JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

^{1}H NMR Quantitative Determination of Photosynthetic Pigments from Green Beans (*Phaseolus vulgaris* L.)

JUAN VALVERDE* AND HERVÉ THIS

Equipe INRA de Gastronomie Moléculaire, UMR 214, (INRA, Institut des sciences du vivant et de l'environnement [AgroParisTech]), 16 rue Claude Bernard, 75005 Paris

Using ^{1}H nuclear magnetic resonance spectroscopy (1D and 2D), the two types of photosynthetic pigments (chlorophylls, their derivatives, and carotenoids) of "green beans" (immature pods of *Phaseolus vulgaris* L.) were analyzed. Compared to other analytical methods (light spectroscopy or chromatography), ^{1}H NMR spectroscopy is a fast analytical way that provides more information on chlorophyll derivatives (allomers and epimers) than ultraviolet-visible spectroscopy. Moreover, it gives a large amount of data without prior chromatographic separation.

KEYWORDS: ***Phaseolus vulgaris* L.; crude extracts; plant pigments; ^{1}H NMR**

INTRODUCTION

Green vegetables are widely consumed all around the world (*1*). Their color is an important factor of appreciation (*2*). Chlorophylls (Chls) and carotenoids (Cars) (**Figure 1**) have been considered to be responsible for the color of fresh green plants (*1, 6, 16, 25*). Other Chls derivatives such as pheophytins (Pheos) are not widely present in fresh green plants (*31*), but their content increases during thermal processing (*1, 2, 11, 32*); their contribution to the color is then important, even when they are present in small quantities, because the molar extinction coefficient of Pheos (in the majority of solvents) is larger than for their respective Chls (*1–5*). The main Cars present in green beans are lutein (Lut) and β-carotene (β-Car) (*9, 12, 16, 27, 28*), but other Cars, such as neoxanthin (Neo) and violaxathin (Vio), have also been previously observed (*16, 25, 28*).

Several methods have been used to determine the content of these pigments in plants: ultraviolet–visible (UV-Vis) spectroscopy, high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC). UV–Vis spectroscopy (*2–5*) is useful for determining the content in Chl a and b and total Cars (TCars) in extracts made from fresh plants; it was recently proposed as an alternative to HPLC (*5*). HPLC has been used to get a lot of information on Chls, their derivatives, and Cars from fresh or thermally processed samples (*6–11*). Although the pigment analysis by some HPLC methods is fast, they can be complex, labor intensive, and time-consuming. When retention time is used for identification, the possibility of the presence of several compounds with similar retention times cannot be excluded, even with the addition of reference compounds (*13*); also, many labile substances undergo decomposition or modification during the chromatographic separation step. In particular, Cars oxidation can be catalyzed by the stationary phase (*30*). Finally, TLC is mainly used for identification (*14*), but it can also be used for direct quantification (*15, 16*).

Since the 1990s, NMR spectroscopy has been widely used to analyze lipids in crude extracts from plant or algae tissues (*13, 17, 30*) without the drawbacks mentioned above. Simple ^{1}H NMR spectroscopy of lipid fractions was used only to get general classes of compounds (cell membrane compounds: lipids, sterols, and fatty acids) because protons from all molecules in the studied extracts have overlapping resonances. ^{13}C NMR and two-dimensional (2D) correlation techniques give better results because the narrower ^{13}C peaks do not overlap for lipophilic mixtures (*13*). Despite the constraints, ^{1}H NMR metabolic profiles of crude extracts from plants can contain huge quantities of complementary information (*17, 18*), which can be selectively treated to analyze only some of the components contributing to the whole spectrum. In particular, lipophilic pigments of plants can be analyzed because their signals are easy to identify in a complex mixture (*18, 30*).

Because NMR spectra contain the resonances of all components with concentrations higher than the detection threshold (around 50–100 μM), it has been used not only for identification (*18, 21, 24, 25, 30*) and structure elucidation but also for quantification (*19, 18*). For this purpose, it can be coupled to HPLC (*21*), but it has also been used without a prior chromatographic separation step to calculate the total mass of primary and secondary metabolites (*20*). This offers several advantages for quantification of chemically labile compounds such as plant pigments: no sample preparation is required after extraction, and no preselection of the experimental conditions is necessary. Also, NMR spectroscopy has been demonstrated to be a powerful technique for identifying and determining the structural properties of Chls derivatives (*22, 23*) and Cars (*24, 25, 30*). The aim of the present study is to directly identify and quantify the main photosynthetic pigments in green bean crude extracts using NMR methods.

* Corresponding author. Telephone: +33 (0)1 44 08 16 60, e-mail: juan.valverde@agroparistech.fr.

