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COMPLEXATION OF CURCUMIN USING WHEY PROTEINS TO ENHANCE AQUEOUS SOLUBILITY, STABILITY AND ANTIOXIDANT PROPERTY

LEVENTE ZSOLT RACZ[a], CSABA-PAL RACZ[a], OSSI HOROVITZ[a], GHEORGHE TOMOAIA[b,c], AURORA MOCANU[a], IRINA KACSO[d], MELINDA SARKOZI[e], MONICA DAN[d], SEBASTIAN PORAV[d], GHEORGHE BORODI[d], MARIA TOMOAIA-COTISEL[a,c,*]

ABSTRACT. The stability and solubility of curcumin, CCM, can be enhanced by complexation with whey protein concentrate (WPC). The CCM-WPC of 1:1 and 1:0.5 molar ratio complexes were encapsulated by two methods, namely spray drying (SD) and freeze drying (FD). The major objective of this work is to determine the stability and solubility of the CCM-WPC complexes encapsulated by SD and FD method. The X-ray diffraction and thermogravimetric analysis were performed on raw CCM and WPC, and on their complexes. The changes in thermal degradation and in crystallinity suggest the formation of these CCM-WPC complexes. Scanning electron microscopy images showed that the used methods influenced the morphology and the properties of the formed complexes. The stability and solubility of curcumin are enhanced by complexation in both encapsulation methods. However, comparing the used molar ratio and drying methods, the results are various. The solubility of samples obtained by FD method was higher than in the case of the SD method, which can explain the difference of their morphology (FD samples are more amorphous than SD samples). The antioxidant property of curcumin and its protein-complexes compared to vitamin C showed that CCM and its complexes have a lower IC_{50} value than vitamin C, i.e., the antioxidant effect of CCM is higher. The addition of WPC improved the antioxidant activity of CCM, probably due to its encapsulation in the protein. Our results suggest that the 1CCM-1WPC_SD complex is the best antioxidant from these studied CCM-WPC complexes.

Keywords: *curcumin, whey protein concentrate, spray drying, freeze drying, solubility, half-life time, antioxidant property*

[a] *Babeş-Bolyai University, Research Center of Physical Chemistry, Faculty of Chemistry and Chemical Engineering, 11 Arany Janos str., RO-400028, Cluj-Napoca, Romania*
[b] *Iuliu Hatieganu University of Medicine and Pharmacy, Department of Orthopedics and Traumatology, 47 Gen. Traian Mosoiu str., RO-400132, Cluj-Napoca, Romania*
[c] *Academy of Romanian Scientists, 3 Ilfov str., RO-050044, Bucharest, Romania*
[d] *National Institute for Research and Development of Isotopic and Molecular Technologies, Department of Molecular and Biomolecular Physics, 67-103 Donath Str., RO-400293, Cluj-Napoca Romania*
[e] *SC. Parapharm SRL, 9 Dacilor Str., RO-335200, Brad, Romania*
** Corresponding author: mcotisel@gmail.com*

INTRODUCTION

As a naturally occurring polyphenol, curcumin (CCM) is an active component of the dietary spice turmeric (Curcuma longa), which possesses physiological and pharmacological properties [1]. CCM's health-promoting properties include like anti-Alzheimer [2, 3], anticancer [4-8], anti-inflammatory [9, 10], antidiabetic [11, 12], antiproliferative [13], antioxidant [14] and antimicrobial effects [15, 16]. However, the therapeutic application is contained by its low water solubility, instability and bio-accessibility [17-19]. To improve these limiting factors, β-Lactoglobulin (βLG), the major protein from whey protein concentration (WPC), can be used, due to its properties to solubilize and bind small hydrophobic molecules [20, 21].

Microencapsulation is a widely used technology for improving stability or solubility, is a process in which tiny insoluble particles are surrounded by a coating to give small capsules, with useful properties. In this case CCM is capsulated with WPC [22]. Various microencapsulation techniques have been applied, such as physical, or physico-mechanical techniques as spray drying, freeze drying, extrusion, and centrifugal extrusion [23] or physico-chemical ones as coacervation or chemical techniques as matrix polymer. Spray drying is an efficient and cheap drying method. It can be used for drying liquid mixtures where the dry matter content is dissolved or suspended. The liquid is broken down into small droplets by the spray head, and the moisture content by the drying air evaporates. The speed of the process is due to the increased evaporation surface. Freeze-drying, also known as lyophilization, is a method in which the humidity is removed by freezing and sublimation. During the process, the material is first frozen, the pressure is reduced, and then the heat loss due to drying is compensated by heating the material, otherwise this would significantly slow down the process. The disadvantage is that the procedure is time consuming and comes with a high price [24].

