Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1054 (2004) 217–226
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Review

Determination of pigments in vegetables

Benoît Schoefs*

Dynamique Vacuolaire et Réponses aux Stress de l'Environnement, UMR INRA-1088/CNRS-5184/UB, Plante–Microbe-Environnement, Université de Bourgogne à Dijon BP 47870, F-21078 Dijon Cedex, France

Available online 13 July 2004

Abstract

Plant pigments are responsible for the shining color of plant tissues. They are also found in animal tissues and, eventually in transformed food products as additives. These pigments have an important impact on the commercial value of products, because the colors establish the first contact with the consumer. In addition plant pigments may have an influence on the health of the consumers. Pigments are labile: they can be easily altered, and even destroyed. Analytical processes have been developed to determine pigment composition. The aim of this paper is to provide a brief overview of these methods.

Keywords: Reviews; Plant pigments; Anthocyanidin; Carotenes; Tetrapyrroles; Alkaloids; Polyphenols; Xanthophylls; Betalains; Phycobilins; Chlorophyll

Contents

* Tel.: +33 3 80 39 39 66; fax: +33 3 80 39 62 87.
E-mail address: benoit.schoefs@u-bourgogne.fr (B. Schoefs).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.05.105

1. Introduction

A large number of vegetables harbor shining colors. These colors are conferred by the pigments contained in the cells. On the basis of their chemical structures, pigments can be classed into four families, i.e. tetrapyrroles (e.g. chlorophyll), carotenoids (e.g. β-carotene), polyphenolic compounds (e.g. anthocyanins), and alkaloids (e.g. betalains). An example of the chemical structure of a member of each family of compounds is presented in Fig. 1. The number of molecules belonging to each family is quite large and, consequently, several volumes would be necessary to describe their particular properties (e.g. [1–7]).

Although animal tissues contain plant pigments, such as carotenoids, these tissues are usually not able to synthesize them. Therefore, the pigments must be obtained from food [8–12]. Once assimilated pigments enter into the biochemical pathways along which they may eventually be modified (reviewed in [13]). For instance β-carotene is splitted into two parts, one of these serving as a precursor of vitamin A [14–16], an important molecule for vision. An increasing number of studies indicates that plant pigments have positive roles on human health ([17–21], but see [22]) and several studies have established the minimum daily intake in these valuable molecules. Because the consumption of fresh food has decreased, while that of processed foods has increased, food colors have become an important aspect of the food formulation process. In order to restore the natural level of pigments in the processed products, extracted pigments are incorporated into the final food products [3,23–25]. Similarly the preparation of fortified products requires addition of pigments to the products [6]. Regardless of the type of final products, the pigments are incorporated either under their natural occurrence or under a chemical modified form. As a consequence of these additional pigment needs, the demand in isolated natural colorants has increased as compared with synthetic dyes [26,27]. However, this need cannot always be satisfied due to the limitation in the supply of raw materials because the production of pigments using conventional plant cultivation methods is influenced by climatic conditions, plant cultivars and varieties (reviewed in [28]). Consequently part of plant pigment research is oriented in finding new sources of pigments. This quest is not only directed in finding natural alternatives for synthetic dyes, but also with the aim to discover new taxons and new procedures for the pigment production, for instance from cell and/or tissue cultures [29–31], or genetic engineering [32–34].

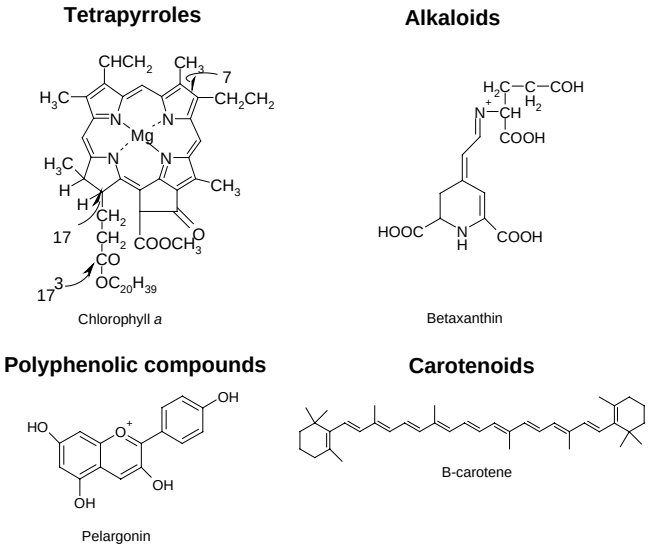

Fig. 1. Example of the chemical structure of a member of each family of pigments synthesized by plants.

For all the reasons cited above, pigment analysis constitutes a real analytical challenge because pigments are often part of complex structures and/or mixtures. Therefore, addressing these issues is never straightforward. To take up this challenge, it is necessary to have at disposal a diversified number of analytical procedures, able to rapidly and precisely quantify the different pigments and their eventual degradation products present (together) in the samples. The development of powerful analytical methods is also of prime importance in the control of quality (e.g. [35]).

In this contribution, the methods for pigment extraction and separation are first briefly reviewed. Then the ways of pigment identification and quantification are described.

2. Pigment extraction

Preparing samples from biological tissues is often tedious and time-consuming because the accuracy of the analysis depends on many parameters linked to the preparation of the sample. Examples are sample storage, process, etc. In addition, the complete extraction of pigments often requires several steps, and may use a mixture of several solvents. This is especially true when the sample contains pigments of different polarities and/or are present in a complex matrix (reviewed in [36,37]). In rare cases, pigments can be extracted in a one step process (e.g. [38]). The aim of the following discussion is not to make an exhaustive list of the extraction and analytical methods described in the literature, but more to provide representative selected examples, which can then be used as reference.

2.1. Chlorophyll and carotenoid molecules

Chlorophyll and carotenoid molecules are usually rather hydrophobic compounds (Fig. 1) and, therefore, they can be extracted with a single or a mixture of organic solvents using a homogenizer. For instance Thompson et al. [39] extracted lycopene from tomatoes using a mixture of hexane, acetone and ethanol (50:25:25 v/v/v). The extraction was repeated until no color was observed in the tissues. All the fractions were pooled in a separatory funnel and treated with diethyl ether. A NaCl solution (e.g. 10% w/v) can be added to assist for the transfer of the pigments into the nonpolar phase. The

water phase is then discarded and the pigmented layer is dried with anhydrous Na_2SO_4 (e.g. 2% w/v).