10.1021/jf070277j CCC: $40.75
Published on Web 12/15/2007

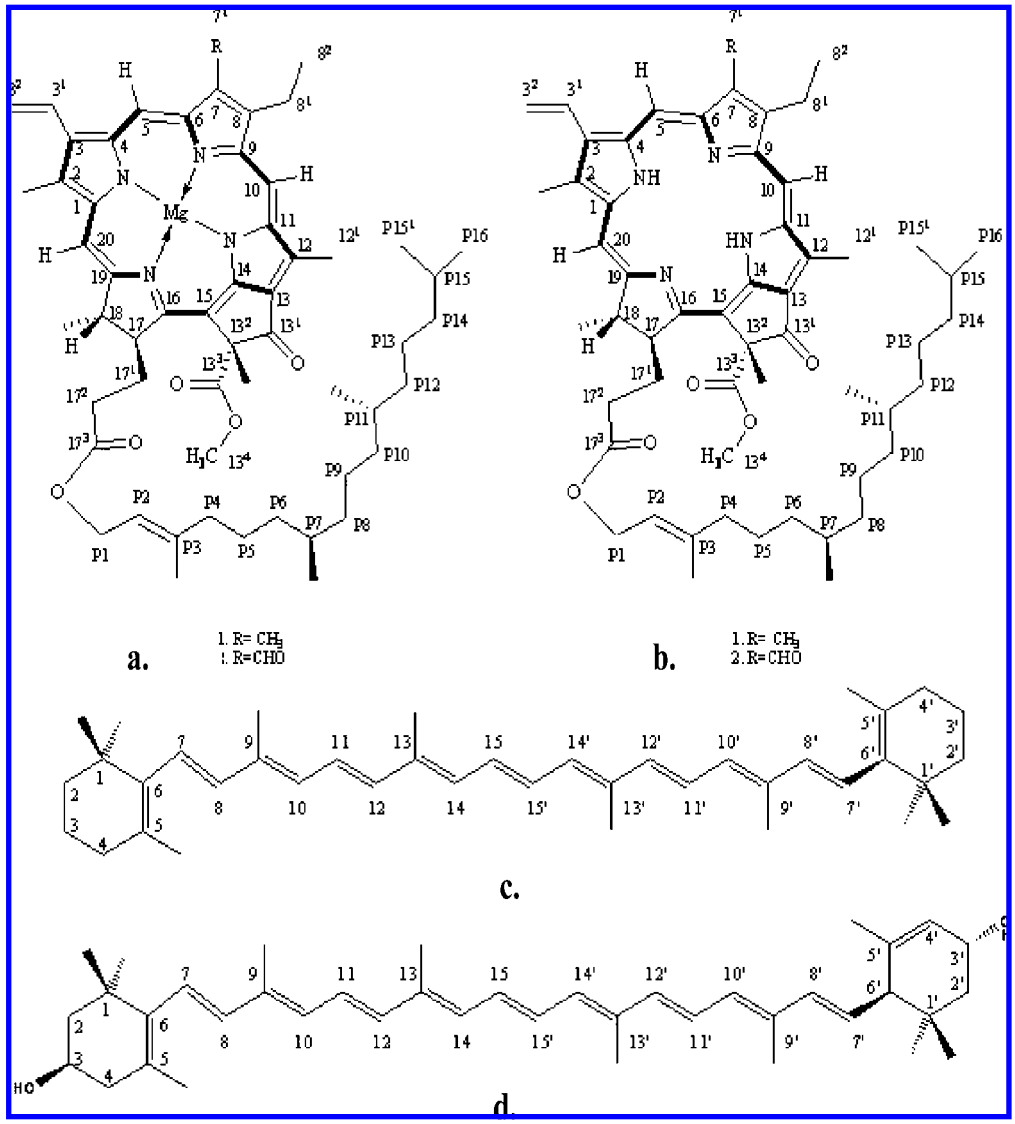

Figure 1. (**a**) Structure and numbering for 13^2 (R)-Chl a and 13^2 (R)- Chl b; (**b**) structure and numbering for 13^2 (R)-Pheo a and 13^2 (R)-Pheo b. Chl a′ and Chl b′ are 13^2(S) corresponding molecules; (**c**) *All trans* β, β–Carotene (β-Carotene); (**d**) (3R, 3′R, 6′R) β,ϵ Carotene 3,3′ diol.

MATERIAL AND METHODS

Extraction of Pigments from Green Beans. Fresh green beans were purchased from a local market, and frozen green beans (*Calisto cultivar*) were obtained from Gelagri Inc. (Loudéac, France). The method used for pigment extraction (*16*) is modified from a previously published extraction method (*14*). This modification consists in adding a liquid–liquid extraction step after the extraction of the pigments from the plant tissues, to concentrate the final extract. Under subdued light (*2*), ca. 15 g of green beans are combined with ca. 15 g of anhydrous sodium sulfate, 15 g of magnesium carbonate, and 75 g of sand (all masses were precisely weighted). Sodium sulfate is added to eliminate water from the extracting solvent because water can form a photosensitive reactive adduct with Chls (*3*). Magnesium carbonate is used for the neutralization of the acids that could come from vegetable tissues or from the sea sand (*3*). Then, 150 mL of cold acetone are added to the mixture (this solvent can penetrate the plant tissues, but it also dissolves water; hence the use of sodium sulfate). After cooling with liquid nitrogen, the mixture is ground in a Waring blender (15 min). Separation of soluble impurities from acetone and concentration of pigments are accomplished through liquid–liquid partition with 5.0 mL of cyclohexane (*6*). The separation of the two phases is achieved by adding distilled water (15.0 mL). The upper organic layer (epiphase) is washed twice with additional distilled water (15.0 mL) to remove acetone. At this stage, centrifugation is performed to separate pigment extracts from suspended particles (*6*), to achieve a better separation between phases, and, finally, to speed up the extraction step. After combining the epiphases, the solvent is removed in a rotary evaporator.

The dried residue is weighted and redissolved in deuterated acetone (500.0 μL). The solution is transferred at low temperature (ca. −20 °C) into an NMR tube (5 mm).

When samples seem to be too concentrated (peak broadening, suspension of small lipid particles in the NMR tube), they are diluted by the addition of controlled volumes (100.0 μL) of deuterated acetone (*29*). After each addition, a new spectrum is recorded and compared with the previous ones.

Identification of Pigments. 64 K scans, one-dimensional (1D) ^{1}H NMR spectra were recorded using a 300 MHz spectrometer (Bruker Biospin): 6 kHz spectral width, 5.3 s acquisition time, zg = 90°, 3 s relaxation delay. Spin–lattice relaxation times were measured previously (*25*), but the values were for purified Chl samples at high dilution (16 mM) and not for crude extracts. The value of the relaxation delay that was used in our study was determined after preliminary tests using the progressive saturation technique (*26*) to determine the spin–lattice relaxation time (T1).

