densitometry
f	1. Stored fruits 2. Sauces (C, DHC)	1. Oven dry, 55- 80°C, ground  2. No	EtOH-H <sub>2</sub> O (50:50), sonication 60 min, r.t.	CE: Silica capillary (50 cm x 50 µm ID x 375 µm OD); Buffer: 15 mM Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> - 0.05 % Tween 20 - 2.2 mM sodium dodecylsulfate (pH = 10.1)

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g	Commercial oleoresins	No	MeOH-H <sub>2</sub> O (60:40), sonication 15 min	HPLC: Hypersil C18 (250 x 4.6 mm, 5 µm),  (C, DHC, NDHC, HC, HDHC, octanoyl vanillylamid, nonanoyl vanillylamid)
h	Pericarp	No	Acetone, then acetone-petroleum ether (1:1), followed by partition against H <sub>2</sub> O, concentration <i>in vacuo</i> , and TLC (silica gel, petroleum ether-EtOAC-MeOH (75:20:5)).	HPLC: Eurospher 80 (C18), MeCN-H <sub>2</sub> O; linear gradient from (10:90) to (90:10), 30 min

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j	Ground pepper (C, DHC)	Ground	Acetone, Soxhlet 5 h. Extract evpd. and dissld. in MeCN, filtered through Sep-	HPLC: $\mu$ -Bondapak C18 (300 x 4 mm), MeOH-H <sub>2</sub> O (60:40) Pak C18
k, l	1. Fresh fruits: pericarp, placenta, seeds	1. No	1. MeOH, r.t	HPLC: Inertsil ODS-3v (400 <sup>3*</sup> x 4.6 mm, 4 $\mu$ m), MeCN-0.5% formic acid aq. (45:55) <sup>k</sup>
	2. Dry fruits	2. Ground	2. MeOH, sonication 20 min, r.t.	LC/MS: Eclipse XDB-C18 (150 x 4.6 mm, 3.5 $\mu$ m), the same mobile phase <sup>k</sup>
	3. Canned fruits	3. Freeze dry	3. Same	HPLC: Inertsil ODS-3v (250 x 4 mm, 5 $\mu$ m),
	4. Sauces (C, DHC, NDHC, HC, HDHC,	4. Homogenized	4. Same	MeCN-0.5% formic acid aq.; nonlinear gradient from (31:69) to (80:20), <sup>100</sup> °C, 1 h

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nonivamide) 100 min<sup>l</sup>

m	Fresh fruits (C, DHC)	Blendered	EtOH, stirred for 30 s, boiled at 78°C, 30 min	HPLC: Pinnacle II (C18) (250 x 4.6 mm, 5 µm), MeCN-1% acetic acid aq.; nonlinear gradient from (50:50) to (80:20), 11 min
n, o	Fresh fruits (C, DHC, NDHC)	Blendered	MeOH, 30 min, r.t.	HPLC: Lichrospher RP-18e (250 x 4 mm, 5 µm), MeCN-H <sub>2</sub> O-acetic acid (50:50:0.5)
p-r	Fresh fruits (C, DHC, HC, HDHC)	Deseeded, blendered	1. Microwave extraction with MeOH, EtOH, acetone, EtOAc, H <sub>2</sub> O, 5-30 min, 50-200°C <sup>p</sup>	1. HPLC: Chromolith Performance RP-18e (100 x 4.6 mm) <sup>4*</sup> , H <sub>2</sub> O (0.1% acetic acid)-MeOH (0.1% acetic acid); nonlinear gradient from (0:100) to (100:0), 20 min <sup>p</sup>

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2. H<sub>2</sub>O or EtOH  
pressurized to 100  
atm, 50-200 °C <sup>q,r</sup>

2. LC/MS: Luna (150 x 3  
mm, 5 µm), the same  
mobile phase <sup>q,r</sup>

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<sup>\*</sup>TCC: Total capsaicinoid content; C: Capsaicin; DHC: Dihydrocapsaicin; NDHC: Nordihydrocapsaicin; HC: Homocapsaicin; HDHC: Homodihydrocapsaicin. <sup>\*\*</sup>r.t: room temperature. <sup>3\*</sup> Two columns (250 and 150 mm) in series. <sup>4\*</sup> Monolithic column.

a: Thomas et al., 1998; b: Peña-Alvarez et al., 2009; c: Antonious et al., 2007; d: Monforte-González et al., 2010; e: Monforte-González et al., 2007; f: Liu et al., 2010; g: Maillard et al., 1997; h: Perucka and Oleszek, 2000; j: Korel et al., 2002; k: Kozukue et al., 2005; l: Choi et al., 2006; m: Davis et al., 2007; n: Kirschbaum-Titze et al., 2002; o: Kirschbaum-Titze et al., 2002a; p: Barbero et al., 2006; q: Barbero et al., 2006a; r: Barbero et al., 2008

**Table 2** Summary of analytical protocols for the determination of capsinoids and capsiconinoids in pepper.

I efs.	Matrix	Compounds determined	Sample preparation	Extraction	Analysis
, b	Fresh fruits	capsiconiate, dihydrocapsiconiate, capsiate,	Freeze dried, blended	Acetone then ethyl acetate, r.t. eluted through a Sep-Pak C18 cartridge	HPLC: µ-Bondapack- C18 (150 mm x 3.9 mm, 10 µm), MeOH-H <sub>2</sub> O (70:30)

		capsaicin, dihydrocapsaicin	blendered	a Sep-Pak C18 cartridge	$\mu\text{m}$ ), MeOH-H <sub>2</sub> O (70:30)
c	Fresh fruits	capsiate, dihydrocapsiate	Homoge nized	MeCN, 4°C, 24 h, then homogenized for 2 min	HPLC: C18 monolithic column (100 x 4.6) (Phenomenex), MeCN-H <sub>2</sub> O (60:40)
					GC: DB-5 MS (30 m x 0.25 mm i.d., 0.25 $\mu\text{m}$ ), 90- 290°C
c	1. Placenta	phenylalanin, cinnamic acid, <i>p</i> -coumaric acid, ferulic acid, vanillin, capsiate, dihydrocapsiate	Freeze dried, ground	1. Ethyl acetate, 1h. The residue was extracted with 80% EtOH, 1h x 2.	HPLC: Fluofix IEW 425 (250 mm x 4.6 mm), MeCN-0.1% TFA; gradient from (7:93) to (100:0), 70 min, 40°C
	2. Pericarp, seeds			2. 80% EtOH.	

a: Tanaka et al., 2009; b: Tanaka et al., 2010; c: Singh et al., 2009; d: Sutoh et al., 2006.