When necessary, the pigments can be saponified with KOH–methanol (10% w/v) and left for some time with periodic shaking. The pigments are then transferred to diethyl ether by adding distilled water and the organic phase is washed with water until neutrality is reached. Lastly the aqueous phase is removed. The organic phase is filtered on a bed of anhydrous Na_2SO_4 to dry the pigments.

A few carotenoids such as those contained in saffron are water-soluble. To extract carotenoids from saffron stigma the plant tissues are shaked during 30 min in cold water. The solution is heated to 60 °C during 30 min and allowed to stand in the dark for 24 h. The clear supernatant contains the pigments [40].

The most popular methods to extract carotenoids and chlorophylls use organic solvents. Most of these solvents are toxic and expensive. Several methods such as supercritical fluid extraction have been suggested as alternative methods for carotenoid extraction because they are unexpensive and nontoxic. Since these methods can be hyphenated to specific chromatographic methods, they will be described together below.

2.2. Anthocyanins

Anthocyanins are water-soluble pigments because the basic skeleton is often acylated with one or more polar side chains such as glucosides. In addition, and depending on pH, the oxygen atom of the heterocycle may be positively charged (Fig. 1). Extraction with water, acetone or chloroform is recommended [41,42]. The addition of a small amount of hydrochloric acid or formic acid is recommended to prevent the degradation of the nonacylated compounds [4]. A comparison of the extraction efficacy of different solvents indicates that the best solvent is 1% hydrochloric acid in methanol [43,44]. However, attention should be paid on the decrease of pH value when extracts are concentrated by evaporation. The report by Revilla et al. [45] compares various protocols for anthocyanin extractions from red grapes. The authors concluded that solvents containing up to 0.12 mol L^{-1} hydrochloric acid can cause partial hydrolysis of acylated anthocyanins.

After the extraction, it is advisable to pre-purify the extract by solid-phase extraction on Sephadex- or Amberlite XAD-7- or acidic methanol-activated C18-minicolumns [46,47]. Anthocyanins as well as other phenolics are adsorbed onto the phase, while sugars, acids and other water-soluble molecules are washed away with 0.01% aqueous HCl. Addition of ethyl acetate eluates phenolic compounds other than anthocyanins, which are eluted using acidified methanol (0.01% HCl v/v) [48].

2.3. Betalains

These pigments are also water-soluble and usually localized in a unique organelle of plant cell: the vacuole. So far, betalain synthesis has only been observed in plants belonging to the taxonomic group of Centrospermeae (red beet, etc.). Because betalains and anthocyanins are chemically (but not biochemically) related, extraction and prepurification methods used for anthocyanins can also be applied to the preparation of betalains.

3. Chromatographic separation of vegetable pigments

Pigments are able to absorb visible light because the chromophore—i.e. the part of the molecules, which is involved in light absorption—is made of several conjugated double bonds. This unique property, however, has a negative side: pigments are highly sensitive to acid, base, oxygen, heat and light (anthocyanins [49]; betalains [50]; carotenoids [51–53]; tetrapyrroles [54]). Therefore, special care should be then taken during and after pigment extraction.

3.1. Open column

Various phases like powdered sucrose, DEAE-Sepharose, cellulose or MgO/Hyflosupercel have been used to achieve chlorophyll and carotenoid separation [55–57]. The all-*trans*-β-carotene can be separated from 9-*cis*- and 13-*cis*-isomers using an open column filled up with calcium hydroxide. For phycobilins, an hydroxylapatite column can be used [58]. Anthocyanins are able to self-aggregate. Under this form, they are more stable and their light-isomerization is prevented. To separate these aggregates gel filtration has been used [59,60]. Nowadays although open column chromatography is mostly used to clean extracts or for preparative purposes, it may be still used for analytical methods. For example Somers and Evans [61] reported that the formation of aggregates of glucoside anthocyanins is typical for wine aging and is related to color stability. This natural process can be strongly accelerated by addition of acetaldehyde. Therefore, upon addition of this compound to a young wine, it can “appear” old. In an attempt to find an analytical method, which would allow to establish the “real” age of a wine, Johnson and Morris [62] used a silica gel column together with a gradient of formic acid to separate different glucoside anthocyanin aggregates from red wines. Using this technique, the anthocyanins were eluted into four fractions. The two first fractions contained free anthocyanins, whereas the two last ones contain polymerized and condensed anthocyanins, respectively [63]. Comparing the amounts of anthocyanins in each fraction from the nontreated and the treated wine, a clear difference appeared, because treated wines contain more aggregates of monoglucoside anthocyanins than the nontreated ones. This may be explained by the fact that acetaldehyde accelerates preferentially the condensation of monoglucoside pigments, but only slightly diglucoside ones [62].

3.2. Thin-layer chromatography

Thin-layer chromatography is often used as a fast, effective and relatively unexpensive analytical method. For instance Hornero-Méndez and Minguez-Mosquera [64] separated carotenoids from *Capsicum annuum* on silica gel GF60 plates in a presaturated chamber. The elution mixture was composed by petroleum ether (65–95 °C), acetone and diethylamine (10:4:1 v/v/v). Special care should however be taken when the separation is done on silica gel, because its slight acidity may trigger pigment degradations such as epoxide–furanoxide rearrangement in carotenoids [65] and chlorophyll-pheophytinization. Therefore, it is necessary to first neutralize the acidity of silica before pigment separation. Unfortunately, the separation of compounds with similar structures with thin-layer chromatography is usually difficult. This is well illustrated by the comparison of the chromatograms obtained from the pigments extracted from pumpkin seed oil and separated by thin-layer chromatography or HPLC [66,67]. Using thin-layer chromatography, four bands are found: the two green bands correspond to protochlorophyll and protopheophytin (protochlorophyll molecules, which have lost their Mg^{2+} atom) pigments, respectively, while the two yellow bands reflect the presence of carotenoids. When the same sample was analyzed by HPLC, 14 peaks were obtained.

As a consequence of the relatively "weak" resolution power of thin-layer chromatography, these methodologies have been progressively supplemented by more efficient separation techniques, such as HPLC [68]. Nevertheless both, open column chromatography and thin-layer chromatography methods, are still of use for cleaning extracts and/or to prepare large amounts of pigments (e.g. [69]).