Major photosynthetic pigments (Chl a, Chl b, Pheo a, β-Car, and Lut) were identified by peak assignment using ^{1}H NMR spectra from the pigment extract, from pure samples, and by comparison with previously published data (*18, 22–26*). Pigment extract samples were spiked with appropriate standards to confirm that the chemical shifts were identical. Further confirmation was performed through 2D

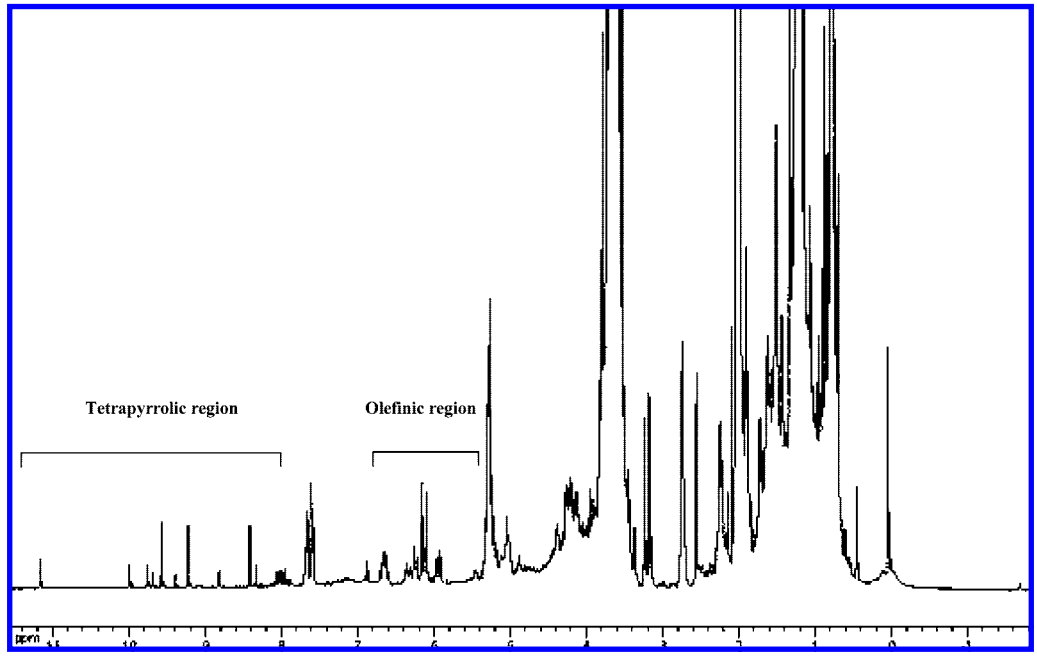

Figure 2. ^{1}H-NMR spectrum (300 MHz, δ [ppm] from internal HMDSO) for frozen green beans extracts in acetone-d_6. The different regions where the pigment identification and quantification are performed are indicated on the graph.

(correlation spectroscopy [COSY] and total correlation spectroscopy [TOCSY]) experiments.

The choice of the solvent is important because chemical shifts for protons belonging to Chls and Chls derivatives are extremely sensitive to the solvent composition, as well as to concentration and temperature (*18*). This is mainly because of the molecular structure of Chls and of certain derivatives, which have a tendency to aggregate (*23*); the central magnesium (Mg) atom of Chls, being electrophilic, has a strong affinity for one or both axial positions to be occupied by an electron-donor ligand. Monomer species of Chls (Chl L_1 or Chl L_2) are obtained with monofunctional polar solvents (acetone, methanol, pyridine, THF, etc.). The self-aggregation is observed in NMR spectra; the chemical shifts of the methyne bridge protons in the macro-cycle are moved toward higher fields; also, the signal of the C-3^2 proton is broadened, which also distorts the baseline, affecting the signals from the macro-cycle protons. Deuterated chloroform is a common solvent for NMR, but it is not polar enough to avoid Chls self-aggregation. A mixture of $CDCl_3$ in excess of methanol-d_4 (v/v 3:2) can also be used, because this mixture seemed to dissolve the samples better. However, it was not used in our study because methanol can oxidize Chls into allomers (*23*). Finally, the solvent mainly used in our study was acetone-d_6. Hexamethyldisiloxane (HMSDO) was used as internal reference for the calibration of chemical shifts (*19*).

To confirm peak assignment, some extracts were acidified with one drop of a 10% solution of hydrochloric acid in deuterated water to transform Chls into Pheos through a reaction called pheophytinization (*1, 2*), in which the Mg^{2+} ion in the center of the tetrapyrrole macrocycle is replaced by two protons. In this way, the replacement of Chls signals by Pheos signals can be followed. Pheophytinized pigments were recovered through liquid–liquid partition using cyclohexane (equal phase volumes). The epiphase was washed three times with cold (0 °C) distilled water and evaporated until completely dry. The solid residue was redissolved in 500 μL of deuterated acetone and placed in an NMR tube.

Confirmation of the initial peaks identification was done through 2D-NMR (^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSY).

^{1}H-^{1}H COSY was carried out under the following conditions: spectral width 3 kHz, in both dimensions; 2048 data points in f2 and 512 increments in f1; and no water presaturation during relaxation delay. For confirmations, ^{1}H-^{1}H COSY spectra were compared to previously published results (*18*).

^{1}H-^{1}H TOCSY spectra were recorded in the time-proportional phase increment (TPPI) phase-sensitive mode, with no water presaturation during relaxation delay, a spectral width of 3 kHz in both dimensions, 2 s relaxation delay, and 2048 data points in f2 and 512 increments in f1. Several mixing times were tested in the range recommended for Cars (*24*); a range of values arbitrarily chosen between 20 and 200 ms were tested to determine the mixing time giving the best resolution. Following these experiments, a mixing time of 150 ms was chosen as the most suitable value.

Quantification of Pigments. Hexamethyldisiloxane (HMDSO) was used for chemical shift calibration and for quantification of absolute concentrations of pigments; a 0.005% HMDSO solution in deuterated acetone was placed in a sealed capillary tube that was introduced in the NMR tube (tetramethylsilane was tested but its volatility was a problem during flame sealing of capillary tubes). HMDSO is an interesting internal standard; it can be obtained very pure, it is soluble in the chosen solvent, it is stable for a long time under the experimental conditions, and it gives a singlet in a usually signal-free region of the NMR spectra (0.045 ppm). The HMDSO signal was cross-calibrated with a secondary standard before being used for quantitative purposes. The exact concentration in HMDSO was calculated with two methods.