**Table 3** Spectroscopic and chromatographic characteristics for pigment identification.

Pigment	Spectral data, $\lambda_{\max}$ , nm <sup>*</sup>	Colour on TLC	$R_f$ <sup>**</sup>	
			Eluent A <sup>a</sup>	Eluent B <sup>b</sup>
<u>Chlorophylls</u>				
chlorophyll <i>b</i>	457, 646 (A) <sup>a</sup>	yellow-green	<sup>3*</sup> 0.36 <sup>g</sup>	0.39
chlorophyll <i>a</i>	430, 662 (A) <sup>a</sup>	blue-green	<sup>3*</sup> 0.45 <sup>g</sup>	0.46
pheophytin <i>b</i>	428, 652 (B) <sup>c</sup>			
pheophytin <i>a</i>	408, 505, 534, 667 (A) <sup>a</sup>	gray	<sup>3*</sup> 0.51 <sup>g</sup>	
pheophorbide <i>a</i>	409, 665 (C) <sup>d</sup>			

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## Carotenoids

neoxanthin	416, 438, 470 (D) <sup>e</sup>	yellow	0.23
capsorubin	(452) 482, 512 (D) <sup>e</sup>	red-brown	0.31
violaxanthin	418, 442, 472 (D) <sup>e</sup>	yellow	0.34
capsanthin	472, (498) (D) <sup>e</sup>	intense red	0.39
lutein	423, 447, 475 (E) <sup>f</sup>	yellow	0.41
anteraxanthin	422, 448, 478 (D) <sup>e</sup>	yellow	0.42
zeaxanthin	426, 452, 482 (D) <sup>e</sup>	yellow-orange	0.44
capsolutein	424, 448, 476 (D) <sup>e</sup>	yellow	0.47
cryptocapsin	482, (518) (D) <sup>e</sup>	pale red	0.51
$\beta$ -cryptoxanthin	428, 454, 482 (D) <sup>e</sup>	yellow	0.57
$\beta$ -carotene	(428) 452, 480 (D) <sup>e</sup>	yellow-orange	0.96      0.93

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\* Data in parentheses denote the shoulder. Solvents: (A) acetone; (B) 85-95% MeOH; (C) MeOH-MeCN-0.25M pyridine aq.-acetone; (D) 90-95% acetone; (E) MeCN (0.05% triethylamine)-MeOH-ethyl acetate. \*\*Eluent A: Petroleum ether-acetone-diethylamine (10:4:1); Eluent B: hexane- acetone (7:3). <sup>3\*</sup>Eluent: Cyclohexane- acetone-diethylamine (10:4:1).

a: Mínguez-Mosquera and Hornero-Méndez, 1993; b: Quach et al., 2004; c: Gokmen et al., 2002; d: Suzuki and Shioi, 2003; e: Sheer, 2006; f: de Azevedo-Meleiro and Rodriguez-Amaya, 2009; g: Valverde et al., 2007.

**Table 4** HPLC conditions used in the analysis of pepper pigments.

Refs.	Column	Elution mode, t (run time, min)	Mobile phase composition (v/v)	Compounds separated*	Similar methods**
a	Spherisorb ODS-2 (250 x 4.6 mm, 5μm)	Linear gradient 75→100% A t = 17	A: Acetone B: H <sub>2</sub> O, F = 1.5	Chlorophyll fraction: Chlide <i>a</i> , Pheide <i>a</i> ; Chl <i>a</i> , Chl <i>b</i> , OH Chl <i>a</i> , OH Chl <i>a'</i> , Carotenoid fraction: Crb, V, Cps-5,6-epoxide; Cps, cis- Cps, Ant, Cuc A, Z, cis-Z, β-	g-m

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Cry,

$\beta$ -Car.

b	Vydac 201TP54 (250 x 4.6 mm, 5 $\mu$ m), t = 25	Stepwise gradient 25→75→100% A	A: MeCN- 0.05 M $\text{CH}_3\text{COONH}_4$ (MeOH)- $\text{CH}_2\text{Cl}_2$ -triethylamine (75:20:5:0.05) modified with 0.1% BHT  B: MeOH (0.1% BHT)  F = 1.5	Crb, Cps, Z, $\beta$ -Cry, $\beta$ -Car, $\alpha$ -Car	n, o
c	YMC C30 (250 x 4.6 mm, 5 $\mu$ m)	Stepwise gradient 90→90→80→30→90 →90% A t = 25	A: MeOH  B: MTBE  F = 1	$\beta$ -Cry, L, Z, Lyc, $\beta$ -Car, Ant, NX, V, cis-V, Crb, Cps	p, q
d	Spherisorb C18 ODS (3.9 x 300 mm, 10 $\mu$ m)	Isocratic	MeCN-MeOH (95:5)  F = 1; T = 25	$\beta$ -Car, Cps, $\beta$ -Cry, Z	r, s
e	LiChrospher 100 RP-18	Stepwise gradient	A: MeOH-H <sub>2</sub> O (80:20)	(all-E)-NX, (9'Z)-NX, (all-E)-V, (all-E)-L epoxide, (all-	