3.3. Countercurrent chromatography

As supercritical fluid extraction, countercurrent chromatography constitutes an alternative method to solvent extractions and allows the analysis of the large-scale preparation of samples. In fact countercurrent chromatography is an automated version of liquid–liquid extraction, comparable to the repeated partitioning of an analyte between two immiscible solvents by vigorous mixing in a separatory funnel. As each liquid chromatographic technique, countercurrent chromatography operates under gentle conditions and allows a nondestructive isolation of labile natural compounds such as pigments. Due to the absence of any solid stationary phase, adsorption looses are minimized and, consequently, the sample recovery is close to 100% (reviewed in [70]). Countercurrent chromatography has been successfully applied to the isolation of carotenoids from *Gardenia* [7] and anthocyanins from several sources [7,70].

3.4. High-performance liquid chromatography

Photosynthetic pigments, chlorophylls and carotenoids, have a clear hydrophobic character and are usually analyzed by C18-reversed-phase (RP) columns. More recently, a C30-RP appeared on the market. The C30-RP is particularly efficient for the separation of carotenoids because the interactions of the pigments and the stationary phase are maximized by their similar size. With this phase, many *cis*-isomers of the same carotenoid are separated from each other [71–73]. This C30-RP has been successfully applied to the determination of saponified carotenoids in orange juice [74]. However, when a mixture is complex, coelutions may become rapidly limited. When the total amount of *cis*- and *trans*-carotenoid isomers are needed, less selective stationary phases, such as the C18-RP, are therefore preferably used [75,76].

The mobile phase used for the separation of hydrophobic molecules is usually made up of organic solvents, except when polar molecules such as glycosyl esters of carotenoids or chlorophyll c are present in the mixture. In this later case, a polar organic solvent, mixed with a small amount of water is recommended [77,78].

To improve pigment separation, heating of the column is sometimes proposed (carotenoids [79]; anthocyanins [80]). This procedure is, however, not recommended in the case of chlorophylls and carotenoids, because heating can trigger carotenoid isomerization and, chlorophyll epimerization and allomerization, as well. These modifications are not detected using usual HPLC methods [81]. There are many HPLC methods described in the literature for carotenoid analysis (e.g. [1,36,39,64,82,83] and the references therein). Despite of this abundance, when the carotenoid composition is very complex, such as in passiflora fruit, it might be necessary to first separate the different groups of carotenoids, for instance using an open column packed with alumina [84]. Using this method three fractions were obtained: fraction 1, which contained carotenes and epoxi-carotenoids, was eluted with petroleum ether; fraction 2, which was composed by monohydroxy- and keto-carotenoids, was eluted with 70–90% diethyl ether in petroleum ether; and fraction 3, made up of polyhydroxy-carotenoids was eluted with 0–30% ethanol in ether. The pigments contained in individual fractions could further be separated using particular thin-layer chromatography or HPLC methods.

In any case, care should be taken to employ proper chromatographic conditions to ensure that the pigments or degradation products do not escape the analyses. To illustrate this possibility, the HPLC elution profiles of pigments from pumpkin seed oil using two different reversed-phase HPLC methods [75,85] recommended for the analysis of plant pigments are compared (Fig. 2). HPLC has also been used to evaluate the purity of commercially available food colorants deriving from natural plant pigments [82,86,87].

While most of the methods used for separation of photosynthetic pigments only require organic solvents, the elution mixtures necessary to separate more hydrophilic compounds, such as anthocyanins and betalains, are usually a mixture of organic and aqueous solvents (e.g. [88]). Before analysis of anthocyanins by HPLC, it is recommended

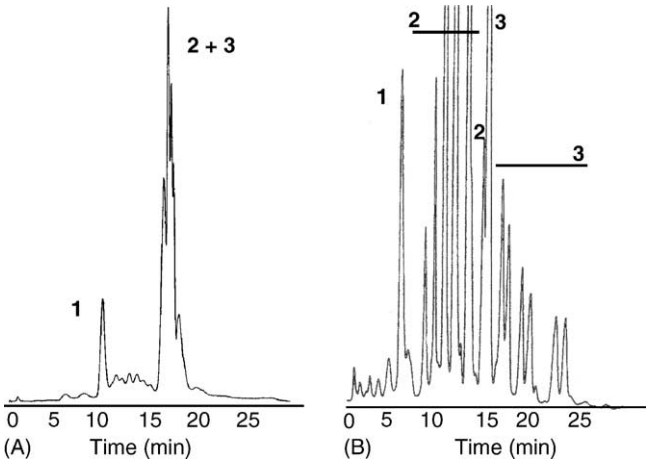

Fig. 2. Chromatogram recorded at 440 nm of the pigments from pumpkin seed oil. (A) HPLC (methanol, acetonitrile, methylene chloride, see [67]). (B) HPLC (methanol, methanol–hexane, see [85]). (1) Lutein; (2) protochlorophyll; (3) protopheophytin.

to purify the extract on a C18-RP cartridge previously activated with acidified methanol [29,46]. Using a similar protocol Takeoka et al. [89] analyzed the anthocyanin content of seed coat from black bean by HPLC. Three anthocyanins were found: delphinidium 3-glucose, petunidin 3-glucose and malvidin 3-glucoside. Because the A_{ACYL}/A_{VIS} ratio reflects the molar ratio of the cinnamic acid and anthocyanin [90], the use of a photodiode array detector allows to obtain additional information about the acylation or the glycosylation patterns of anthocyanins [46]. For instance the absence of an absorbance peak between 300 and 350 nm in the absorbance spectra of the eluted pigments suggested that none of them was acylated with aromatic amino acids [91]. HPLC analyses of anthocyanins from radish have established that the eight different anthocyanins separated were acylated [92]. The comparison of the absorption spectra of acylated and nonacylated compounds revealed that acylation shifts the anthocyanin color to the shorter wavelengths [92,93]. HPLC elution programmes for anthocyanins are constantly improved and today it is possible to separate up to 15 different anthocyanins within a single 30 min run [94]. However, determination and quantification of anthocyanins by HPLC suffer from the general lack of availability of pure anthocyanin standards.