First, the capillary tube was calibrated with another sealed capillary tube with a known concentration of 3-(trimethyl-silyl)propionic-2,2,3,3-d.4. acid sodium salt (TSP) in deuterated water (10.0 mM). Both capillary tubes were placed inside a NMR tube with 500 μL of deuterated acetone. The capillary tube containing TSP had been previously cross-calibrated with calibration curves of C_1H-α glucose and (C3H, C4H)-β + C3H-α fructofuranose (*20*) in deuterated water.

Another calibration was performed by placing the capillary tube in NMR tubes containing solutions of Pheo a, obtained from purified Chl a (*3*), at various concentrations. Pheo a was chosen as a standard because it has a similar molecular weight, a similar chemical structure, and similar molecular properties, and it is not as chemically labile as Chl a. Phasing was performed automatically, and integrations of the characteristics peaks of Pheo a in the downfield region (8–10 ppm) of the spectrum were compared to the integration of the peak of HMDSO in the high-field region (see **Figure 3**). These peaks of Pheo a were chosen for integration because they are isolated from neighboring signals.

The comparison of each pigment peak area (selected resonances) to the internal reference peak area allowed the estimation of their absolute concentrations. For Chls, the integration accuracy was estimated by calculating the standard deviation of the peak area of three methyne bridge proton peaks.

The quantification was repeated five times for five different samples.

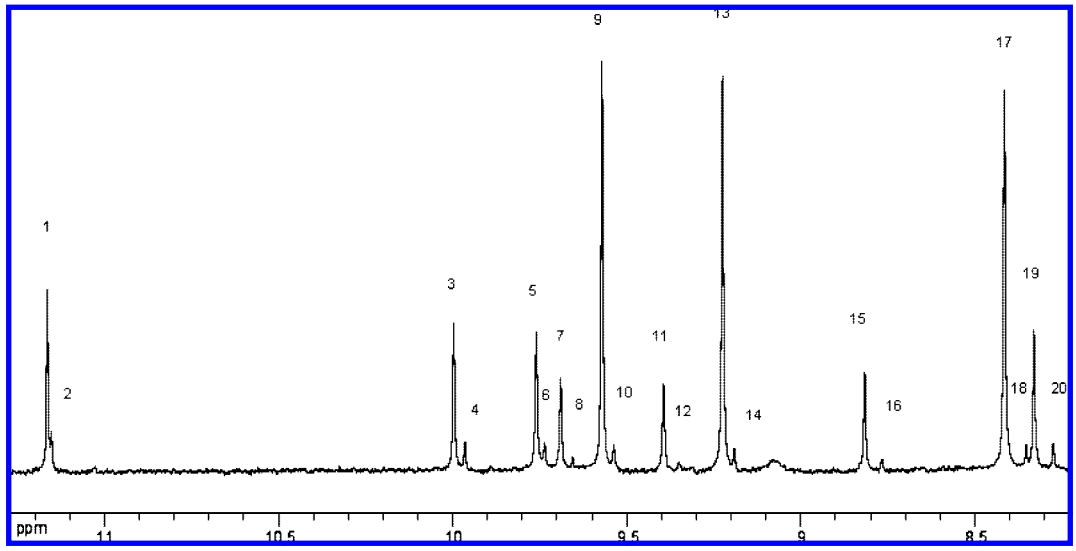

Figure 3. Detail of the δ 11.3−8.2 ppm region of the [1]H NMR spectrum (300 MHz, from internal HMDSO) of green beans extract. This region includes peaks from Chls and derivatives of Chls.

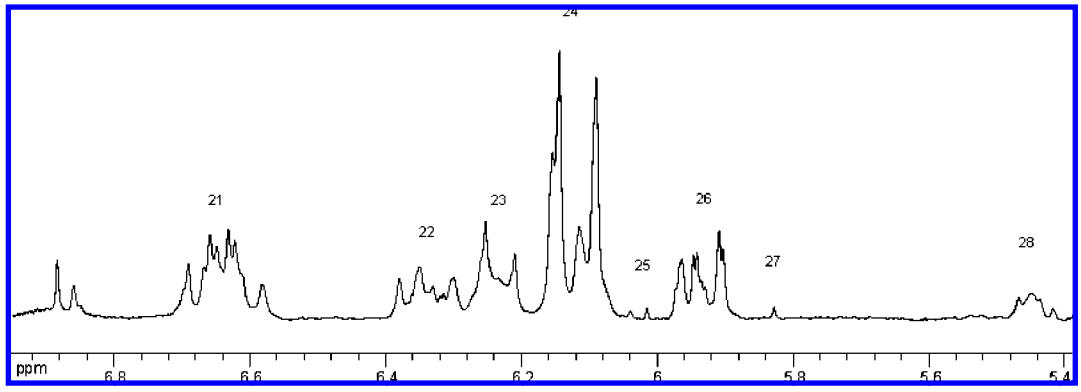

Figure 4. Detail of the δ 6.8–5.7 ppm region of the [1]H NMR spectrum (300 MHz, from internal HMDSO) of green beans extract. This region includes peaks from Cars and other molecules having conjugated double bonds (*17*).

Chemicals. For pigment extraction, acetone (99.8%) was from Carlo Erba (Ródano, Italy). Cyclohexane (analytical grade), Fontainebleau sand, sodium sulfate (99%), magnesium carbonate (99%), and hydrochloric acid (35.5%) were obtained from SDS (Peypin, France).

The standards for pigment identification were commercially purified β-Car, Chl a, Chl b, and Lut and were purchased from Sigma-Aldrich (β-Car, C4582; Chl b, C5878; Chl a, C5753, Lut, X6250).

For NMR measurements, deuterated acetone (99.8%) and $CDCl_3$ (99.8%) were purchased from SDS (Peypin, France), and HMDSO (99.5%) was purchased from Spectrometrie Spin et Techniques (Paris, France).

