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Characterization of Lycopene Nanoparticles Combining Solid-State and Suspended-State NMR Spectroscopy

JÜRGEN WEGMANN,† MANFRED KRUCKER,† STEFAN BACHMANN,† GERD FISCHER,† DANIEL ZEEB,† ANNETTE LIENAU,† TOBIAS GLASER,† FRANK RUNGE,‡ ERIK LÜDDECKE,‡ AND KLAUS ALBERT*,†

Institut für Organische Chemie der Universität Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany, and BASF Aktiengesellschaft, D-67056 Ludwigshafen, Germany

The core−shell structure of lycopene micronizates can be verified by employing a combination of solid-state and suspended-state NMR spectroscopy. The type of molecular aggregation of carotenoid nanoparticles can be clearly determined from their characteristic fingerprint pattern in the solid-state NMR spectra.

KEYWORDS: Aggregation; carotenoids; CP/MAS NMR spectroscopy; HR/MAS NMR spectroscopy; matrix effects; NMR spectroscopy

INTRODUCTION

In modern nutrition, the supplementation and fortification of foods with nutrients such as vitamins and carotenoids is becoming increasingly important for the prevention of chronic diseases of aging, such as heart disease, cancer, and Alzheimer's disease. A well-known example is the mandatory fortification of flour in the United States with folic acid for the prevention of severe birth defects in infants, viz. spina bifida.

There is a general recommendation for an increased consumption of fruit and vegetables, rich in carotenoids and other phytochemicals. Particularly rich sources of the carotenoids lutein and zeaxanthin are spinach and broccoli, and the most important sources of lycopene are tomatoes and tomato products (*1*−*3*). Carotenoid nanoparticles, optimized with respect to bioavailability, can be used in human and animal nutrition if they are stabilized by special techniques such as microencapsulation. The resulting properties are primarily determined by the morphology and structure of the formulated particles. The evaluation of the complex composition is the aim of different investigation methods, each of which can explain only some aspects of the overall composition. The present paper deals with the first applications of NMR techniques to characterize carotenoid micronizates.

The nutritional and physiological effects of carotenoids are partly determined by their bioavailability. Coarse particles of crystalline carotenoids purified from foods and from chemical synthesis are poorly available. An increased bioavailability of poorly soluble compounds such as carotenoids can be achieved by forming nanoparticles with a proper particle size. These nanoparticles have to be adjusted to the desired area of application by extensive research and development activities (*4*−*6*). Due to the high sensitivity of carotenoids to oxidation, the nanoparticles must be prepared extremely carefully. Therefore, the lipophilic carotenoids are coated with a hydrophilic matrix, protecting them against oxidation and, at the same time, against particle agglomeration. Within aqueous media the protecting matrix will dissolve, thereby releasing the carotenoid nanoparticles.

Carotenoid nanoparticles with a size of 100−500 nm consist of a carotenoid core and a shell of adsorbed protective colloid, e.g., gelatin. They are formed using a precipitation process by mixing a carotenoid solution in alcohol and an aqueous gelatin solution (so-called mixing chamber micronization) (*4*, *5*). Other processes to prepare these submicroscopic particles involve special grinding and emulsification techniques. The color of the carotenoid nanoparticles (which are often used as colorants) is determined by the particle size and the agglomeration behavior of the carotenoid molecules (*6*). The structure of the nanoparticles has so far been investigated by using UV/vis absorption spectroscopy, quasi-elastic light scattering, and wide-angle X-ray diffraction (*7*).

In this paper, crystalline lycopene (sample a) and three samples of dried lycopene powders formulated with gelatin/sugar are investigated: coarsely milled lycopene of about 1 μm (sample b), ultrafinely milled lycopene of about 0.3−0.4 μm (sample c), and an ultrafinely precipitated lycopene micronizate of about 0.1−0.2 μm (sample d). By employing a combination of solid-state and suspended-state NMR spectroscopy, it is possible to obtain information about the stereochemistry of the carotenoid lycopene within the protective colloid gelatin. Additionally, full NMR spectroscopic characterization of the formulated lycopene nanoparticles has been achieved for the first time.