	(244 x 4 mm, 5 $\mu$ m),	80→77.5→50→20→ 0→80% A	B: EtOAc F = 1; T = 30  t = 47	E)-L, NL A, NL B, (all-E)- $\beta$ -Car.  Chl <i>a</i> , Chl <i>b</i> , Phe <i>a</i> , Phe <i>b</i>	
e	YMC C30  (250 x 4.6 mm, 5 $\mu$ m),	Linear gradient  100→0% A	A: MeOH-THF (95:5) B: EtOH-H <sub>2</sub> O-THF (5:90:5)  F = 1.5  t = 60	cis/trans- $\beta$ -Car	t
f	Waters Acquity HSS C18  (100 x 2.1 mm, 1.8 $\mu$ m) <sup>4*</sup>	Stepwise gradient  25→25→5→5→0%  A  t = 11	A: MeCN- <i>i</i> -PrOH-H <sub>2</sub> O (39:53:8)  B: MeCN- <i>i</i> -PrOH (60:40)  F = 0.75	NX, V, Z, $\beta$ -Car, Cps, capsorubin, Ant, $\beta$ -cry	

\* Ant: anteraxanthin; Car: carotene; Chl: chlorophyll; Chlide: chlorophyllide; Cps: capsanthin; Crb: capsorubin; Cry: cryptoxanthin; Cuc A: cucurbitaxanthin A; Lyc: lycopene; L: lutein; NL A(B): neolutein A(B); NX: neoxanthin; Phe: pheophytin; Pheide: pheophorbide; V: violaxanthin; Z: zeaxanthin. \*\* Separation conditions differ insignificantly (column brand, flow rate, minor changes in mobile phase composition) from a given method. <sup>3\*</sup> Unless otherwise mentioned, the analysis was carried out at r.t. or the temperature was not reported. <sup>4\*</sup> UPLC.

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a: Roca et al., 2006; b: Howard et al., 2000; c: Guil-Guerrero et al., 2006; d: Kim et al., 2008; e: Larsen and Christensen, 2005; f: Guzman et al., 2010; g: Topuz and Ozdemir, 2007; h: Gallardo-Guerrero et al., 2010; i: Hornero-Méndez et al., 2002a; j: Raffo et al., 2008; k: Matsufuji et al., 2007; l: Deli et al., 2001; m: Mínguez-Mosquera and Hornero-Méndez, 1993; n: Aruna and Baskaran, 2010; o: Russo and Howard, 2002; p: Pérez-López et al., 2007; q: Burns et al., 2003; r: Kim et al., 2004; s: Simkin et al., 2003; t: Kidmose et al., 2006.

**Table 5** Spectrophotometric assays for the analysis of phenolic compounds<sup>\*</sup>.

Assay <sup>**</sup>	PAs	Fons +	Fols +	Anths	Interfering compounds <sup>3*</sup>	Refs.
		Fanos	F3ols			
FC	+	+	+	+	AA, R.Sug	a
AlCl <sub>3</sub> <sup>4*</sup>	-	+ (Fons)	+	+	-	b, c
DMACA	-	-	+	-	-	d
SBC	-	+	+	+	-	e

2,4-DNPH	-	+(Fanos)	-	-	AA, DHAA	c
pH dif.	-	-	-	+	-	b

\*PAs: Phenolic acids; Fons: Flavones; Fanos: Flavanones; Fols: Flavonols; F3ols: Flavan-3-ols; Anths: Anthocyanins;

\*\*FC: Folin-Ciocalteau; DMACA: *p*-dimethylaminocinnamaldehyde; SBC: Sodium borohydride-chloranil; 2,4-DNPH: 2,4-dinitrophenylhydrazine; pH dif: pH differential method. <sup>3\*</sup>AA: Ascorbic acid; DHAA: dehydroascorbic acid; R.Sug: Reducing sugars; <sup>4\*</sup>There are two versions of the AlCl<sub>3</sub> method, without (b) and with (c) potassium acetate.

a: Singleton and Rossi, 1965; b: Kevers et al., 2007; c: Chang et al., 2002; d: Gorinstein et al., 2009; e: He et al., 2008.

**Table 6** HPLC conditions used in the analysis of phenolics.

Refs.	Column	Elution mode, t (run time, min)	Mobile phase composition (v/v)	Compounds separated	Similar Refs*
			F (Flow rate, ml/min),		

T (Temperature, °C) <sup>**</sup>					
a	Supelcosil LC-18 (250 x 4.6 mm, 5µm)	Linear gradient 10→90% A t = 60	A: MeOH B: 5% formic acid aq. F = 1; T = 30	hydroxycinnamic acid derivatives; quercetin and luteolin glycosides	c, d, h
b, c	Lichrocart C18 (250 x 4.6 mm, 5µm) (LC/MS/MS)	Isocratic t = not reported	0.1% formic acid aq. F = 1	caffeic, coumaric, sinapic and ferulic acid derivatives; glycosides of quercetin, luteolin, apigenin and chrysoeriol	
d	STR ODS II (Shinwa) (150 x 2.1 mm) (LC/MS)	Stepwise gradient 30→90% A t = 25 min	A: MeOH B: 0.2% CH <sub>3</sub> COOH aq. F = 0.2; T = 40	caffeic acid, hesperidin, myricetin, quercitrin, quercetin, apigenin	
e	Eurospher 80 (RP-18) (250 x 4.6 mm, 5µm)	Linear gradient 0→40% A t = 45	A: MeCN B: 1% H <sub>3</sub> PO <sub>4</sub> aq. F = 1	glucopiranosides of ferulic and sinapic acids and glucopiranosides, arabino-piranosides, rhamno-piranosides of quercetin, luteolin and apigenin	i-m

f	Prodigy ODS 3 (250 x 4.6 mm, 5µm)	Stepwise gradient 17→25→35→50% A t = 20	A: MeCN B: H <sub>2</sub> O-THF-TFA (98:2:0.1)	glycosides of quercetin, kaempferol, luteolin, apigenin, and cyanidin
g	Zorbax XDB-C18 (250 x 4.6 mm; 5 µm)	Triple stepwise gradient (90:6:4, A:B:C) → (0:15:85) t = 60	A: 0.05% TFA (H <sub>2</sub> O) B: 0.05% TFA (MeOH) C: 0.05% TFA (MeCN)  F = 1	quercetin, kaempferol, luteolin

\* The same as in Table 4; \*\* The same as in Table 4.

a: Raffo et al., 2008; b: Marín et al., 2008; c: Marín et al., 2004; d: Ren et al., 2001; e: Materska et al., 2003; f: Arabbi et al., 2004; g: Chassy et al., 2006; h: Raffo et al., 2007; i: Kevers et al., 2007; j: Pegard et al., 2005; k: Materska and Perucka, 2005; l: Sun et al., 2007; m: Baranowski et al., 2004.