Capillary tubes 20 μL micropipettes were made of borosilicate glass (Corning 7099S-20).

RESULTS AND DISCUSSION

Identification of Pigment. In the 1D-[1]H NMR spectra (**Figures 2** and **3**), peaks corresponding to methyne bridge protons for Chl a, a′, b, and b′, Pheo a and b, and the terpene chain of Cars were assigned after previously published data (*18, 22–26, 30*).

The signals from Chls and Pheos (**Figure 3**) are easily identified as they are either isolated in the downfield region (between 11.2 and 8.5 ppm) or in the upfield (negative values of chemical shift) for the peaks corresponding to the N−H protons of the tetrapyrrole ring (Pheo a, a′, b, and b′, **Figure 4**). In the upfield region (−1.5 to −2.0 ppm), Pheo a, a′, b, and b′ have peaks similar to the corresponding Chl peaks, but they are shifted toward lower fields (*22*). Chl b is represented in the spectra by one additional peak at lower field because of its C-7[1] aldehyde group; this peak is slightly more intense than the rest of the proton signals from the macrocycle because of some overlapping of the epimeric signal of Chl b′. As mentioned above, the assignment was confirmed by adding appropriate standards to the extracts.

In general, Cars have a chain of conjugated double bonds; isomers that are all trans are prevalent in fresh green beans, and the quantities of cis isomers increases after thermal processing (*6, 12*). The protons present in these double bond chains give [1]H NMR signals in the range 6.7–6.0 ppm. Accordingly, [1]H NMR spectra of Cars have the terpenic chain in common (**Figure 4**); they differ mainly by protons in the distal part of the molecules.

The unassigned peaks from distal groups of Cars were analyzed by 2D-NMR spectroscopy. The homonuclear proton *J*-couplings of the conjugated double bonds of the central isoprenoid chain of Cars and the C-3 group of Chls and Chls derivatives were observed by COSY.

Small amounts of xanthophylls, in relation to the amounts to Lut and β-Car, were already identified in green beans in previous literature (*6, 16, 27, 26*). Lut and β-Car can be distinguished by the characteristic protons C-H-4′ and C-H-6′ of non conjugated double bonds in one of the distal groups. These signals of these protons are observable in 3 correlated signals according to the TOCSY experiment (data not shown).

Table 1. Chemical Shifts of Pigments in Green Beans Extract[a]

peak number	assignment	frequency (ppm)
1	Chl b (CHO-7)	11.16
2	Chl b′ (CHO-7)	11.15
3	Chl b (CH-5)	9.99
4	Chl b′ (CH-5)	9.96
5	Chl b (CH-10)	9.76
6	Chl b′ (CH-10)	9.73
7	Pheo a (CH-5)	9.69
8	Pheo a′ (CH-5)	9.65
9	Chl a (CH-5)	9.57
10	Chl a′ (CH-5)	9.54
11	Pheo a (CH-10)	9.39
12	Pheo a′ (CH-10)	9.35
13	Chl a (CH-10)	9.22
14	Chl a′ (CH-10)	9.19
15	Pheo a (CH-20)	8.81
16	Pheo a′ (CH-20)	8.76
17	Chl a (CH-20)	8.41
18	Chl a′ (CH-20)	8.35
19	Chl b (CH-20)	8.33
20	Chl b′ (CH-20)	8.27
21	Cars (CH-11/11′), Cars (CH-15/15′).	6.69–6.59
22	Cars (CH-12/12′)	6.39–6.31
23	Cars (CH-14/14′)	6.26–6.22
24	Cars (CH-7/7′), Cars (CH-8/8′), Cars (CH-10/10′)	6.16–6.10
25	Neo (*C*=C=CH-8)	6.02
26	na	5.97–5.92
27	Vio (C-H-7; C-H-7′)	5.83
28	total Lut (C-H-7′)	5.45

[a] 300 MHz, δ [ppm] from internal HMDSO; na stands for not assigned.

Some weak signals observed between 6.0 and 7.0 ppm were previously observed in vegetable extracts (*18*). Although these signals were not properly assigned they might belong to Cars.

The results of peak assignments (**Figures 3** and **4**) for pigments in which quantification was done are given in **Table 1**. The signals of pigments were not fully resolved because they are overlapped with other compounds (mainly lipids and fatty acids).

Peaks in group 21 were attributed to C-H-11, C-H-15, C-H-11′, and C-H-15′ of the terpenic chain that Cars have in common. Peak groups 22, 23, and 24 were also attributed to the terpenic chain of Cars with overlapping peaks of Chls and Chls derivatives (vinyl group in C-H-3^2 and C-H-$3^{2'}$ and proton in C-13^2). Peak group 22 contains signals from C-H-12 and C-H-12′. Peak group 23 contains the signals from C-H-14 and C-H-14′. Peak group 24 contains the signals from C-H-7, C-H-8, C-H-10, C-H-7′, C-H-8′, and C-H-10′, all from the terpenic chain of Cars.

The 2D experiments (COSY and TOCSY) and the ^{13}C –NMR spectra showed that peak group 26, which was not fully assigned, could have peaks belonging to phenolic compounds (*33*). These peaks were not systematically present. Choung et al. highlighted the fact that the anthocyanin profile of the kidney bean seed coats is highly dependent on the cultivar type of the seed. Delphinidin 3-glucoside is the major anthocyanin for red kidney beans, whereas no anthocyanins were detected in white kidney beans. The seeds in the immature pods of *Phaseolus vulgaris* L (green beans) are generally white−pale green (no anthocyanins), but sometimes red or black seed coating can be found. This reinforced the assumption that these peaks could possibly belong to anthocyanins.

Quantification of Pigments. Using the method given in the Materials and Methods section, the quantity of the various pigments was determined in extracts. The results for fresh and frozen (previously blanched) green beans are given in **Table 2**.