† Universität Tübingen.
‡ BASF Aktiengesellschaft.

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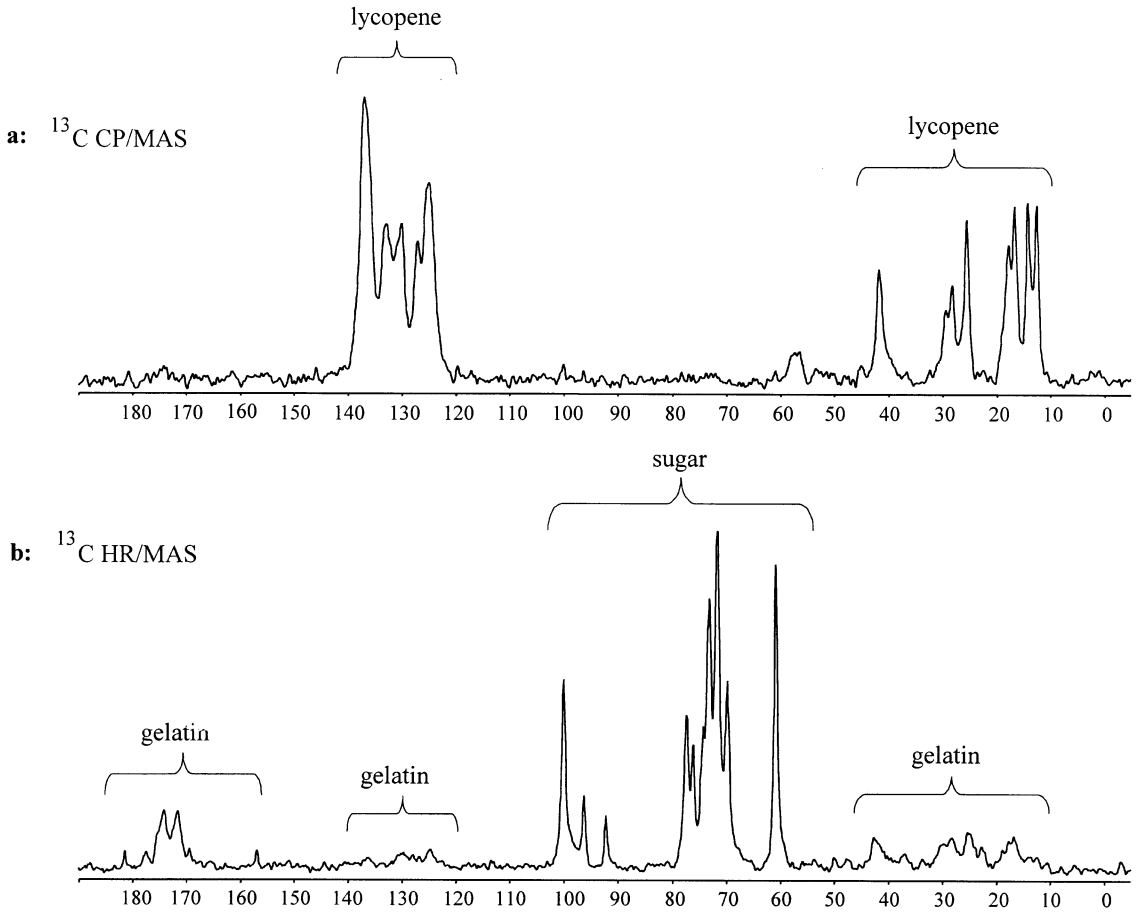

Figure 1. NMR spectra of lycopene micronizates (sample d). (a) ^{13}C CP/MAS NMR spectrum of solid micronizates. (b) ^{13}C HR/MAS NMR spectrum of micronizates suspended in D_2O.