**Table 7** Chromatographic methods used in the analysis of free sugars\* in peppers.

Refs.	Method**	Column	Elution mode	Mobile phase composition (v/v)

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F (Flow rate, ml/min), T  
(Temperature, °C)

a	GC of trimethylsilyl ethers <sup>3*</sup>	SE-30 or SE-54		
b	HPLC-RID	Aminex HPX-87C (300 x 7.8 mm)	Isocratic	H <sub>2</sub> O F = nr; T = 85
c	HPLC-RID	CARBOSep CHO-682 LEAD (300 x 7.8 mm)	Isocratic	H <sub>2</sub> O F = 0.4; T = nr
d	HPLC-RID	RCM Monosaccharides (dimensions nr)	Isocratic	5 mM H <sub>2</sub> SO <sub>4</sub> aq. F = nr; T = 80
e	HPLC-RID	Pinnacle II Amino (150 x 4.6 mm, 5 µm) <sup>4*</sup>	Isocratic	MeCN-H <sub>2</sub> O (80:20) F = 1; T = 30
f	HPLC-RID	Tosoh TSK-gel Amide-80 (250 x 4.6 mm, 5 µm)	Isocratic	MeCN-H <sub>2</sub> O (85:15) F = 1; T = 80
g	HPLC-UV post-column derivatization <sup>5*</sup>	Supercosil LC-NH <sub>2</sub> (250 x 4.6 mm, 5µm)	Linear gradient 16→23% A	A: H <sub>2</sub> O B: MeCN

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	run time nr	F = 0.8; T = 30
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nr: not reported.

<sup>\*</sup>glucose, fructose, and sucrose unless otherwise specified. <sup>\*\*</sup>GC: gas chromatography; HPLC-RID: HPLC with refractive index detector; HPLC-UV: HPLC with UV-detector. <sup>3\*</sup> EtOH-solvable sugars, no other details reported. <sup>4\*</sup> can be replaced with LiCrospher 100 NH<sub>2</sub> (Martínez-Ballesta et al., 2004; Navarro et al., 2006) or Luna 5μ NH<sub>2</sub> 100 A (Bernardo et al., 2008). <sup>5\*</sup> Tetrazolium blue, 90°C, volume of a reaction coil ~ 1 ml; glucose, fructose, sucrose, ribose, xylose, maltose were analyzed.

a: Priya Sethu et al., 1996; b: Gallardo-Guerrero et al., 2010; c: Flores et al., 2009a; d: Demir et al., 2008; e: Raffo et al., 2007; f: Matsufuji et al., 2007; g: Kamilova et al., 2006.

**Table 8** Fractionation of cell wall material.

Fractionation step	Choice of extractant <sup>*</sup>	Dominant polysaccharide	Hydrolysis
Water extraction	Water <sup>a,b,c</sup>	highly methylated low molecular weight pectic substances	A-D

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Chelator extraction	0.5% (NaPO <sub>3</sub> ) <sub>6</sub> <sup>a</sup> 0.5% Na-EDTA <sup>b</sup> 0.1 M Tris-HCl + 0.2% EDTA <sup>c</sup> 0.5% EDTA (70°C) <sup>d</sup> 0.05 M CDTA <sup>3*</sup> + 0.05 M CH <sub>3</sub> COONa <sup>e</sup>	low methylated pectin chains cross-linked by Ca <sup>2+</sup> ions **	A-D
Diluted alkali extraction	0.05 M NaOH, r.t. <sup>a,b,c</sup> 0.05 M Na <sub>2</sub> CO <sub>3</sub> + 0.002 M CDTA + 0.1 M NaBH <sub>4</sub> <sup>e</sup>	highly methylated high molecular weight pectic substances	A-D
Strong alkali extraction	10% NaOH <sup>d</sup> 8 M KOH + 0.1 M NaBH <sub>4</sub> <sup>e</sup>	hemicelluloses	A, B
Insoluble residue		cellulose	A

\* Extraction is carried out at r.t. unless otherwise mentioned. \*\* sometimes called calcium pectate. <sup>3\*</sup> *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid.

a: Howard et al., 1997; b: Gu et al., 1999; c: Gallardo-Guerrero et al., 2010; d: Priya Sethu et al., 1996; e: Harpster et al., 2002.

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A: 12 M H<sub>2</sub>SO<sub>4</sub> (35°C, 1 h), followed by 2 M H<sub>2</sub>SO<sub>4</sub> (100°C, 1 h) (Villanueva-Suárez et al., 2003); B: 2 M TFA (121°C, 1 h) (Melton and Smith, 2005); C: incubation with pectinase and cellulase (55-58°C, pH 4.5, 20 h) (López-Hernández et al., 1996); D: 0.012 Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> in H<sub>2</sub>SO<sub>4</sub> (conc) (100°C, 5 min) (Conforti and Zinck, 2002). Method D is used for the hydrolysis step in the *m*-hydroxydiphenyl assay of galacturonic acid (see explanation in text).

**Table 9** HPLC analysis of water-soluble vitamins.

Refs.	Column	Elution mode, t (run time, min)	Mobile phase composition (v/v) F (Flow rate, ml/min), T (Temperature, °C) <sup>*</sup>	Comments
<b>Vitamin C</b>				
a-g	C18-type column** (250 x 3-4.6 mm, 5μm)	Isocratic	5-50 mM H <sub>2</sub> SO <sub>4</sub> or H <sub>3</sub> PO <sub>4</sub> aq., or 0.2 M KH <sub>2</sub> PO <sub>4</sub> buffer pH = 2.2 F = 0.7-1; T = 35°C <sup>a</sup> , T = r.t. <sup>b-g</sup>	
h	YMC-Pro C18	Isocratic	50 mM phosphate buffer, pH = 4.5	