The aim of this paper was to enhance the solubility and stability of CCM by encapsulation with WPC. Moreover, the comparison of the two used drying methods and their effects on the CCM's thermal stability, antioxidant activity, crystallinity and morphology were also explored in this work.

RESULTS AND DISCUSSION

Thermogravimetric analysis (TG/DTG)

In our research, we performed TG/DTG measurements of CCM, WPC and their complexes (Figures 1-6) prepared by two different methods.

During the measurements, different thermal decomposition of the substances was observed, which provides evidence for the formation of the curcumin and whey protein complex.

Derivation of the curves allows the evaluation of fine details. The weight loss of CCM takes place in two steps between 200-410°C (42.34%) and 410-550°C (58.42%). In the keto form of CCM, a highly activated carbon atom leads to the loss of hydrogen atoms, which initiates the degradation of it [1].

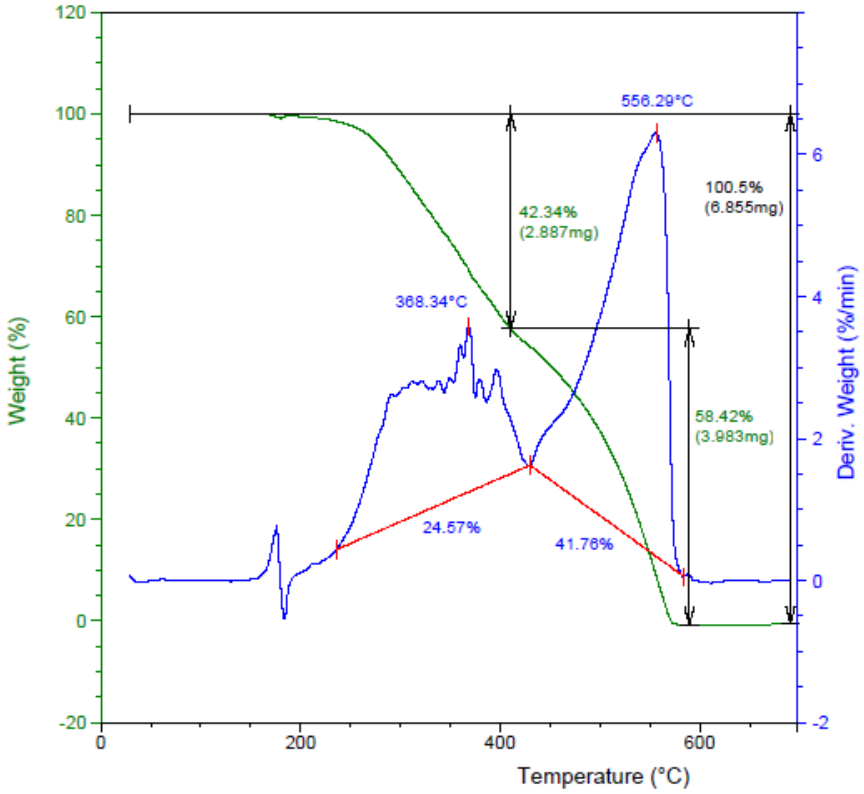

Figure 1. TG/DTG analysis of CCM

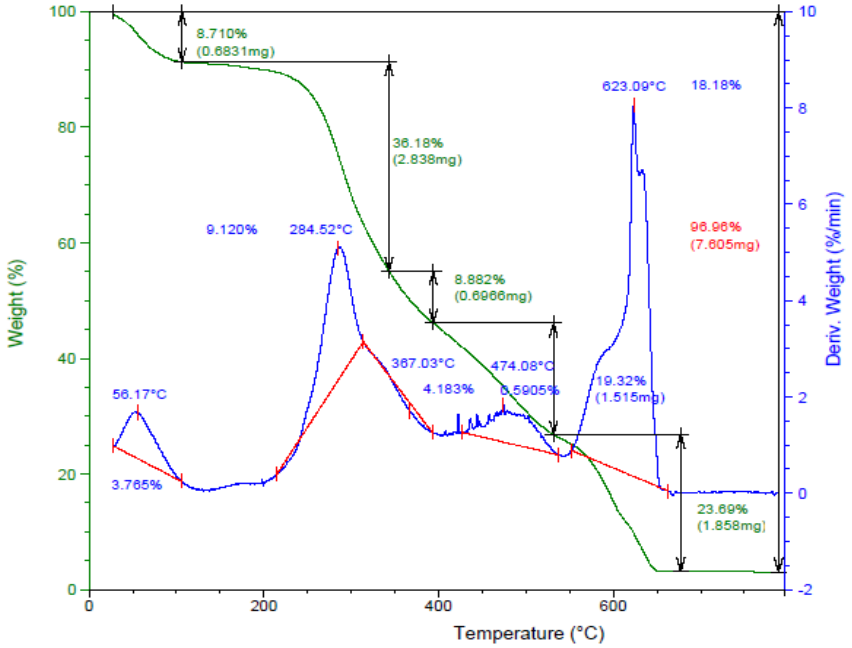

Figure 2. TG/DTG analysis of WPC

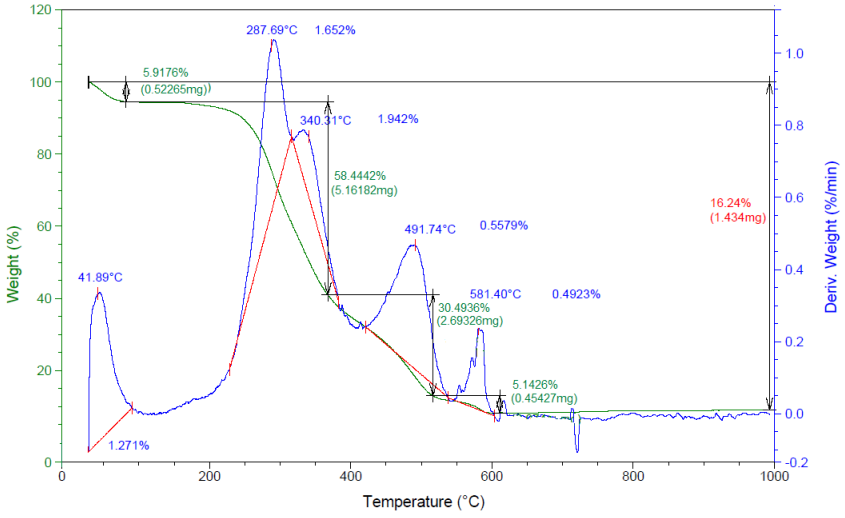

Figure 3. TG/DTG analysis of 1CCM-0.5WPC_SD

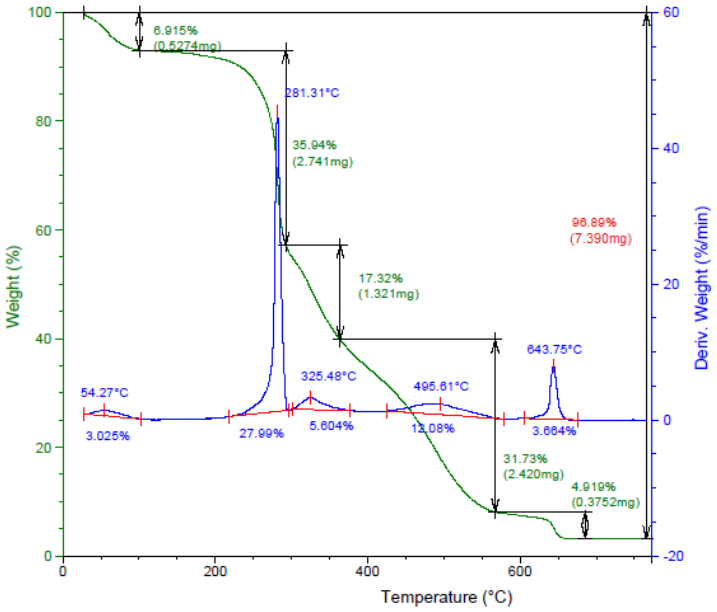

Figure 4. TG/DTG analysis of 1CCM-1WPC_SD

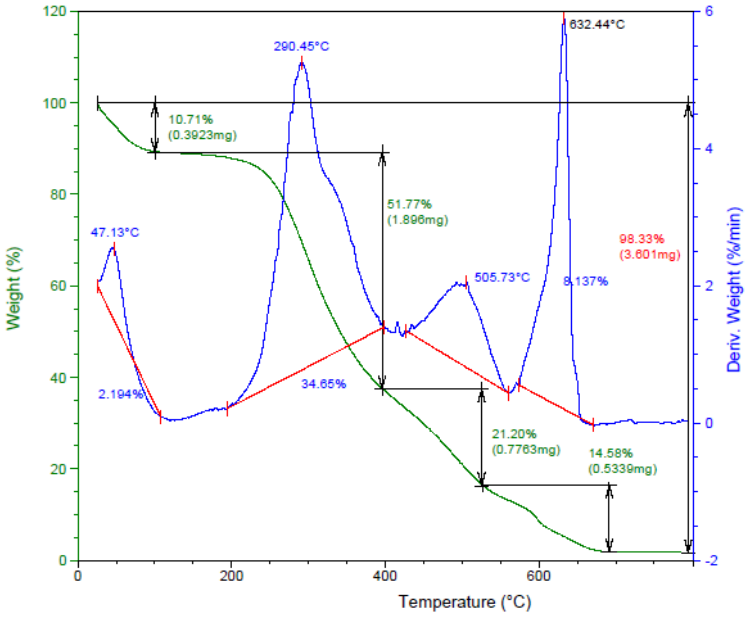

Figure 5. TG/DTG analysis of 1CCM-1WPC_FD

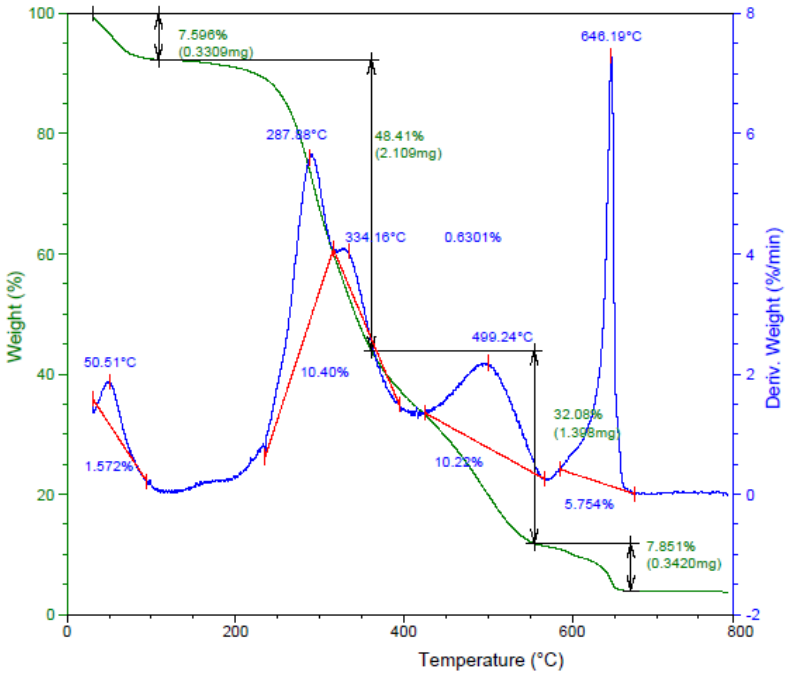

Figure 6. TG/DTG analysis of 1CCM-0.5WPC_FD

The thermal decomposition of WPC takes place in 5 steps between 50-110°C (8.71%), which is attributed to water evaporation, ~200-350°C (36.18%), 350-400°C (8.88%), 400-540°C (19.32%) and 540-670°C (23.69%), similarly in cases of its complexes.

X-ray diffraction analysis (XRD)

The XRD patterns of CCM, WPC and their complexes for studying their crystallinity are shown in Figure 7 WPC showed classic amorphous XRD patterns indicating that the amorphous nature of WPC was not changed by complexation with CCM.

On the contrary, the XRD pattern of free curcumin displayed different intense diffraction peaks between 5° and 39° attributed to its highly crystallized structure [20, 25]. However, as represented in the diffractograms of CCM-WPC samples, most of the characteristic peaks related to the crystalline structure of curcumin disappeared in the complexes, which suggest that the complexation process occurs [19].