MATERIALS AND METHODS

Materials. The lycopene samples were provided by BASF Aktiengesellschaft, Ludwigshafen, Germany. Crystalline lycopene (sample a), as well as three specially prepared laboratory samples of lycopene dry powder formulations based on gelatin/sugar, i.e., coarsely milled lycopene of about 1 μm (sample b), ultrafinely milled lycopene of about 0.3−0.4 μm (sample c), and an ultrafinely precipitated lycopene micronizate of about 0.1−0.2 μm (sample d), were investigated. The formulations were prepared by wet-milling in aqueous media and by controlled precipitation (mixing chamber micronization). These technologies have been described in detail (*4*−*7*).

NMR Methods. All NMR spectra were obtained on a Bruker DSX 200 NMR spectrometer.

^{13}C suspended-state NMR measurements were conducted by suspending the micronizates in D_2O. Magic angle spinning was carried out with 4-mm double-bearing rotors of ZrO_2 and a spinning rate of 4000 Hz at a probe temperature of 295 K. The ^{13}C 90° pulse length was 4.7 μs. A total of 10K transients were accumulated at a time domain size of 3K data points with an acquisition time of 78 ms and a delay time of 10 s.

^{13}C solid-state NMR spectra were obtained with the cross-polarization/magic angle spinning (CP/MAS) technique. Magic angle spinning was carried out with 4-mm double-bearing rotors of ZrO_2 and a spinning rate of 10 000 Hz at a probe temperature of 295 K. The proton 90° pulse length was 4.7 μs, and the contact time and delay time were 3 ms and 3 s, respectively. A total of 10K transients were accumulated at a time domain size of 2K data points with an acquisition time of 41 ms. For the measurements of cross-polarization curves, 11 different contact times in the range of 0.1−12.5 ms were chosen.

RESULTS AND DISCUSSION

The core−shell structure of the lycopene micronizate (sample d) can be verified by two different types of ^{13}C NMR measurements, as shown in **Figure 1**. Two complementary NMR techniques were utilized, the ^{13}C cross-polarization/magic angle spinning (CP/MAS) method and the ^{13}C high-resolution/magic angle spinning (HR/MAS) method. The ^{13}C CP/MAS technique is employed for the registration of NMR spectra of solid amorphous powders (*8*); the ^{13}C HR/MAS technique allows the acquisition of NMR spectra of samples suspended in a solvent (*9*). Both use the magic angle spinning (MAS) technique to eliminate dipole−dipole and chemical shift anisotropy interactions in the solid state and susceptibility distortions in the suspended state. Because NMR signal line width is more affected by dipole−dipole and chemical shift anisotropy than by susceptibility interactions, high spinning rates of 10 000 Hz are employed in the solid-state measurement. HR/MAS suspended-state measurements are performed at a spinning rate of 4000 Hz.

For the acquisition of solid-state NMR spectra, the cross-polarization (CP) technique allows the registration of ^{13}C NMR spectra at reasonable pulse repetition times in the dimension of seconds. Here, the magnetization is transferred from protons to the ^{13}C atoms of the sample, whereas in the ^{13}C HR/MAS technique the carbon atoms are observed by direct excitation. The ^{13}C signals of the rigid, heavily aggregated lycopene cores in lycopene micronizate (sample d) are preferentially excited with cross-polarization experiments and can, therefore, be seen in the CP/MAS NMR spectrum (**Figure 1a**). ^{13}C signals of the mobile sugar and the gelatin protection shell, on the other hand, can be recorded with the ^{13}C HR/MAS suspended-state NMR technique (**Figure 1b**). Here, ^{13}C signals of the rigid lycopene core cannot be detected due to the presence of strong dipole−dipole interactions.