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	(150 x 3.9 mm)		F = 0.5; T = 30	
i	Kromasil 100 C18 (250 x 4.6; 5µm)	Isocratic	MeOH-50 mM KH <sub>2</sub> PO <sub>4</sub> (pH = 4.5) (5:95) modified with 5 mM cetyltrimid F = 0.9	pre-column derivatization of DHAA with 1,2- <i>o</i> -phenylenediamin
j	Supelcogel C-610H <sup>3*</sup> (210 x 7.8 mm)	Isocratic	0.1 % H <sub>3</sub> PO <sub>4</sub> F = 0.5; T = 30	
k	Supelcosil LC18 (250 x 4.6 mm)	Isocratic	MeCN-H <sub>2</sub> O (30:70) pH = 2.8 adjusted with H <sub>3</sub> PO <sub>4</sub> F = 1	
l	Lichrosorb C18 (240 x 4.6 mm; 10 µm)	Isocratic	10 mM KH <sub>2</sub> PO <sub>4</sub> -MeOH-tetrabutyl ammonium hydroxide (97:3:0.1) pH = 2.8 F = 1	
m	Spherisorb C18 (250 x 4.6mm; 10 µm)	Isocratic	10 mM KH <sub>2</sub> PO <sub>4</sub> -MeOH-tetrabutyl ammonium hydroxide (97:3:0.05) pH = 2.85	post-column derivatization of DHAA with 1,2- <i>o</i> -phenylenediamin

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F = 1

n	Purospher RP-C18 (250 x 4.6 mm; 5 µm)	Isocratic	MeCN-MeOH-THF (45:50:5) F = 1
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o	YMC-Pack Polyamine II (250 x 4.6 mm)	Isocratic	MeCN-NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> <sup>4*</sup> aq. (70:30) F = 1
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## Folates

p	Luna C18 (150 x 2.1 mm; 4 µm)	Stepwise gradient <sup>5*</sup> 3→5→30→100→ 100% A	A: 1% formic acid (MeCN) B: 1% formic acid (H <sub>2</sub> O) F = 0.2	LC-MS
		t = 22		

\* Unless otherwise mentioned, the analysis was carried out at r.t. or the temperature was not reported; \*\* Reprosil-Pur-C18-AQ<sup>a</sup>, Nucleosil C18<sup>b</sup>, Prontosil 120-3-C18<sup>c</sup>, Lichrospher 100 RP-18<sup>d</sup>, Chromsil C18<sup>e</sup>, Kromasil KR 100-5 C18<sup>f</sup>, and Atlantis T3 C18<sup>g</sup>; <sup>3\*</sup>ion-exchange resin; <sup>4\*</sup>Concentration was not reported; <sup>5\*</sup> the last step is isocratic.

a: Topuz and Ozdemir, 2007; b: Raffo et al., 2008; c: Flores et al., 2009; d: Oboh and Rocha, 2007; e: Navarro et al., 2006; f: Nazzaro et al., 2009; g: Isabelle et al., 2010; h: Wahyuni et al., 2011; i: Marín et al., 2008; j: Serrano et al., 2010; k: Sgrosso

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and Pereyra, 2009; l: Daood et al., 2006; m: Márkus et al., 1999; n: Saha et al., 2010; o: Kim et al., 2008a; p: Phillips et al., 2006.

**Table 10** HPLC analysis of vitamin E.

Refs.	Column	Elution mode, t (run time, min)	Mobile phase composition (v/v)	Comments
a	Shodex Sil 5B (250 x 4.6 mm; 5µm)	Isocratic	<i>i</i> -PrOH-hexane (1:99) F = 1	
b, c	YMC C30 (250 x 4.6 mm; 5µm)	Triple stepwise gradient (95:5:0, A:B:C) → (80:5:15) → (30:5:65) t = 30	A: MeOH B: 0.2% CH <sub>3</sub> COONH <sub>4</sub> (20% MeOH aq.) C: TBME F = 1; T = 25°C <sup>b</sup> ; 40°C <sup>c</sup>	tocopherols and carotenoids
d, e	Silica <sup>**</sup>	Isocratic	EtOH-hexane (0.4:99.6)	

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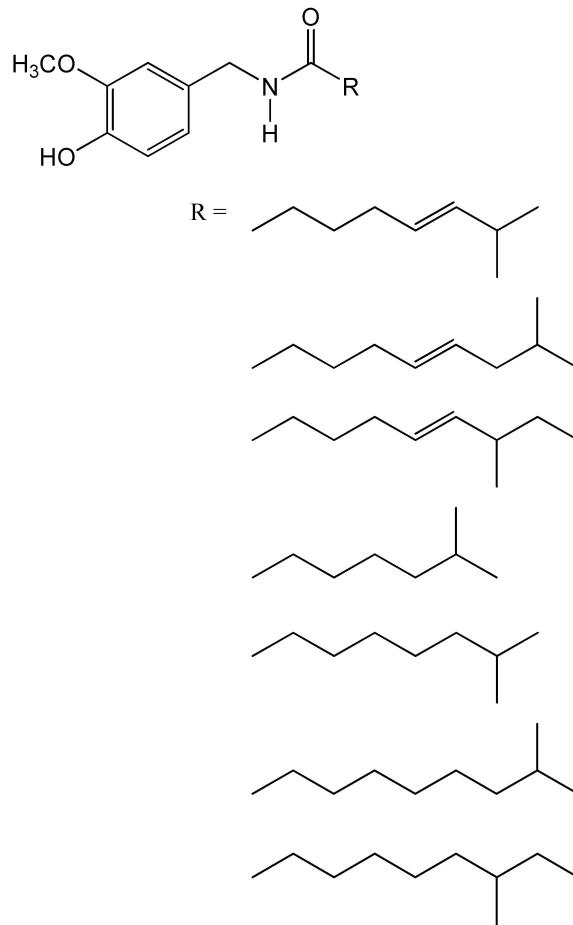
	(240 x 4.6 mm; 10µm)	F = 1.2
f	Lichrospher 100 Diol	Isocratic TBME-isooctane (4:96)
	(250 x 3 mm; 5µm)	F = nr
g	Spherisorb ODS1 (250 x 4.6 mm; 4µm)	Linear gradient <sup>3*</sup> 0→100→100% A t = 18 A: MeOH:EtOAc (68:32) B: MeCN:MeOH:0.1 M Tris-buffer (pH = 8) (84:2:14)
		F = 1.2
h	2-column system 1: Zorbax SB-C18 (150 x 4.6 mm; 5 µm) 2: Partisphere 5 C18 (110 x 4.7 mm; 5µm)	Linear gradient 80→60% A t = 40 A: MeCN B: MeOH F = 1; T(column1) = 30°C; T(column2) = 4°C tocopherols, carotenoids, capsaicinoids