Table 2. Pigment Content in Fresh and Frozen (Previously Blanched) Green Beans Expressed in mg of Pigments for 100 g of Pods[a]

pigment	mg/100 g of fresh green beans	mg/100 g of frozen green beans
Chl a	7.9 ± 0.4	8.98 ± 0.27
Chl a′	ND	0.39 ± 0.09
Chl b	3.3 ± 0.2	3.19 ± 0.18
Chl b′	ND	0.41 ± 0.08
Pheo a	0.10 ± 0.03	2.13 ± 0.13
Pheo a′	ND	0.10 ± 0.05
β-Car	1.03 ± 0.15	3.13 ± 0.31
total Lut	1.38 ± 0.2	3.54 ± 0.14
Vio	0.12 ± 0.02	0.10 ± 0.02
Neo	0.09 ± 0.03	0.11 ± 0.03

[a] Mean and standard deviation for five measurements. All data for carotenoids are for the total amount between cis and trans isomers. Limit of detection was 0.1 mg for chlorophyllic pigments and 0.08 mg for carotenoids. ND stands for not detected.

The quantities of Chls and Chls derivatives are calculated from the various well-separated peaks. In **Table 2**, the average quantities are given both for peaks of the same spectrum and for repetitions of the measurements for various samples (fresh and frozen green beans). The content in all pigments increases in frozen green beans, perhaps because the extractability of pigments increases with the blanching process to which they are submitted to prior to the freezing process, as well as the freezing process itself (*2, 3*). For Cars, except β-Car, the assigned peaks given in **Table 1** were used for quantification. For β-Car, quantification was performed by subtraction; Total Lut ($E + Z$ isomers), Vio, and Neo quantities were subtracted from the TCar quantity given by the range of peaks at 6.7–6.3 ppm.

The obtained values (**Table 2**) were compared with previous literature (**Table 3**). The a/a′ and b/b′ ratios for Chls and Pheos are in the range of those given in previously published literature (*3, 10, 11, 27, 26*). The formation of Chl a and a′ could be explained by solvent mediated epimerization (which can increase the enolic form of 13^2 C). Mild heating or blanching processes (*1, 2*) were also reported as a cause of epimerization of Chls. The Pheo content in processed green beans is higher than in fresh plant tissues, probably because of thermal degradation of Chls during blanching.

The Chl a/Chl b ratio can vary considerably in function of the cultivar (*28*) and, within one cultivar, of the storage conditions. The Car content is the most variable. All authors except Cruz-Garcia et al. (*10*) and Lopez-Hernandez et al. (*27*) agree that Lut is present in higher quantity than β Car. In our analysis, we find significantly higher quantities of Cars than other publications. In particular, frozen green beans contain high Car quantity, perhaps because of the better extractability of these pigments in food processed samples (*2, 24*). Cruz-Garcia et al. (*10*) already reported big amounts of Cars in green beans.

The precision of pigment determination by quantitative NMR (qNMR) spectroscopy can be compared to other methods (**Table 4**).

First, the precision of qNMR is on the same order of magnitude as other methods. UV–vis spectrometry gives only pigment concentration for Chl a, Chl b, and TCars; the precision is better than for qNMR, but Chl epimers and different Cars cannot be distinguished. HPLC methods give comparable precisions (2–11%) as qNMR, and in certain cases the precision for Cars determination is even better (for example, precision is better for Neo and Vio). However, the precision for HPLC is ob-

Table 3. Comparison of the Pigment Amount in Green Beans (Intersample Repetitions Average and Standard Deviation) for Various Methods for the Quantitative Determination of Photosynthethic Pigments[a]

	concentration of pigments in green beans (mg/100 g edible food)								
pigment	A	B	C	D	E	F	G	H	I
Chl a	11.37 ± 0.10	5.4	ND	3.66−10.50	17.85 ± 0.52	8.42 ± 0.86	2.22 ± 0.92	ND	ND
Chl b	7.59 ± 0.10	1.7	ND	0.22−0.46	2.73 ± 0.07	1.91 ± 0.02	1.41 ± 0.38	ND	ND
Pheo a	0.7 ± 0.10	ND	ND	0.08−0.23	0.31 ± 0.01	0.55 ± 0.06	ND	ND	ND
b-Car	ND	ND	ND	1.32−2.32	0.31 ± 0.01	0.07 ± 0.01	0.81 ± 0.06	0.60 ± 0.07	0.20 ± 0.01
Lut	ND	ND	0.418	0.47−0.99	0.62 ± 0.01	0.25 ± 0.01	0.52 ± 0.10	0.69 ± 0.03	0.36 ± 0.07
Epoxy-Lut	ND	ND	ND	ND	0.41 ± 0.01[b]	0.13 ± 0.01[b]	ND	0.19 ± 0.04	ND
Vio	ND	ND	ND	ND			ND	0.23 ± 0.07	ND
Neo	ND	ND	ND	ND			ND	0.13 ± 0.02	ND

[a] A: Turkmen et al., 2006; B: Wrolstad et al., 2005; C: Humphries et al., 2003; D: Cruz-Garcia et al., 1998; E: Monreal et al., 1999 (Perona cv); F: Monreal, 1999 (Boby cv); G: Lopez-Hernandez et al., 1993, H: Khachick et al., 1992; I: Granado et al., 1992. ND stands for not detected or quantified. [b] Total minor xanthophylls.