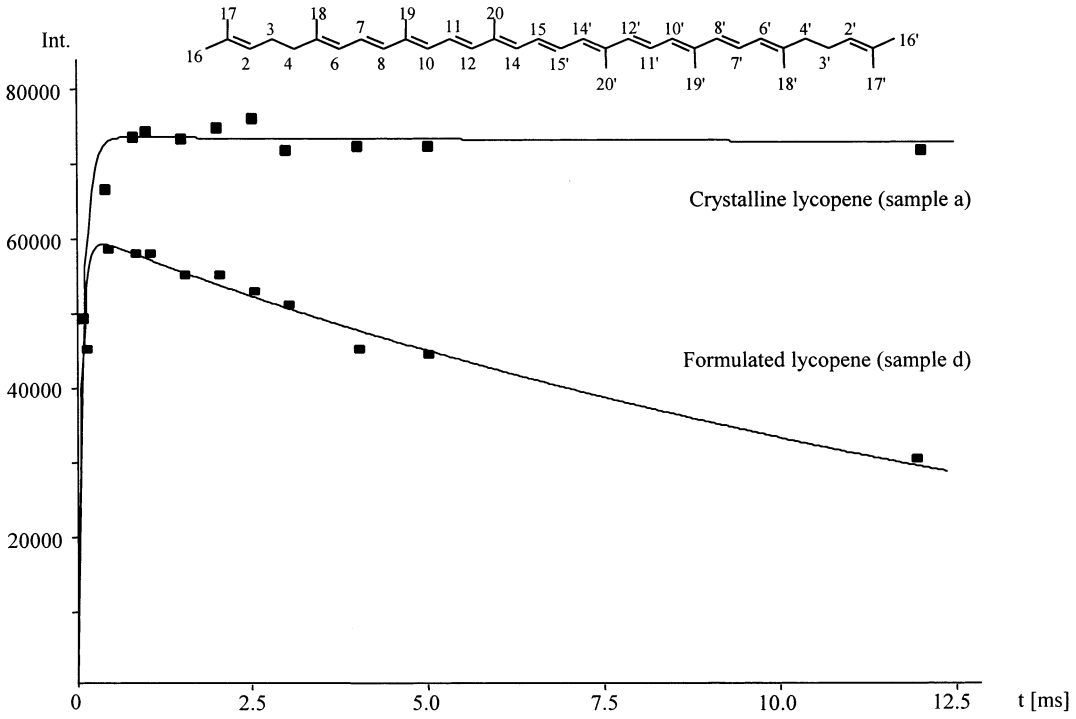

Figure 2. Cross-polarization curves of carbon atom C-4 of crystalline lycopene (sample a) and of formulated lycopene micronizate (sample d).