3.5. Supercritical fluid chromatography

Supercritical fluid extraction has successfully been used to isolate β-carotene from carrots [95] and capsanthin from paprika [96]. Favati et al. [97] isolated β-carotene and lutein from leaf concentrates at 40 °C at pressures ranging between 29 and 70 MPa. Although some laboratory equipments can work with less than 2 g, a major limitation of the use of supercritical fluid extraction is the high amount of material needed (kg range, see [36]). From the analytical point of view, supercritical extraction is compatible with supercritical fluid chromatography, because the two techniques can share the mobile phase and some devices, favoring the development of extraction and separation methodologies. Because the elution strength of pure CO_2 with respect to carotenoids is rather weak, it is necessary to add cosolvents, to increase the carotenoid solubility in CO_2. The solubility parameter theory predicts that the maximum solubility of a compound is reached, when the solubility parameter of the solvents equals that of the solute. For instance, β-carotene can be extracted at 43 °C and approximately 70 MPa [97]. Unfortunately, some equipments do not allow elution at such an elevated pressure. Therefore, new conditions of pressure and temperature should be found using a diagram connecting the variations of pressure to gas densities for different temperatures [98]. At lower temperatures, densities similar to those reached at higher temperatures, can be obtained for lower pressures. These conditions are referred to as subcritical conditions. Ibanez et al. [99] successfully separated β-carotene from lycopene in less than 10 min under these conditions. The best separation was obtained at 10 °C under a pressure of approximately 35 MPa, with a home-packed capillary column containing deactivated silonal groups with an octamethyl-cyclotetrasiloxane reagent. This column cannot be used at higher temperatures, because the structure of the phase may be broken at high temperatures, forming O–Si–CH_3 bonds with the silica phase.

In many cases the experiments were focused on the determination of the optimal pressure, temperature, and separation steps to achieve carotenoid extraction. In contrast the carotenoid concentration profile in the supercritical step received a much lower attention. Ambrogi et al. [100] reported the variation of carotenoid concentration along the process. Under their conditions of pressure (30 MPa), temperature (60 °C) and CO_2 flow (8–9 kg/h), 75% of the total carotenoid were recovered involving only 15% of the total CO_2 needed for the total extraction of the pigments. In addition, the HPLC analyses of the carotenoids extracted at selected steps of the process indicate that the different types of carotenoids, i.e. free xanthophylls, xanthophyll monoesters, xanthophyll polyesters, and β-carotene from paprika fruits were extracted in the same proportion.

3.6. Capillary electrophoresis

Pigments with very similar structure might be difficult to separate using classical RP-C18 HPLC columns. This is the case for zeaxanthin and lutein. To overcome this difficulty, a particular HPLC solvent program should be applied [101]. Alternatively other separation methods such as capillary column can be used. The main interest of capillary electrophoresis lies to the fact that it allows fast separations of the components at relatively low costs, because the amounts of solvent and waste are strongly reduced. Capillary electrophoresis was successfully used in the separation of zeaxanthin and lutein from eye humor [102]. The good separation and the fast elution of the pigments suggest that

capillary electrophoresis is suitable for routine analysis of tiny samples. Capillary electrophoresis was employed for the separation of anthocyanin pigments from strawberry, elderberry, and blackcurrant fruits [103,104].

Micellar electrokinetic chromatography has been used to analyze the anthocyanin content of several food samples [105].

4. Detection of pigments

Once separated the eluted pigments have to be identified and quantified. While pigment identification is often based on spectral properties and the chromatographic behavior of individually eluted molecules, quantifications are mostly based on absorbance measurements.

Details on the molecular structure of the eluted molecules can be obtained using sophisticated analytical methods such as mass spectrometry (MS) and multidimensional NMR. In the following, several methods for the identification and the quantification of vegetable pigments are presented. A short discussion about the standards has also been provided.

4.1. UV-Vis spectroscopy

The absorbance spectra of pigments can be considered as its fingerprint (for limitations see below). Therefore, absorbance spectroscopy constitutes the simplest way to identify and quantify the major pigments present in a mixture [66,82,87,107,108]. Several possibilities, ranging from manual measurements using a colorimeter to diode-array detectors, can be used. Here, I wish to emphasize the importance of the conditions needed for an accurate determination of pigments.

Because the environment of the pigment (e.g. solvent, temperature, ligation to protein, etc.) may strongly influence the position of the absorbance maximum and the shape of the spectrum, an accurate measurement of pigment concentration requires that the pigment is dissolved into a solvent for which specific (or molar) absorbance coefficients have been determined. With these data, it is always possible to establish a new equation set, adapted to the particular situation, such as complex elution mixture used in HPLC. The precision of the measurements depends on the type of the device used, on the exact determination of the position of the absorbance maxima and, of course, on the accuracy of the absorption coefficient used for the calculation. It is therefore advisable to check regularly the literature for new values. The major limitation in the identification of pigments on the sole basis of the absorbance spectrum is the overlapping of the absorbance bands of individual pigments present in the mixture. When two pigments coelute, it is usually possible to establish a set of equations, which will allow the calculation of the concentrations of both pigments (for example, see [101]). This method, however, is much less efficient when the number of pigments is higher. For completeness, it is necessary to add that the *cis*-carotenoid isomers can be recognized, because the absorbance spectrum presents an additional band in the UV-region. The position of the *cis*-double bound is reflected in ratio A_{cis}/A_{MAX}. The total phenols in an anthocyanin preparation can be routinely determined using the Folin–Ciolcoteau procedure [109].

Fluorescence spectroscopy can be more diagnostically helpful due to the selective excitation of pigments, such as chlorophylls and bilins, but useless in case of carotenoids, since their fluorescence is very weak [110].

4.2. IR and CD spectroscopies

In some occasions, other spectroscopic methods can be used to identify pigments on the basis of a particular structural feature. For instance infrared (IR) spectroscopy was used to reveal the presence of an allylic group in the carotenoids fucoxanthin, alloxanthin, and bastaxanthin (reviewed in [36]). IR spectroscopy was also employed to establish the details of the light-induced oxygen-dependent bleaching of the food colorant chlorophyllin [54] (reviewed in [111]).

In conclusion, spectroscopic methods usually permit a crude identification of the pigments present in an extract, but in most cases the specific composition can remain obscure. Therefore, obtaining detailed information about the composition of a mixture of pigments requires additional analyses. These analyses often involve the separation of the mixture into its components using methods such as chromatography.