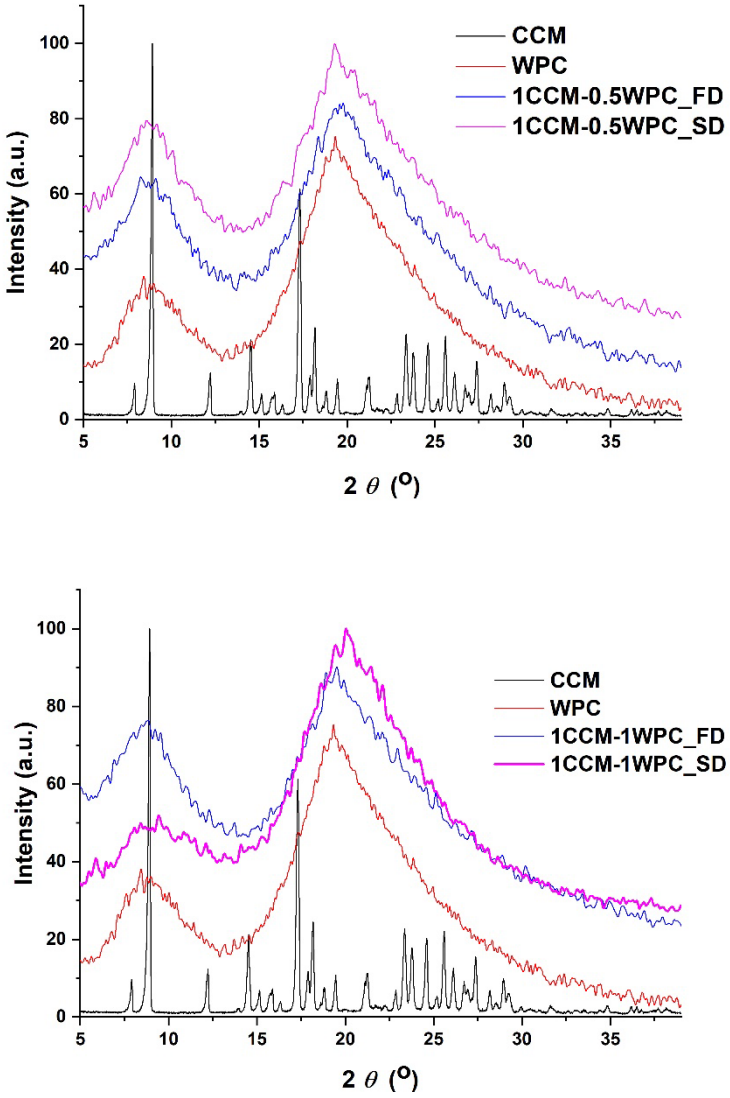

Figure 7. XRD diffractograms of CCM, WPC and their complexes.

Scanning electron microscopy (SEM)

The morphological structures of microparticles examined by SEM are presented in *Figure 8*.

Following the two drying methods, different morphological characteristics resulted. Spray dried microparticles were more spherical and regular in shape, which are typical of spray-dried powders, and their surface appeared relatively smoother than the freeze-dried ones, which exhibited more cracks or fractures [23]. The SEM images of the complexes shown that the curcumin was protected within the wall matrix, which is based on the fact, that the scaly and abrupt-edged structure of CCM it is absent in the microcapsules [19].