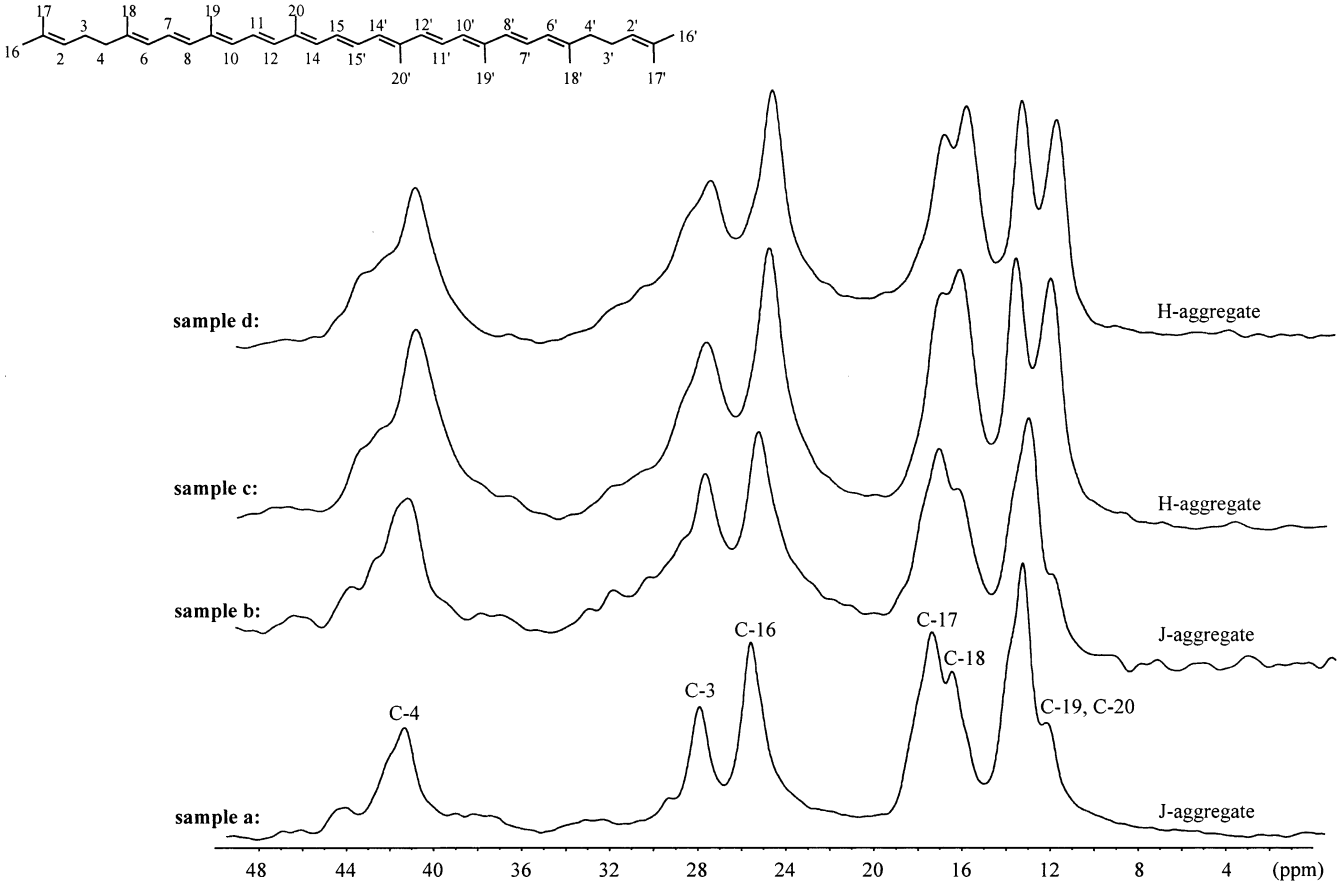

Figure 3. Comparison of the ^{13}C CP/MAS NMR spectrum (aliphatic region) of crystalline lycopene (a) with the spectra of the coarsely milled product (b), the ultrafinely milled product (c), and the lycopene micronizate (d).

The formulation of lycopene particles changes the relaxation behavior of both carbon and proton nuclei of lycopene. The cross-polarization curves of CP/MAS measurements are derived by plotting the recorded signal intensities against the contact times, specified in the following equation (*10*):

$$I(t) = A(1 - T_{CH}/T_{1\rho H})^{-1} [\exp(-t/T_{1\rho H}) - \exp(-t/T_{CH})]$$