None of the methods described above is entirely suitable for elucidation of the structures of pigments. Although spectral fingerprint contains enough information to allow identification of the chromophore, it does not always contain enough information to determine the complete structure of the pigment (e.g. [67,87,108]). In many cases, especially with anthocyanins, the structure of the pigment is much different from that of the chromophore. Although the missing information can be partly deduced from the chromatographic behavior and from the comparison of the obtained retention data with literature, other methods such as mass spectrometry have to be employed to fully characterize the structure of the molecule [112] (reviewed in [108]).

4.3. Mass spectrometry

Chlorophylls and carotenoids have long presented special analytical challenges to MS, because of their high mass, low volatility and thermal instability. The use of methods such as chemical ionization, secondary ion MS, fast-atom bombardment, field-, plasma- and, recently, matrix-assisted laser desorption for which the necessity of sample vaporization prior to the ionization is suppressed opened ways to the molecular ion detection and, thus, to direct molecular weight determination. Unfortunately, these methods

produced additional peaks in the region of lower masses, reflecting the presence of chlorophyll degradation products, such as 10-OH chlorophyll *a* (*m*/*z* 908) or Pheo *a* (*m*/*z* 870) and additional signals at *m*/*z* 482, 556 and 615. From these results, it is difficult to decide, whether the additional signals reflect molecules present in the sample before the analysis or the presence of additional peaks results from pigment degradation(s) during sample preparation and/or analysis [82] (reviewed [108]). The most recent progress in MS analysis of tetrapyrroles has been obtained with the development of atmospheric ionization methods, i.e. atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). The APCI technique, in combination with RP-HPLC, is proved to be efficient for detecting chlorophyll *a* and its nine degradation products at low nanogram levels. The procedure is approximately 1000 times more sensitive than the thermospray ionization [113]. To demonstrate the potential of electrospray MS in the chlorophyll research, ESI interface was employed with an ion trap mass spectrometer as mass analyzer [112,114,115]. ESI is a mild ionization technology feature, allowing the formation of a high proportion of the chlorophyll *a*-protonated molecular ion $(M + H)^+$, *m*/*z* 893.5. It is remarkable that the spectrum was obtained with approximately 2 pmol of the compound. Further structural information has been obtained with the unique MS^N capability of the ion trap analyzer, allowing consecutive dissociation of side chain functional groups from the selected precursor ion (up to eight steps [112]). The MS^N procedure is highly selective and enables consecutive cleavage of at least seven functional groups around the porphyrin, providing valuable structural information about the tetrapyrrole. Using MS methods, chlorophyll allomers and their derivatives, produced during fruit and vegetable processing [116], have been isolated and their structures elucidated [82].

Despite the facts that at low pH anthocyanins are positively charged molecules and are very soluble in water and alcohol, their high MW, which ranges from a few hundred to a few thousands [117], makes their analysis by MS-methodologies difficult. As in the case of tetrapyrrole pigments, the ESI method appears especially suitable for ionization of this family of labile, non-volatile polar compounds. Using this method, Giusti et al. [41,42,94] obtained structural information on a minor anthocyanin component of grape juice and red cabbage. A similar method was used to analyze the anthocyanins present in extracts of grape skins and red wine [118] as well as blackcurrant fruit [104].

A limitation of MS in the analysis of anthocyanins and alkaloids arises from its inability to differentiate between various diasterisomeric forms of sugars. Therefore, this method cannot provide information on the exact glycosidic substitution(s) other than the number of carbon atoms and the presence of methyl side group(s) bound to the sugar moiety. To obtain more information on the structure of these substitutions, it is necessary to use MS–MS technology. When applied to the anthocyanins from grape and red cabbage, typical patterns were obtained [94]. The MS–MS resulted in the cleavage of glycosidic bonds only between the flavilium ring and the sugar directly attached to it. In the case of acylated anthocyanins, the fragmentation pattern allows a rough determination of the localization of the acylating group [94].

ESI–MS has also been successfully employed for determination of the molecular structure of other water-insoluble pigments, such as the polyphenolic yellow molecules, contained in turmeric extracted from the rhizome of *Curcuma longa* [69].

4.4. NMR spectrometry

Additional information on the structure of pigment molecules can also be obtained by NMR. For instance, Choung et al. [80] used NMR to identify the HPLC-separated anthocyanins from the seed coats of 60 kidney beans cultivated in Korea. The study revealed that red and brown seed coats contain several anthocyanins: cyanidin 3,5-diglucoside, delphinin 3-glucoside, cyanidin 3-glucoside, and pelargonin 3-glucoside, but not petunin 3-glucoside. The latter anthocyanin is only present in black seed coats. White seed coats are devoid of anthocyanin. NMR has also been recently used to establish the structure of anthocyanins, such as gentiodelphin and 5-carboxypyranopelargonin [119–123], betacyanins [124]. NMR is a very powerful tool for the determination of the structures of pigments. However, it has two major intrinsic limitations:

- its requires a relatively high amount of samples (mg range);
- data acquisition may be long, especially in the case of 2D-NMR. Therefore, the analyst should be certain that the pigment under study would remain stable during the whole period of analysis. For instance betalains are labile under highly acidic conditions [125]. Under these conditions, pigment degradation proceeds very quickly resulting in complex NMR spectra and also in the loss of the sample. To overcome these difficulties, Stintzing et al. [124] tested several combinations of solvents. They found that the most suitable solvent is deuterium oxide (D_2O) at 25 °C in the pH range 5–7 because it allows to achieve long-term stability of the pigments and is compatible with ^{13}C-NMR requirements. Under these conditions, the solubility of betalains is sufficient and little overlapping of system and sample signals occurred. Alternatively, stop flow NMR hyphenated to HPLC (LC-NMR) can be used for rapid identification of natural products in extracts, pre-purified fractions or labile compounds [126–128].

The 3D conformation of anthocyanin pigments can be investigated using the nuclear overhauser enhancement spectroscopy (NOESY) technique.