\* The same as in Table 9; \*\* The last step is isocratic; <sup>3\*</sup> Nucleosil 100<sup>d</sup> and Lichrosorb<sup>e</sup>.

a: Matsufuji et al., 2007; b: Burns et al., 2003; c: Wahyuni et al., 2011; d: Daood et al., 2006; e: Márkus et al., 1999; f: Koch et al., 2002; g: Lizarazo et al., 2010; h: Isabelle et al., 2010.

**FIGURE CAPTIONS**

**Figure 1.** Chemical structures of main capsaicinoids.



*trans*-Capsaicin

Homocapsaicin I

Homocapsaicin II

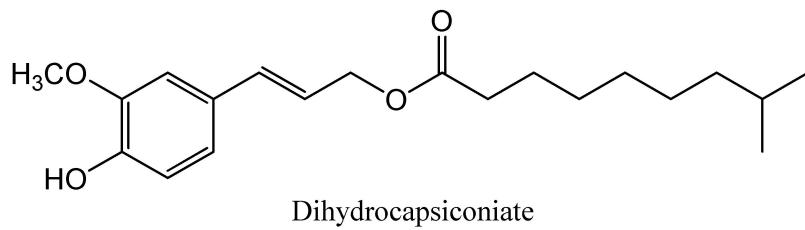
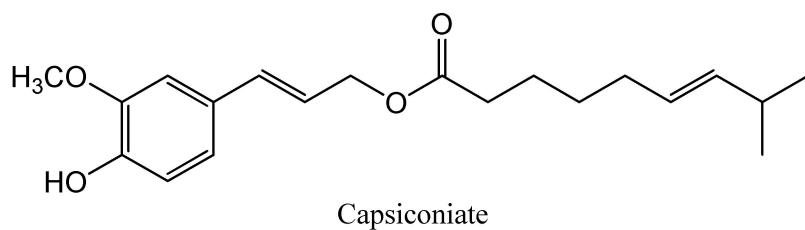
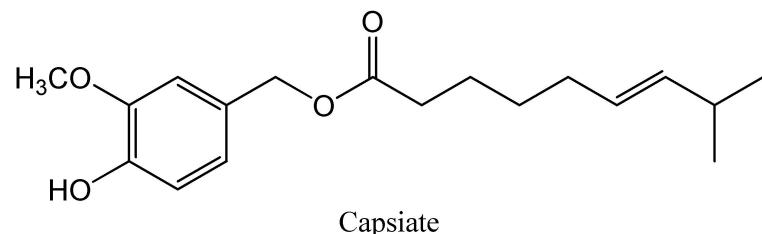
Nordihydrocapsaicin