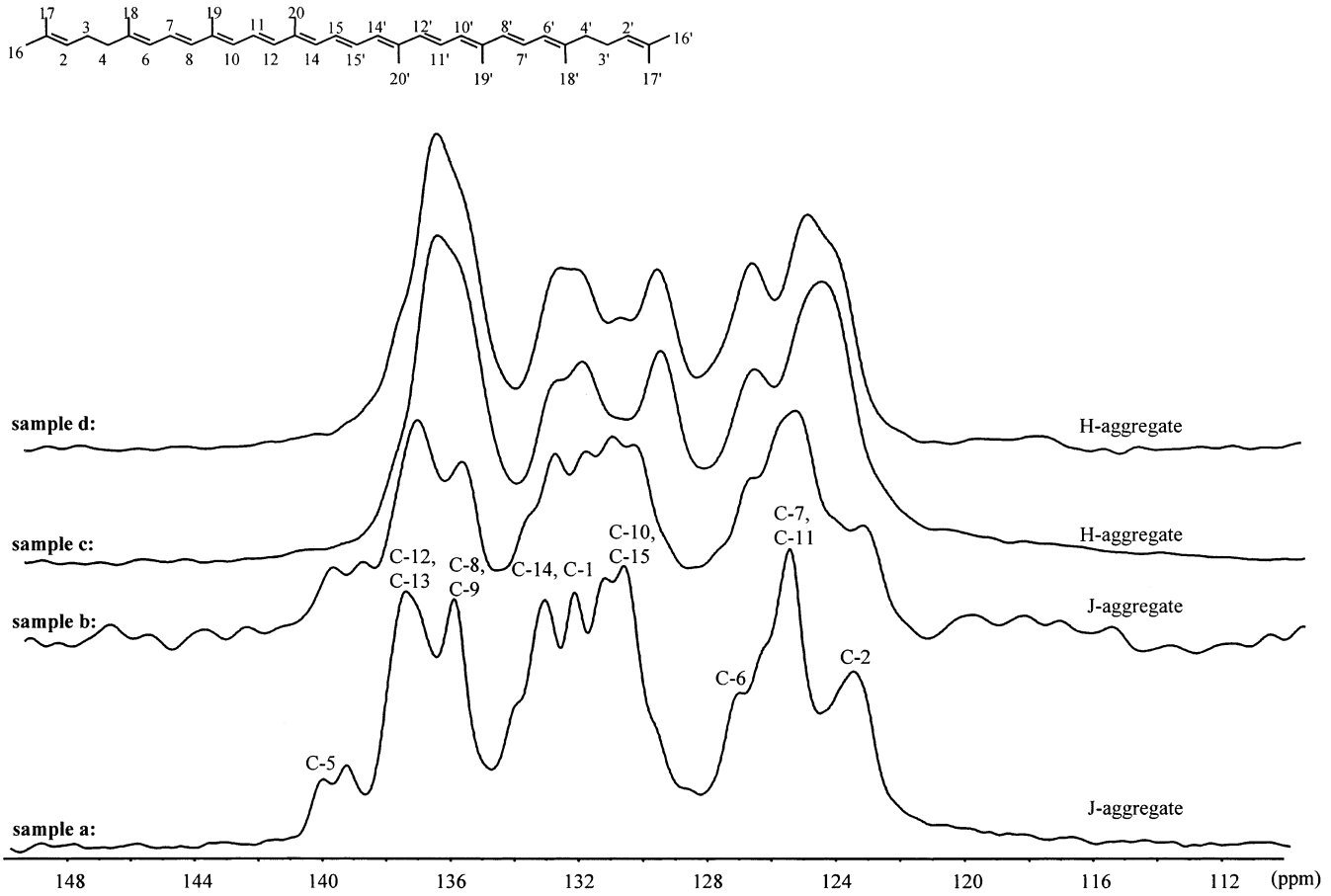

Figure 4. Comparison of the ^{13}C CP/MAS NMR spectrum (olefinic region) of crystalline lycopene (a) with the spectra of the coarsely milled product (b), the ultrafinely milled product (c), and the lycopene micronizate (d).

Table 1. Chemical Shifts of the Olefinic Carbon Atoms in the NMR Spectra of the Different Samples

carbon atom	crystalline lycopene (sample a)	coarse grinding (sample b)	ultrafine grinding (sample c)	micronizate (sample d)
7, 11	125.7	125.9	125	125.4
2	123.6	123.9	125[a]	124.7[b]
6	127.2	127.3	127.2	127.1
15	130.8	130.9	130.1	130.2
10	131.5	131.6	*c*	*c*
1	132.4	132.4	132.5	132.6
14	133.3	133.4	133.4	133.3
8, 9	136.1	136.3	136.3[b]	136.2[b]
12, 13	137.5	137.7	137	136.9
5	139.5, 140.3	139.4, 140.4	*d*	*d*

[a] Signal coincides with the signals of the carbon atoms C-7 and C-11. [b] Shoulder. [c] Signal coincides with the signal of the carbon atom C-15. [d] Signal coincides with the signals of the carbon atoms C-12 and C-13.

The cross-polarization constant, T_{CH}, describes the magnetization transfer and determines the rise of the intensity at short contact times, whereas the spin lattice relaxation time of the protons in the rotating frame, $T_{1\rho H}$, is responsible for the decrease of the signal intensity at longer contact times. **Figure 2** shows the cross-polarization curves for the C-4 atoms (41 ppm) of crystalline lycopene (sample a) and of formulated lycopene micronizate (sample d). At short contact times, both curves show an identical steep increase in intensity. However, clear differences can be seen at longer contact times. The long $T_{1\rho H}$ time observed in the contact time curve of pure crystalline lycopene causes a plateau in the cross-polarization curve. This feature has also recently been described for β-carotene (*11*) and seems to be characteristic of crystalline carotenoids. In contrast, the $T_{1\rho H}$ time observed in the contact time curve of the micronizate is much shorter, leading to a decrease in intensity. This phenomenon occurs due to neighborhood interactions of the lycopene cores with the outer mobile shell, accelerating the relaxation of the protons.