5. Pigment identification and quantification: the problem of standards

In the preceding sections, the different analytical methods, which can be used to identify pigments, were presented. One crucial point is the calibration of the signal detector. This question requires standards, ideally identical to each pigment under consideration. Those standards are not always commercially available and should therefore be prepared. Thinking to this problem, Schiedt and Liaaen-Jensen [65] have defined the minimum criteria for identification of carotenoids: (1) the absorbance spectra in the UV–vis region, obtained in at least two different solvents, should be in agreement with the chromophore suggested; (2) the chromatographic properties of the putative pigment and standard must be identical in thin-layer chromatography (R_f) and HPLC (t_R): both compounds should co-elute; (3) a mass spectrum should be obtained, which allows at least the confirmation of the molecular mass. Although such "rules" were not specified for the identification of other natural pigments, similar criteria are suggested. Even when the identification and detector calibration have been performed according to these rules, pigment quantification may not be straightforward. For instance, the amount of pigments in a heated sample may be higher than before. Such a paradox, which was frequently reported in blanched and cooked vegetables or fruits (e.g. [128]), has been attributed to the greater extractability of the pigments after cooking because heating would trigger cell-wall rupture, then facilitating the release of pigments from the cells.

6. Future trends

Research on plant pigments is fascinating. It involves many methods and a deep knowledge in several fields. Plant pigment studies should be continued, because they may procure several immediate interesting impressions as well as commercial and health advantages. For these advantages it is important to have at disposal the most powerful methodologies to analyze the pigment composition of samples. These methods could involve techniques such as infrared spectroscopies [111], NMR spectroscopies [129] etc., which, in the past, were more restricted to basic research.

Acknowledgments

B.S. is grateful to Mrs. S. Vucsak for her careful reading of the manuscript.

References

[1] G. Britton, The Biochemistry of Natural Pigments, Cambridge University Press, Cambridge, 1988.
[2] J. Gross, Pigments in Vegetables. Chlorophyll and Carotenoids, Van Nostrand Reinhold, New York, 1991.
[3] G. Britton, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, vol. 1B: Spectroscopy, Birkhäuser, Basel, 1995, pp. 13–62.
[4] J.M. Kong, L.-S. Chia, N.-K. Got, T.-F. Chia, R. Brouillard, Phytochemistry 64 (2003) 923.
[5] P. Markakis, Anthocyanins as Food Colors, Academic Press, New York, 1992.
[6] J. Smith, Food Additive User's Handbook, Van Nostrand Reynold, New York, 1991.
[7] A. Degenhardt, P. Knapp, P. Winterhalter, in: T. Ames, T. Hofmann (Eds.), Chemistry and Physiology of Selected Food Colorants. ACS Symposium Series, vol. 775, American Chemical Society, Washington, DC, 2001, p. 22.
[8] T.P.C. Coultate, Food: The Chemistry of its Components, third ed., Royal Society of Chemistry, Cambridge, 1991.
[9] D.H. Baker, Amino Acids 2 (1992) 1.
[10] S. Depee, C.E. West, D. Permaesih, S. Martuti, A. Muhilal, J.G.A.J. Hautvast, Am. J. Clin. Nutr. 68 (1998) 1058.
[11] J.J.M. Castenmiller, C.E. West, J.P.H. Linssen, K.H. van Het Hof, A.G.J. Voragen, J. Nutr. 129 (1999) 349.
[12] D.-I. Corol, I.I. Drobantu, N. Toma, Roum. Biotechnol. Lett. 8 (2003) 1069.
[13] T.M. Parkinson, J.P. Brown, Ann. Rev. Nutr. 1 (1981) 175.
[14] G. Cao, R.L. Prior, Clin. Chem. 45 (1999) 574.
[15] T. Lapidov, S. Harel, R. Granit, J. Kanner, J. Agric. Food Chem. 46 (1998) 4297.
[16] B.C. Goswani, A.B. Barua, J. Labelled Compd. Radiopharm. 46 (2003) 843.
[17] H. Gerster, Int. J. Vitam. Nutr. Res. 63 (1993) 93.
[18] J.T. Kumpulainen, J.T. Salonen, Natural Antioxidants and Food Quality in Atherosclerosis and Cancer Prevention, The Royal Society of Chemistry, Cambridge, 1996.
[19] S. Franceschi, E. Bidoli, C. Vecchia, R. Talamini, B. D'Avanzo, E. Wegni, Int. J. Cancer 59 (1994) 181.
[20] S.T. Mayne, D.T. Janerich, P. Greenwald, S. Chorost, C. Tucci, M.B.. Zaman, R. Melamed, M. Kiely, M.F. McKneally, J. Natl. Cancer Inst. 86 (1994) 33.
[21] J.S. Shim, J.H. Kim, H.-Y. Cho, Y.N. Yum, S.H. Kim, H.-J. Park, B.S. Shim, S.H. Choi, H.J. Kwon, Chem. Biol. 10 (2003) 695.
[22] J.P. Groten, W. Butler, V.J. Feron, G. Kazianowski, A. Renwicks, R. Walker, Regul. Toxicol. Pharmacol. 31 (2000) 77.
[23] G.W. Burton, K.U. Ingold, Science 244 (1984) 569.
[24] P. Palozza, N.I. Krinsky, Free Radic. Biol. Med. 11 (1991) 407.
[25] C. Garcia-Viguera, J.C. Espin, C. Soler, H.J. Wichers, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 88.
[26] D.E. Pszczola, Food Technol. 52 (1998) 70.
[27] J. Joppen, Food Eng. Ingredients 28 (2003) 12.
[28] D.B. Rodriguez-Amaya, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 252.
[29] R. Timmaraju, N. Bhagyalakshmi, M.S. Narayan, G.A. Rawishankar, Process Biochem. 38 (2003) 1069.
[30] I. Konczak-Islam, S. Okuno, M. Yoshimoto, O. Yamakuwa, Biochem. Eng. J. 14 (2003) 155.
[31] N. Plata, I. Konczak-Islam, S. Jayram, K. McClelland, T. Woolford, P. Franks, Biochem. Eng. J. 14 (2003) 171.
[32] M. Yamazaki, Y. Makita, K. Springob, K. Saito, Biochem. Eng. J. 14 (2003) 191.
[33] X. Ye, S. Al-Balili, A. Kloti, J. Zhang, P. Lucca, P. Berger, I. Potrykus, Science 287 (2000) 303.
[34] G. Sandmann, Chem. Biol. 10 (2003) 478.