Table 4. Comparison of the Precision (Intersample Repetitions, Relative Standard Deviation, %) for Various Methods for the Quantitative Determination of Photosynthethic Pigments[a]

pigment\	qNMR	HPLC			UV–vis	TLC/densitometry
method	a	b	c	d	e	f
Chl a	3	2.3–3.3	2.55	ND	<1.0	4.1
Chl a′	22	ND	ND	ND	ND	ND
Chl b	5	2.3–3.3	2.3	ND	<1.0	5
Chl b′	21.1	ND	ND	ND	ND	ND
Pheo a	6.2	2.3–3.3	ND	ND	ND	5.4
Pheo a′	40	ND	ND	ND	ND	ND
β-Car	9.6	2.3–3.3	1.7	12	∼1.0	9.5
Lut	4	2.3–3.3	1.8	4.3		10
Epoxy-Lut	ND	NQ	ND	21		ND
Vio	24.3	NQ	ND	30		14
Neo	30	NQ	ND	15		15

[a] The letters correspond to the following literature: a, present work; b, Cruz-Garcia et al., 1998; c, Lopez-Hernandez et al., 1993; d, Khachick et al., 1992 (calculated for data given); e, Wellburn, 1994; f, Valverde et al., 2006. ND stands for not detected, and NQ stands for detected but not quantified.

tained only if repetitions of experiments are made; on the other hand, qNMR standard deviation can be determined using the various peaks of these compounds. Moreover, some chemical information (Chls aggregation and π-stacking of Pheos) is missing in the papers discussing the question of precision. TLC is a very efficient method (*16*), but the standard process does not show epimers; Neo and Vio concentration can be determined but with less precision (about 10 – 15%), although they have lower detection limits.

Finally, qNMR gives good results for Chl a, a′, b, b′, Pheo a, a′, Lut, and β-Car. Neo and Vio determination could be improved using higher magnetic field (*20*) but probably not with a larger number of scans; in our tests, spectra were generally based on 64 scans (about 15 min), but longer acquisitions were tested (88, 240, and 880); a larger number of scans seemed to improve the signal-to-noise ratio of the spectra (data not shown) but not the spectral resolution. This signal-to-noise ratio is not enough to significantly improve the detection limit. Of course, ^{13}C NMR could also be used to improve precision because resonances are much thinner in these spectra (*13*). However, the processing time is then longer (more than 12 h), and the lower natural abundance of the ^{13}C isotope has a direct effect on the detection limit, which is lower than for ^{1}H NMR spectra (1% vs 100% for ^{1}H).

This study focused on the quantitative determination of photosynthetic pigments from green beans, but the same method could be used without modification for any other plant tissue. The precision of qNMR techniques depends on several factors, such as the mass of plant tissue, the pigment concentration in the sample, the observed resonant nucleus, the magnetic field strength of the spectrophotometer, the pulse sequence used for acquisitions, and the number of acquisitions that are collected. With ^{1}H NMR, good results can be obtained very efficiently because the sample preparation is fast and easy.

ACKNOWLEDGMENT

We are grateful to Marc Vignolle and Marie-Dominique Quignon for support (Marie Groupe UNIQ), Dror Warchawski (Institut de Biologie Physico-Chimique, Paris), Anne Cazor and Robert Meric (Collège de France, Paris), Rachel Edwards Stuarts (Nottingham University), and Audrey Tardieu (AgroParisTech) for technical support, and Catherine Deborde (INRA, Bordeaux) and Douglas Rutledge (AgroParisTech) for their helpful discussions.

LITERATURE CITED

(1) Belitz, H.-D.; Grosch, W.; Schieberle, P. *Food Chemistry*, Third ed.; Springer-Verlag: Berlin, Germany, 2004; p 1070.
(2) Wrolstad, R. E. *Handbook of Food Analytical Chemistry*, First ed.; John Wiley & Sons, Inc.: Hooboken, New Jersey, 2005; Vol. 2, p155–163.
(3) Lichtenthaler, H. K. Chlorophyll and Carotenoids: Pigments of Photosynthetic Biomembranes. *Methods Enzymol.* **1987**, *148*, 350–382.
(4) Wellburn, A. R. The spectral Determination of Chlorophylls a and b, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution. *J.Plant Physiol.* **1994**, *144*, 307–313.
(5) Kupper, H.; Seibert, S.; Parameswaran, A. Fast, Sensitive, and Inexpensive Alternative to Analytical Pigment HPLC: Quantification of Chlorophylls and Carotenoids in Crude Extracts by Fitting with Gauss Peak Spectra. *Anal. Chem.* **2007**, *79*, 7611–7627.
(6) Khachick, F.; Mudlagiri, B.; Beecher, G. R.; Holden, J.; Lubsy, W. R.; Tenorio, M. D.; Barbera, M. R. Effect of Food preparation on Qualitative and Quantitative Distribution of Major Carotenoid Constituents of Tomatoes and Several Green Vegetables. *J. Agric. Food Chem.* **1992**, *40*, 390–398.
(7) Gauthier-Jacques, A.; Bortlik, K.; Hau, J.; Fay, L. B. Improved Method to Track Chlorophyll Degradation. *J. Agric. Food Chem.* **2001**, *49*, 1117–1122.
(8) Minguez-Mosquera, M. I.; Garrido-Fernandez, J. Chlorophyll and Carotenoid Presence in Olive Fruit (*Olea europaea*). *J. Agric. Food Chem.* **1989**, *37*, 1–7.
(9) Canjura, F. L.; Watkins, R. H.; Schwartz, S. J. Color Improvement and Metallo-Chlorophyll Complexes in Continuous Flow aseptically Processed Peas. *J. Food Sci.* **1999**, *64*, 987–990.
(10) De La Cruz-Garcia, C.; Gonzalez-Castro, M. J.; Oruña-Concha, M. J.; Simal-Lozano, J. A.; Simal-Gandara, J. The effects of various culinary treatments on the pigment content of green beans (*Phaseolus Vulgaris*, L.). *Food Res. Int.* **1997**, *30* (10), 787–791.