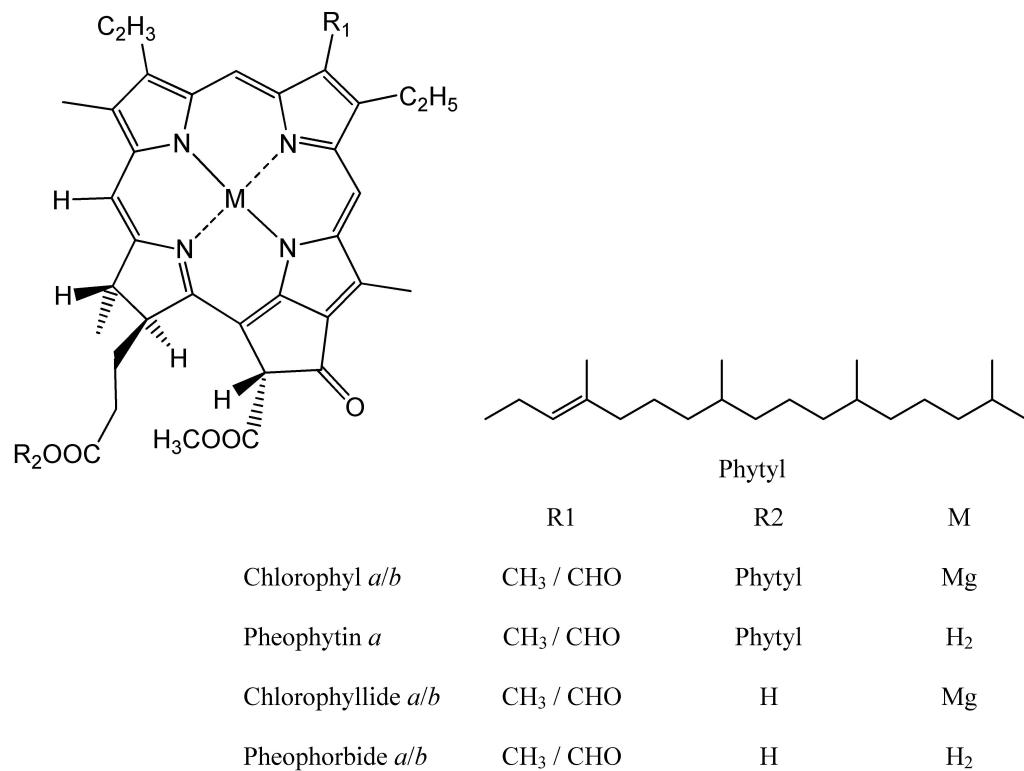
Dihydrocapsaicin

Homodihydrocapsaicin I

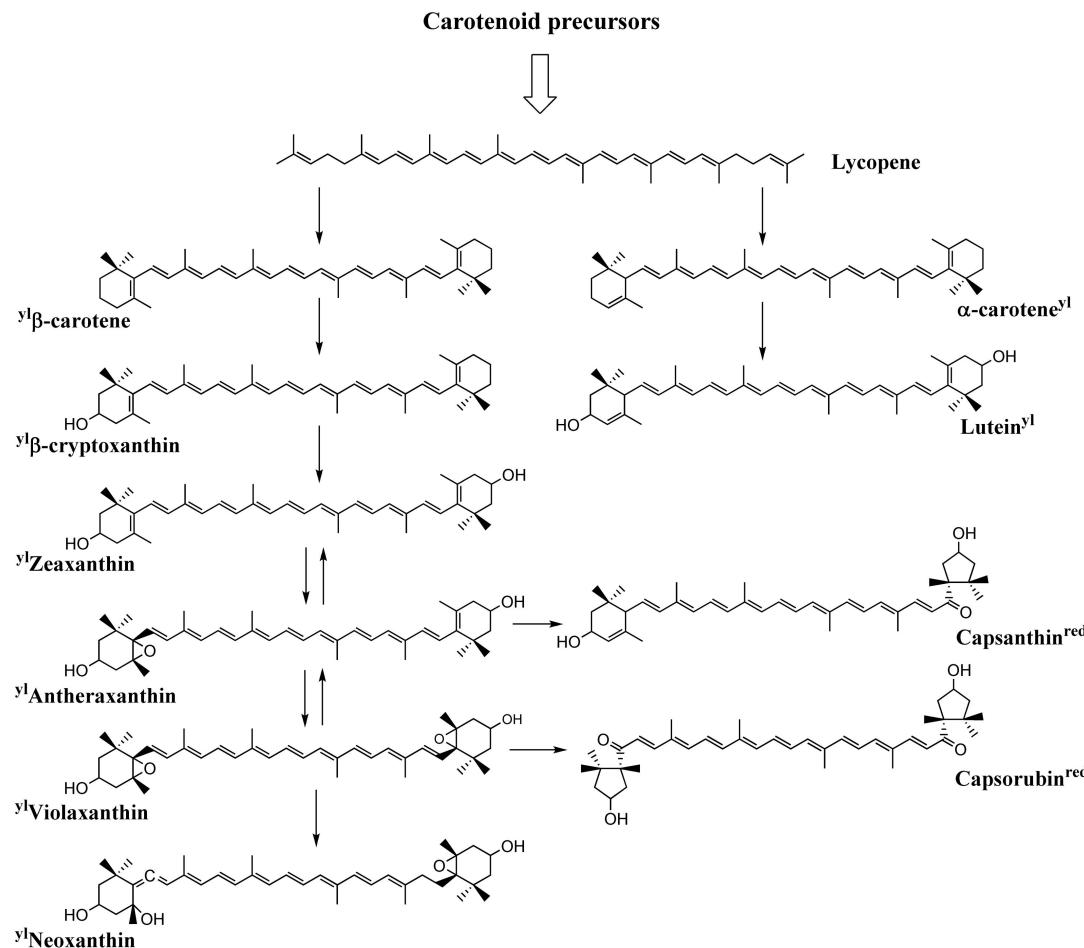
Homodihydrocapsaicin II

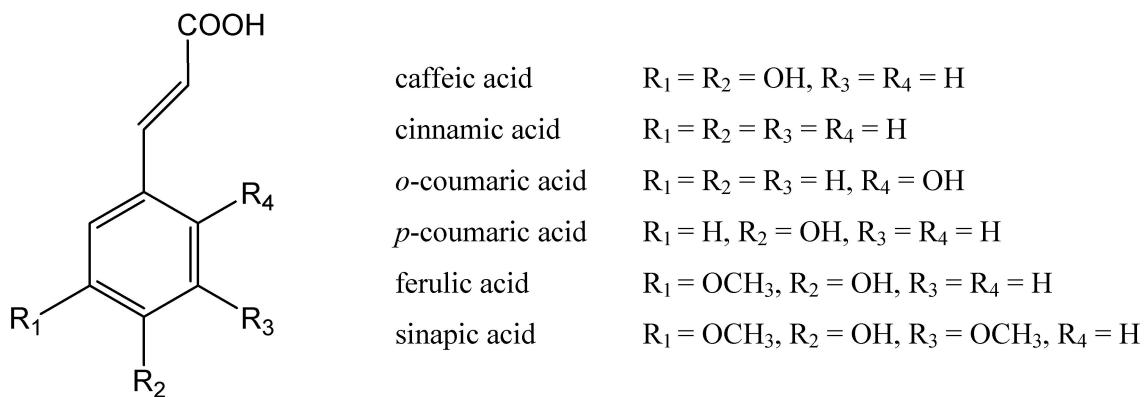
**Figure 2.** Chemical structures of capsiconinoids.

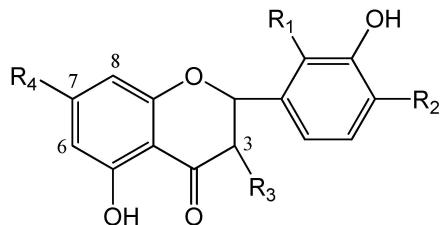


**Figure 3.** Chemical structures of chlorophyll species.

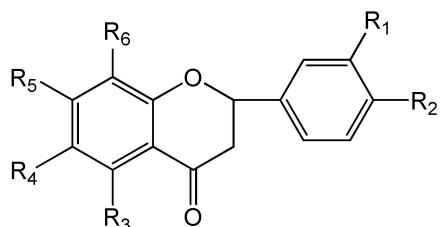
**Figure 4.** Major carotenoids. Superscripts “yl” and “red” denote yellow and red pigments, respectively.



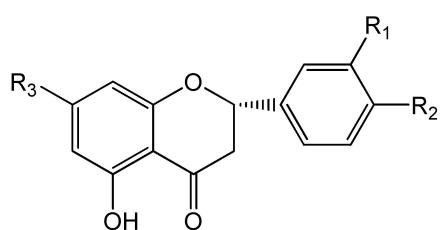
**Figure 5.** Chemical structures of phenolic acids.**Figure 6.** Chemical structures of major pepper flavonoids.