[35] M. Anese, P. Falcone, V. Fogliano, M.C. Nicolli, R. Massini, J. Food Sci. 67 (2002) 3442.
[36] G. Britton, S. Liaaen-Jensen, H. Pfander, Carotenoids, vol. 1–3, Birkhaüser, Basel, 1995.
[37] A.K. Taungbodhitham, G.P. Jones, M.L. Wahqvist, D.R. Briggs, Food Chem. 63 (1998) 577.
[38] J. Kopecky, B. Schoefs, K. Loest, D. Stys, O. Pulz, Arch. Hydrobiol. Algol. Stud. 98 (2000) 153.
[39] K.A. Thompson, M.R. Marshall, C.A. Sims, C.I. Wei, S.A. Sargent, J.W. Scott, J. Food Sci. 65 (2000) 791.
[40] E. Tsatsaroni, M. Liakopoulou-Kyriakides, I. Eleftheradis, Dyes Pigments 37 (1998) 307.
[41] M.M. Giusti, D.E. Wrolstad, J. Food Sci. 61 (1996) 322.
[42] M.M. Giusti, D.E. Wrolstad, J. Food Sci. 61 (1996) 688.
[43] L. Gao, G. Mazza, J. Food Sci. 61 (1996) 600.
[44] A. Gürenç, A. Karaali, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 135.
[45] E. Revilla, J.M. Ryan, G. Martin-Ortega, J. Agric. Food Chem. 46 (1998) 4592.
[46] V. Hong, R.E. Wrolstad, J. Agric. Food Chem. 38 (1990) 708.
[47] C. Garcia-Viguera, P. Zaffrilla, F.A. Tomas-Barberans, Phytochem. Anal. 9 (1998) 274.
[48] J. Oszmianski, C.Y. Lee, Am. J. Enol. Vitic. 41 (1990) 204.
[49] H. Aoki, T. Koda, T. Ichi, M. Nakamura, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 1.
[50] R. Carle, F.C. Stintzing, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 43.
[51] H.-D. Martin, C. Ruck, M. Schmidt, S. Sell, S. Beutner, B. Mayer, R. Walsh, Pure Appl. Chem. 71 (1999) 2253.
[52] T. Anguelova, J. Warthesen, J. Food Sci. 65 (2000) 71.
[53] H. Pfander, B. Traber, A. Häberli, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 213.
[54] M.L. Salin, M.L. Avarez, B.C. Lynn, B. Habulihaz, A.W. Fountain, Free Radic. Res. 31 (1999) 97.
[55] T. Omata, N. Murata, Plant Cell Physiol. 22 (1983) 1093.
[56] H.H. Strain, B.T. Cope, W.A. Svec, Methods Enzymol. 23A (1971) 452.
[57] J.W.F. Wasley, W.T. Scott, S. Holt, Can. J. Bot. 48 (1970) 376.
[58] H.W. Siegelman, J.H. Kycia, in: J.A. Hellebust, J.S. Craigie (Eds.), Handbook of Physiological Methods, vol. 2, Cambridge University Press, Cambridge, 1978, p. 71.
[59] T.C. Somers, Nature 209 (1966) 368.
[60] K. Yoshida, R. Okumo, K. Kameda, M. Mori, T. Kodo, Biochem. Eng. J. 14 (2003) 163.
[61] T.C. Somers, M.E. Evans, J. Sci. Food Agric. 28 (1977) 279.
[62] T.V. Johnson, J.R. Morris, J. Food Sci. 61 (1996) 109.
[63] K. Datzberger, I. Steiner, J. Washuttl, G. Kroyer, Z. Lebensm. Unters. Forsch. 193 (1991) 462.
[64] D. Hornero-Méndez, M.I. Minguez-Mosquera, J. Agric. Food Chem. 46 (1998) 4087.
[65] K. Schiedt, S. Liaaen-Jensen, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, vol. 1A: Isolation and Analysis. Birkhäuser, Basel, 1995, p. 81.
[66] B. Schoefs, Adv. Food Sci. Nutr. (2004), in press.
[67] B. Schoefs, Lebensm. Biotechnol. 2001 (1) (2001) 8.
[68] T. Braun, S. Zsindely, Trends Anal. Chem. 11 (1992) 267.
[69] X.-G. He, L.-Z. Lin, L.-Z. Lian, M. Lindenmaier, J. Chromatogr. A 818 (1998) 127.
[70] M. Schwarz, S. Hillebrand, S. Habben, A. Degenhardt, P. Winterhalter, Biochem. Eng. J. 14 (2003) 179.
[71] C. Emenhiser, L.C. Sander, S.J. Schwartz, J. Chromatogr. A 707 (1995) 205.
[72] K.E. Sharpless, J.B. Thomas, L.C. Sanders, S.A. Wise, J. Chromatogr. B 678 (1996) 187.
[73] T. Lacker, S. Stroschein, K. Albert, J. Chromatogr. A 854 (1999) 37.
[74] R. Rousseff, L. Raley, H.-J. Hofsommer, J. Agric. Food Chem. 44 (1996) 2176.
[75] B. Schoefs, M. Bertrand, Y. Lemoine, J. Chromatogr. A 692 (1995) 239.
[76] B. Schoefs, Y. Lemoine, M. Bertrand, Am. Biotechnol. Lab. 14 (1996) 18.
[77] C. Dufresne, F. Cormier, S. Dorion, U.A. Niggli, S. Pfister, H. Pfander, Enzyme Microb. Technol. 24 (1999) 453.
[78] M. Latasa, K. van Lenning, J.L. Garrido, R. Scharek, M. Estrada, F.F. Rodriguez, M. Zapata, Chromatographia 53 (2001) 385.
[79] L. Vanheukelem, J. Lewitsu, T.M. Kata, N.E. Craft, Mar. Ecol. Prog. Ser. 114 (1994) 303.
[80] M.-G. Choung, B.-R. Choi, Y.-N. An, Y.-H. Chu, Y.-S. Cho, J. Agric. Food Chem. 51 (2003) 7040.
[81] K. Hyvärinen, P.H. Hynninen, J. Chromatogr. A 837 (1999) 107.
[82] B. Schoefs, Trends Food Sci. Technol. 13 (2002) 361.
[83] B. Schoefs, Trends Anal. Chem. 22 (2003) 335.
[84] A.Z. Mercadante, G. Britton, D.B. Rodriguez-Amaya, J. Agric. Food Chem. 46 (1998) 4102.
[85] P. Ilik, R. Kouril, J. Fiala, J. Naus, F. Vacha, J. Photochem. Photobiol. B: Biol. 59 (2000) 103.
[86] S. Chernomorsky, R. Rancourt, D. Sahai, R. Poretz, J. AOAC Int. 80 (1997) 433.
[87] B. Schoefs, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 85.
[88] P. Sarni-Manchado, H. Fulcrand, J.-M. Souquet, V. Cheynier, M. Moutounet, J. Food Sci. 61 (1996) 938.
[89] G.R. Takeoka, L.T. Dao, L.T. Full, R.Y. Wong, L.A. Harden, R.H. Edwards, J.D.J. Berrios, J. Agric. Food Chem. 45 (1997) 3395.
[90] J.B. Harborne, J. Chromatogr. 1 (1958) 473.
[91] O.M. Andersen, Acta Chem. Scand. B 42 (1988) 462.
[92] M.M. Giusti, L.E. Rodriguez-Saona, D. Griffin, D.E. Wrolstad, J. Agric. Food Chem. 47 (1999) 4657.
[93] M.M. Giusti, L.E. Rodriguez-Saona, D. Griffin, D.E. Wrolstad, J. Agric. Food Chem. 47 (1999) 4631.
[94] M.M. Giusti, D.E. Wrolstad, L.E. Rodriguez-Saona, in: J.F. Francis (Ed.), Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery and Cosmetics, SIC Publishing, Hamden, 2000, p. 95.
[95] P.J. Vega, M.O. Balaban, S.F. O'Keefe, J.A. Cornell, J. Food Sci. 61 (1996) 757.
[96] M. Jarén-Galan, U. Nienaber, S.J. Schwartz, J. Agric. Food Chem. 47 (1999) 3558.
[97] F. Favati, J.W. King, J.P. Friedrich, K. Eskins, J. Food Sci. 53 (1988) 1532.
[98] R.M. Smith, Supercritical Fluid Chromatography, RSC Chromatography Monographs, London, 1988.
[99] E. Ibanez, S. Lopez-Sebastian, J. Tabera, G. Reglero, J. Chromatogr. A 823 (1998) 313.
[100] A. Ambrogi, R. Cardarelli, R. Eggers, J. Food Sci. 67 (2002) 3236.
[101] E. Darko, B. Schoefs, Y. Lemoine, J. Chromatogr. A 876 (2000) 111.
[102] A. Karlsen, G. Alexander, R. Blomhoff, T.E. Gundersen, J. Chromatogr. B 795 (2003) 17.
[103] P. Bridle, J. Liq. Chromatogr. Related Technol. 19 (1997) 537.
[104] C.T. da Costa, B.C. Nelson, S.A. Margolis, D. Horton, J. Chromatogr. A 799 (1998) 321.