(11) Turkmen, N.; Poyrazoglu, E. S.; Sari, F.; Velioglu, Y. S. Effects of cooking methods on chlorophylls, pheophytins and colour of selected green vegetables. *Int. J. Food Sci. Tech.* **2006**, *41*, 281–288.
(12) Khachick, F.; Beecher, G. R.; Whittaker, N. F. Separation, Identification and Quantification of Mayor Carotenoid and Chlorophyll Constituents in Extracts of Several Green Vegetables by Liquid Chromatography. *J. Agric. Food Chem.* **1986**, *34*, 603–616.
(13) Pollesello, P.; Toffanin, R.; Eriksson, O.; Kilpeläinen, I.; Hynninen, P. H.; Paoletti, S.; Leo Saris, N. E. Analysis of Lipids in Crude Extracts by^{13}C Nuclear Magnetic Resonance. *Anal. Biochem.* **1993**, *214*, 238–244.
(14) Quach, H., T.; Steeper, R.; Griffin, G. W. An Improved Method for the Extraction and Thin-Layer Chromatography of Chlorophyll a and b from Spinach. *J. Chem. Educ.* **2004**, *81* (3), 385–387.
(15) Sherma, J.; Fried, B. Separation and Determination of Chloroplast Pigments from Spinach by Thin-Layer Chromatography: a Student Laboratory Experiment. *J. Planar Chromatogr.* **2004**, *17*, 309–313.
(16) Valverde, J.; This, H.; Vignolle, M. Quantitative Determination of Photosynthetic Pigments in Green Beans Using Thin-Layer Chromatography and a Flatbed Scanner as Densitometer. *J. Chem. Educ.* **2007**, *84*, 1505–1507.
(17) Fan, T. W.-M. Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *28*, 161–219.
(18) Sobolev, A. P.; Brosio, E.; Gianferri, R.; Segre, A. L. Metabolic profile of lettuce leaves by high-field NMR spectra. *Magn. Reson. Chem.* **2005**, *43*, 425–638.
(19) Pauli, G. F.; Jaki, B. U.; Lankin, D. C. Quantitative^{1}H-NMR: Developement and Potential of a Method for Natural Product Analysis. *J. Nat. Prod.* **2005**, *68*, 133–149.
(20) Cazor, A.; Deborde, C.; Moing, A.; Rolin, D.; This, H. Sucrose, Glucose, and Fructose Extraction in Aqueous Carrot Root Extracts Prepared at Different Temperatures by Means of Direct NMR Measurements. *J. Agric. Food Chem.* **2006**, *54*, 4681–4686.
(21) Schoefs, B. Determination of Pigments in Vegetables. *J. Chromatogr. A* **2004**, *1054*, 217–226.
(22) Smith, K. M.; Goff, D. A.; Abraham, R. J. NMR Spectra of Porphyrins. 27 Proton NMR spectra of Chlorophyll-a and Pheophytin-a. *Org. Magn. Reson.* **1984**, *22* (12), 779–783.
(23) Hyvärinen, K.; Helaja, J.; Kuronen, P.; Kilpeläinen, I.; Hynninen, P. H. ^{1}H and^{13}C NMR Spectra of the Methanolic Allomerization Products of 13^{2} (*R*)-Chlorophyll a. *Magn. Reson. Chem.* **1995**, *33*, 646–656.
(24) Englert, G. NMR of Carotenoids: novel experimental techniques. *Pure Appl. Chem.* **1991**, *63* (1), 59–70.
(25) Putzbach, K.; Krucker, M.; Albert, K.; Grusak, M. A.; Tang, G.; Dolnikowski, G. G. Structure Determination of Partially Deuterated Carotenoids from Intrinsically Labelled Vegetables by HPLC-MS and^{1}H NMR. *J. Agric. Food Chem.* **2005**, *53*, 671–677.
(26) Sanders, J. K. M.; Waterton, J. C.; Denniss, I. S. Spin-lattice relaxation, nuclear Overhauser enhancements, and long range coupling in chlorophylls and metalloporphyrins. *J. Chem. Soc., Perkin Trans. 1* **1978**, *10*, 1150–1157.
(27) Lopez-Hernandez, J.; Vazquez-Oderiz, L.; Vazquez-Blanco, E.; Romero-Rodriguez, A.; Simal-Lozano, J. HPLC Determination of Major Pigments in the Bean Phaseolus vulgaris. *J. Agric. Food Chem.* **1993**, *41*, 1613–1615.
(28) Monreal, M.; De Ancos, B.; Cano, M. P. Influence of Critical Storage Temperatures on Degrdative Pathways of Pigments in Green Beans (*Phaseolus Vulgaris* Cvs. Perona and Boby). *J. Agric. Food Chem.* **1999**, *47*, 19–24.
(29) Sparling, M. L.; Zidovetzki, R.; Muller, L.; Chan, S. I. Analysis of membrane lipids by 500 MHz 1H NMR. *Anal. Biochem.* **1989**, *178* (1), 67–76.
(30) Tiziani, S.; Schwartz, S. J.; Vodovotz, Y. Profiling of carotenoids in Tomato Juice by One- and Two-Dimensional NMR. *J. Agric. Food Chem.* **2006**, *54*, 6094–6100.
(31) Eijckelhoff, C.; Dekker, J. P. A routine method to determine the chlorophyll a, pheophytin a and β-carotene contents of isolated Photosystem II reaction center complexes. *Photosynth. Res.* **1997**, *52*, 69–73.
(32) Lin, S.; Brewer, M. S. Effects of Blanching Method on the Quality Characteristics of Frozen Peas. *J. Food Qual.* **2005**, *28*, 350–360.
(33) Choung, M. G.; Choi, B. R.; An, Y. N.; Chu, Y. H.; Cho, Y. S. Anthocyanin profile of Korean cultivated kidney bean (*Phaseolus vulgaris L.*). *J. Agric. Food Chem.* **2003**, *51* (24), 7040–7043.
(34) Granado, F.; Olmedilla, B.; Blanco, I.; Rojas Hidalgo, E. Carotenoid composition in raw and cooked Spanish vegetables. *J. Agric. Food Chem.* **1992**, *40*, 2135–2140.
(35) Humphries, J. M.; Khachick, F. Ditribution of Lutein, Zeaxanthin, and Related Geometrical Isomers in Fruit, Vegetables, Wheat, and Pasta Products. *J. Agric. Food Chem.* **2003**, *51*, 1322–1327.

Received for review January 31, 2007. Revised manuscript received October 26, 2007. Accepted October 31, 2007.

JF070277J