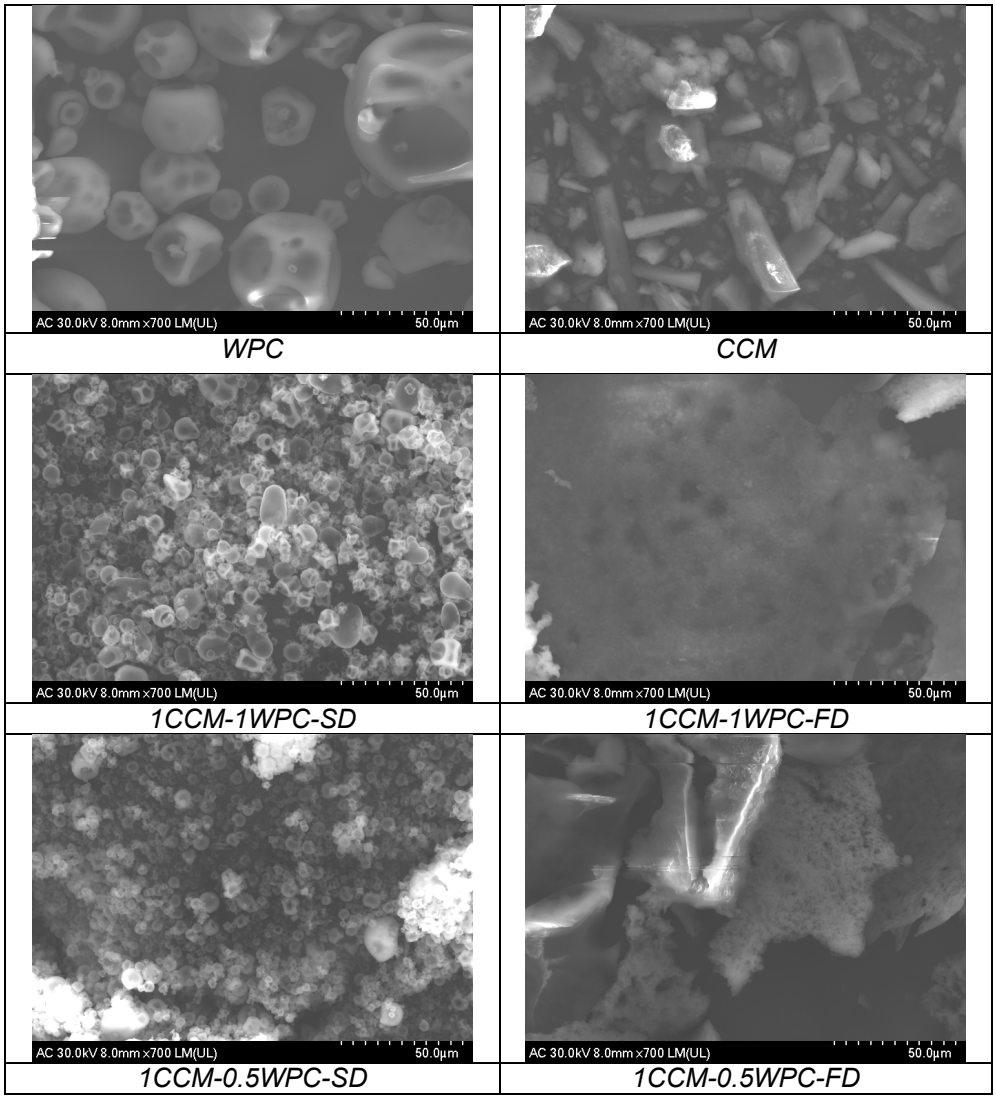

Figure 8. SEM images of WPC, CCM, and CCM-WPC complexes

Solubility in water

For the solubility measurements the absorbance of CCM-WPC complexes was measured by an UV-Vis spectrometer at 425 nm (see in Figure 9).

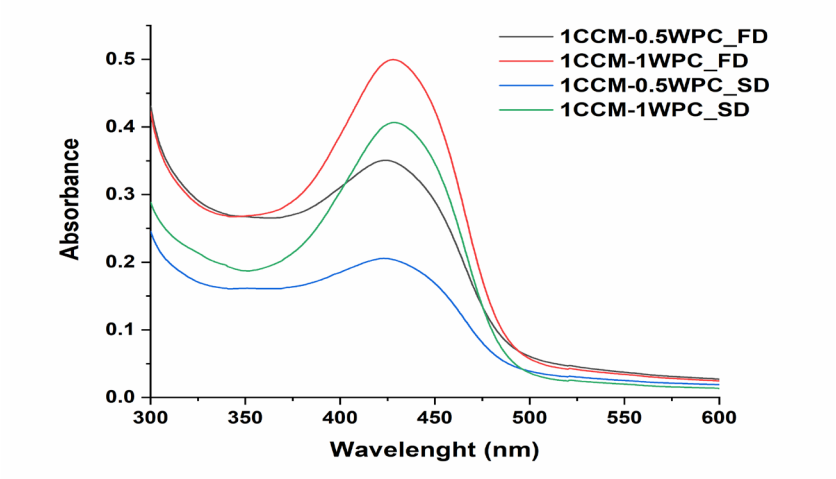

Figure 9. The UV-Vis spectra of complexes.

The CCM concentration was calculated using calibration curve of CCM in ethanol (Figure 10). Absorbance is given in arbitrary units.

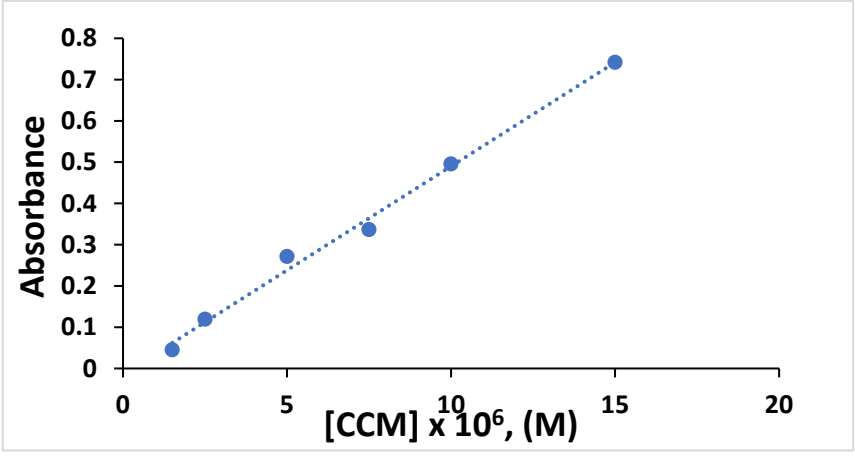

Figure 10. Calibration curve of CCM in ethanol. Characteristics of regression line are: $y = 50361x - 0.0137$, where x is curcumin concentration, and $R^2 = 0.9932$.

Water solubility of curcumin is very low, just 0.00262 mg/ml (Table 1). Complexation of CCM with WPC increases the water solubility of CCM, in all cases and the CCM-WPC complexes (particularly of 1:1 molar ratio) have the higher solubility (Table 1). Comparing the spray drying (SD) and freeze-drying (FD) methods, the prepared complexes with the latter method have shown a higher solubility, namely SD method showed 7.5x increase, while FD method exhibited an 8.9x increase in CCM solubility. SEM images (Figure 8) suggest that FD methods resulted in amorphous particles, which are markedly more soluble than their crystalline counterparts obtain by the SD method, which is supported by the XRD diffractograms too [26].