**Flavonols**

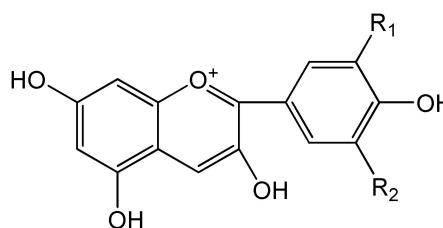
- |            |   |
|------------|---|
| kaempferol | R <sub>1</sub> = R <sub>2</sub> = H, R <sub>3</sub> = R <sub>4</sub> = OH     |
| myricetin  | R <sub>1</sub> = OH, R <sub>2</sub> = H, R <sub>3</sub> = R <sub>4</sub> = OH |
| quercetin  | R <sub>1</sub> = R <sub>2</sub> = R <sub>3</sub> = R <sub>4</sub> = OH        |

**Flavones**

- |          |  |
|----------|--|
| apigenin | R <sub>1</sub> = H, R <sub>2</sub> = R <sub>3</sub> = OH, R <sub>4</sub> = H,<br>R <sub>5</sub> = OH, R <sub>6</sub> = H |
| luteolin | R <sub>1</sub> = R <sub>2</sub> = R <sub>3</sub> = OH, R <sub>4</sub> = H,<br>R <sub>5</sub> = OH, R <sub>6</sub> = H    |

**Flavanones**

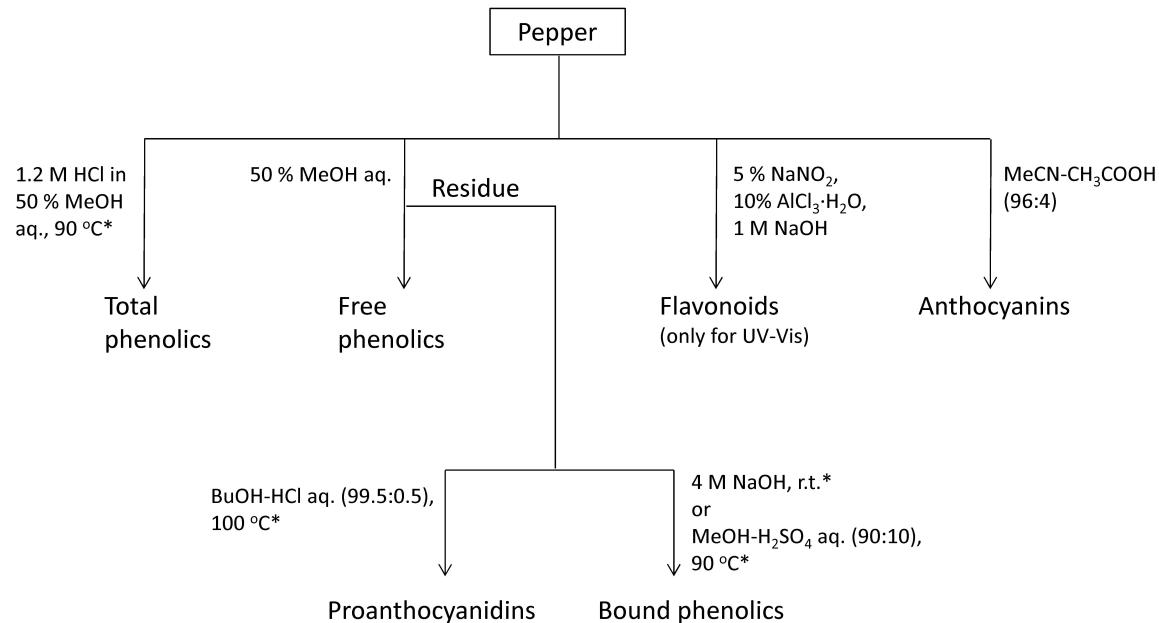
- |            |   |
|------------|---|
| hesperidin | R <sub>1</sub> = OH, R <sub>2</sub> = OCH <sub>3</sub> ,<br>R <sub>3</sub> = <i>O</i> -rutinoside |
| hesperitin | R <sub>1</sub> = OH, R <sub>2</sub> = OCH <sub>3</sub> , R <sub>3</sub> = OH                      |

**Anthocyanidins**

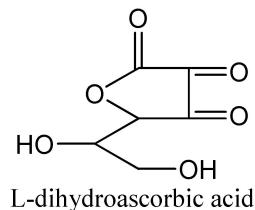
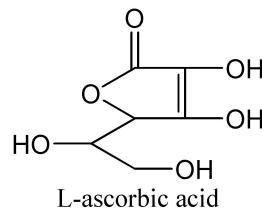
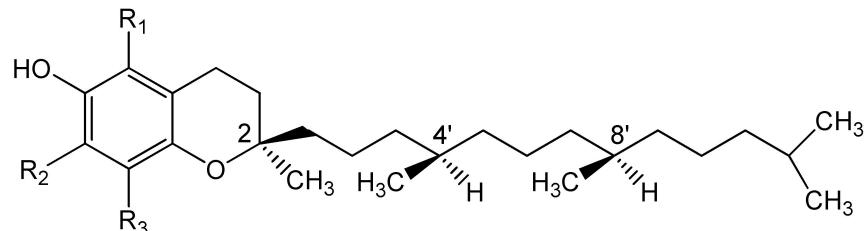
- |          |   |
|----------|---|
| cyanidin | R <sub>1</sub> = OH, R <sub>2</sub> = H |
|----------|---|

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**Figure 7.** A diagram for selection of an extraction protocol. Superscript “star” designates conditions that result in hydrolysis of phenolic conjugates. See text for detail explanation.



**Figure 8.** Chemical structures of vitamins.

**Vitamin C****Vitamin E**

Compound	R1	R2	R3
$\alpha$ -Tocopherol	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>
$\beta$ -Tocopherol	CH <sub>3</sub>	H	CH <sub>3</sub>
$\gamma$ -Tocopherol	H	CH <sub>3</sub>	CH <sub>3</sub>
$\delta$ -Tocopherol	H	H	CH <sub>3</sub>

**Folic acid**