Both the supramolecular aggregation behavior of the lycopene molecules within the core and the core particle size have a significant impact on light absorption and, therefore, on the color of the micronizates. For β-carotene molecules, two specific configurations have been discussed (*7*). The formation of so-called H-aggregates, in which the crystals form a parallel configuration like a deck of cards, leads to a hypsochromic shift in the UV/vis spectra as compared to the absorption maximum of the carotenoid in the dissolved (isolated) state. In the head-to-tail configuration of the molecules, the so-called J-aggregates, both a bathochromic shift and a hypsochromic shift in the UV/vis spectra can be observed. Depending on the production process, lycopene formulations show the same shifts. Larger particles (1 μm and above), such as crystalline lycopene (sample a) or a coarsely milled product (sample b), reveal the characteristic UV/vis absorption bands for J-aggregates, whereas in the absorption spectra of finely milled lycopene and the micronizates (samples c and d, particle size below 0.4 μm), the typical bands for H-aggregates are intensified.

High-resolution ^{13}C solid-state NMR spectroscopy enables differentiation between the individual aggregates. Spectroscopic measurements of testosterone (*12*) and androstenone (*13*) pointed out that the chemical shift of carbon atoms in solid-state NMR

spectra is exceptionally sensitive to conformational changes, molecule geometry, and van der Waals forces with respect to the core. The different fine structure of signals depending on the aggregation type is shown in **Figure 3** (aliphatic region) and in **Figure 4** (olefinic region). Assignment of the signals was achieved with data of conventional high-resolution NMR spectra (*14*). It can be clearly seen that the spectrum of crystalline lycopene (sample a) is identical with that of coarsely milled lycopene (sample b, J-aggregates), whereas these spectra show differences in comparison with the spectra of (ultra)finely milled lycopene (sample c, H-aggregates) and the micronizate (sample d, H-aggregates). In the aliphatic region, the signals of methyl groups C-17 to C-20 reflect the different aggregation behavior (**Figure 3**). While the intensity of signals of C-17 and C-18 changes depending on the aggregate type, the difference in the patterns of signals of C-19 and C-20 is remarkably noticeable. In the case of H-aggregates, two separate signals appear at 12.6 and 14.2 ppm. J-aggregates, on the other hand, show a major signal at 13.7 ppm, which is not symmetrical due to small amounts of H-aggregates in the sample that lead to shoulders at 12.6 and 14.2 ppm.

The signals of carbon atoms C-2 and C-5 also show significant differences (**Figure 4**). In the NMR spectra of H-aggregates these signals are not resolved, whereas in the case of J-aggregates the signal of C-2 is shifted to higher field and the signal of C-5 to lower field. The latter signal is split in two resonances due to packing effects; this feature is often observed in solid-state NMR spectra.

In the spectra of crystalline lycopene (sample a) and the coarsely milled product (sample b), the signals of carbon atoms C-1, C-10, C-14, and C-15 show significantly different fine structures in comparison to the signals of the ultrafinely milled lycopene (sample c) and the lycopene micronizate (sample d) (**Figure 4**). **Table 1** summarizes the different chemical shifts of the individual signals in the olefinic region. Although these differences are sometimes very small, they are nevertheless clearly observable and cannot be ascribed to the measurement conditions because the chemical shift values for carbon atoms C-1, C-6, and C-14 are nearly identical. Due to the characteristic signal pattern in solid-state NMR spectra, an unambiguous classification of the aggregation type of lycopene particles is possible.

Thus, solid-state, together with suspended-state, NMR spectroscopy proves to be a versatile tool for detailed characterization of this important class of compounds.

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