[105] T. Watanabe, A. Yamamoto, S. Nagais, S. Terabes, Anal. Sci. 14 (1998) 839.
[106] M. Bertrand, B. Schoefs, in: M. Pessarakli (Ed.), Handbook of Photosynthesis, first ed., Marcel Dekker, New York, 1997, p. 151.
[107] M. Bertrand, J. Garrido, B. Schoefs, in: M. Pessarakli (Ed.), Handbook of Photosynthesis, second ed., Marcel Dekker, New York, in press.
[108] V.L. Singleton, J.A. Rossi, Am. J. Enol. Vitic. 16 (1965) 144.
[109] Y. Koyama, H. Hashimoto, in: G. Britton, A.J. Young (Eds.), Carotenoids in Photosynthesis, Chapman & Hall, London, 1993, p. 327.
[110] D. Chenery, H. Bowring, Spectrosc. Eur. 15 (2003) 8.
[111] B. Schoefs, Int. Lab. 33 (2001) 34.
[112] C.B. Eckhardt, B.J. Keely, J.R. Maxwell, J. Chromatogr. 557 (1991) 271.
[113] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[114] T. Hutton, H.J. Major, Biochem. Soc. Trans. 23 (1995) 924.
[115] M.I. Minguez-Mosquera, L. Gallardo-Guerrero, D. Homero-Mendez, J. Garrido-Fernandez, J. Food Protec. 58 (1995) 564.
[116] N. Terahara, N. Saito, T. Honda, K. Toki, Y. Osajima, Tetrahedron Lett. 31 (1990) 2921.
[117] I. Revilla, S. Perez-Magarino, M.L. Gonzalez-SanJosé, S. Beltran, J. Chromatogr. A 847 (1999) 83.
[118] M. Fukuchi-Mizutani, H. Okuhara, Y. Fukui, M. Nakao, Y. Katsumoto, K. Yonekura-Sakakibara, T. Kusumi, T. Hase, Y. Tanaka, Plant Physiol. 132 (2003) 1652.
[119] K. Kazuma, N. Naonobu, S. Masahiko, Phytochemistry 64 (2003) 1133.
[120] M.M. Giusti, E.S. Luis, R. James, J. Food Sci. 63 (1998) 219.
[121] M.M. Giusti, H. Ghanadan, R.E. Wolstrad, J. Agric. Food Chem. 46 (1998) 4558.
[122] O.M. Andersen, T. Fossen, K. Toskgerpoll, A. Fossen, U. Hauge, Phytochemistry 65 (2004) 405.
[123] F.C. Stintzing, J. Conrad, I. Klaiber, U. Beifuss, R. Carle, Phytochemistry 65 (2004) 415.
[124] N. Kobayashi, J. Scmidt, M. Nimtz, V. Wray, W. Schliemann, Phytochemistry 54 (2000) 419.
[125] S.C. Bobzin, S. Yang, T.P. Kasten, J. Chromatogr. B 748 (2000) 259.
[126] B. Vogler, I. Klaiber, G. Roos, C.U. Walter, W. Hiller, P. Sandor, W. Kraus, J. Nat. Products 61 (1998) 175.
[127] W. Stahl, H. Sies, J. Nutr. 122 (1992) 2161.
[128] M. Borsari, E. Ferrari, R. Grandi, M. Saladini, Inorg. Chim. Acta 328 (2002) 61.