Table 1. Water solubility (μg/mL) of curcumin and its complexes

Materials	Solubility (μg/mL)
CCM	2.6
1CCM-1WPC_SD	19.5
1CCM-0.5WPC_SD	12.9
1CCM-1WPC_FD	23.1
1CCM-0.5WPC_FD	15.3

Stability and degradation of curcumin and its complexes in different aqueous solutions of various pH values

The chemical instability of curcumin is widely known [27]. In order to investigate the protective effects of the WPC and the influence of the drying methods on the degradation kinetics of curcumin, the kinetic profile of water-mediated curcumin degradation, CCM-WPC complexes were dissolved in buffers with pH values as in simulated gastric (1.5), in intestinal (8.0) or in physiological (7.4) [28] simulated fluids without enzymes. Therefore, beside the protective effect of WPC and preparation technique, the effect of pH was also studied on the stability of CCM. Depending of the used medium, the degradation products and mechanism of CCM are different because of its structure [29, 30].

Consequently, in the following two examples are given as the UV-vis spectra of the decrease in time of CCM absorbance (noted A, a.u.) for 1CCM-0.5WPC_SD and 1CCM-1WPC_FD complexes at pH 7.4 in Fig. 11A and Fig. 11B, respectively.

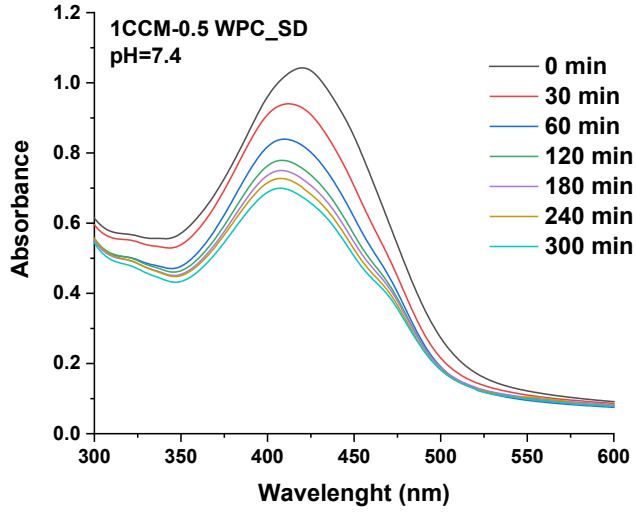

Figure 11.A. The UV-vis spectra of the decrease in time of CCM absorbance in 1CCM-0.5WPC_SD at pH 7.4

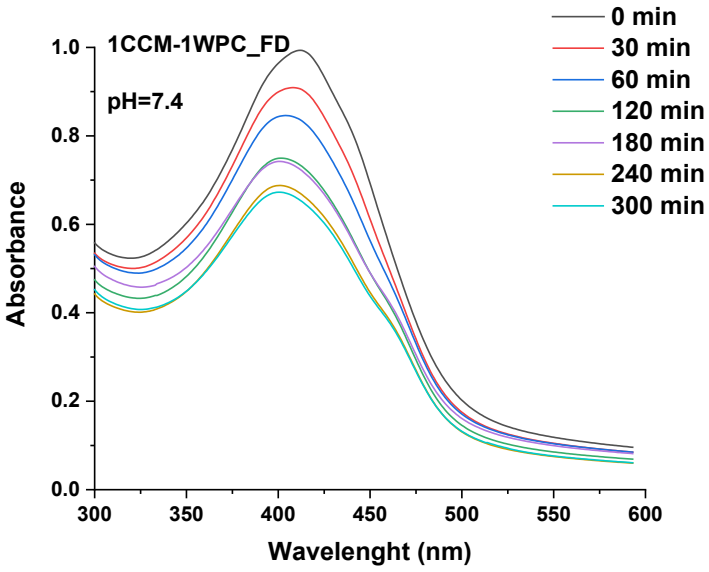

Figure 11.B. The UV-vis spectrum of the decrease in time of CCM absorbance in 1CCM-1WPC_FD complex at pH 7.4

The half-life ($t_{1/2}$) is the time moment on which the initial concentration of CCM is decreased by half of its original value. Depending on the reaction type, the half-life can be calculated with different equations:

- For *first order* reaction:

  R→ $\boldsymbol{Products}$

$$\text{rate} = k[R]$$

where k is the rate constant, and [R] is the molar concentration of the reactant. Since the absorbance, A, is proportional to the concentration, absorbance values can be used in the kinetic equations instead of concentration values.

The integrated form of the *first order* kinetic equation can thus be written as:

$$\ln A = \ln A_o - kt$$

where A values are the absorbance values at different moments and A_o is the initial value (at t = 0).

The rate constant k was determined by plotting of ln(A) versus time;

$$k = -a,$$

where a is the slope of the regression line: y = ax + b, and $t_{1/2}$ was calculated as:

$$t_{1/2} = \frac{0.693}{k}$$

- For *second order* reaction:

  2R→ $\boldsymbol{Products}$ or R + S → $\boldsymbol{Products}$; when [R] = [S]:

$$\text{rate} = k[R]^2$$

Using absorbance values instead of concentrations, the integrated 2nd order kinetic equation is:

$$1/A = 1/A_o + kt$$

The k value was determined, by plotting 1/A versus time, as the slope a of the regression line,

$$k = a.$$

Then, $t_{1/2}$ was obtained from the relation:

$$t_{1/2} = \frac{1}{kA_0}$$

The measured and calculated values are given in Table 2, together with the coefficients of determination, R^2, for the linear regression, represented, as examples, in Fig.12A and Fig.12B.

Table 2. The stability measurements (e.g., absorbance, a.u.) of the 1CCM-1WPC_FD complex in different (1.5, 7.4 and 8.0 pH) buffer solutions

pH 8.0									
t(sec)	Absorb-ance, A	1st order kinetics				2nd order kinetics			
		ln A	a = -k (s^{-1})	b	R^2	1/A	a= k (s^{-1})	b	R^2
0	0.9834	-0.017	-0.0006± 1.22E-4	-0.102± 0.089	0.8518	1.017	0.0009± 1.62E-4	1.1025± 0.117	0.8789
240	0.8358	-0.179				1.196			
480	0.5859	-0.535				1.707			
720	0.5292	-0.636				1.890			
960	0.5131	-0.667				1.949			
1200	0.5021	-0.689				1.992			
pH 7.4									
t(min)	Absorb-ance, A	1st order kinetics				2nd order kinetics			
		ln A	a = -k (min^{-1})	b	R^2	1/A	a = k (min^{-1})	b	R^2
0	0.9423	-0.059	-0.0012± 2.6E-4	-0.138± 0.044	0.8401	1.061	0.0015± 3.23E-4	1.1479± 0.054	0.8653
30	0.8508	-0.162				1.175			
60	0.7885	-0.238				1.268			
120	0.6917	-0.369				1.446			
180	0.6905	-0.370				1.448			
240	0.6596	-0.416				1.516			
300	0.6506	-0.430				1.537			
pH 1.5									
t(min)	Absorb-ance, A	1st order kinetics				2nd order kinetics			
		ln A	a = -k (min^{-1})	b	R^2	1/A	a= k (min^{-1})	b	R^2
0	1.019	0.019	-0.0007± 3.1E-5	0.0082± 0.005	0.9809	0.981	0.0007± 3.48E-5	0.9891± 0.0059	0.9897
30	0.9804	-0.020				1.020			
60	0.9629	-0.038				1.039			
120	0.9291	-0.074				1.076			
180	0.9022	-0.103				1.108			
240	0.8660	-0.144				1.155			
300	0.8229	-0.195				1.215			

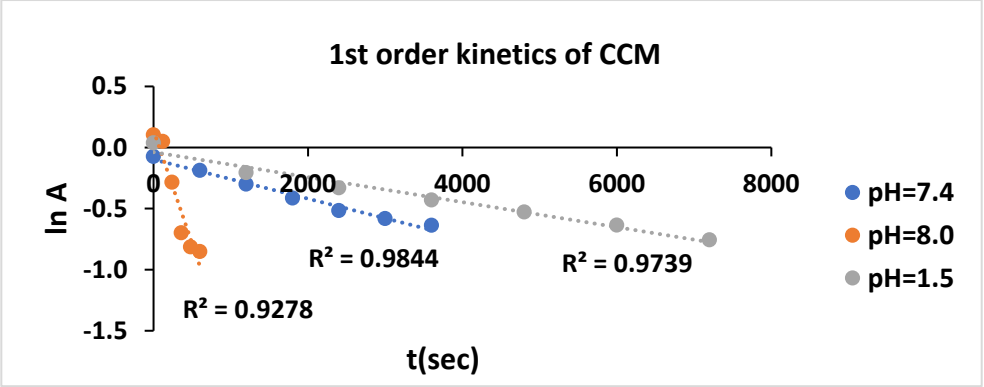

Figure 12 A. The first order degradation plot of CCM at different pH values and at 37°C.

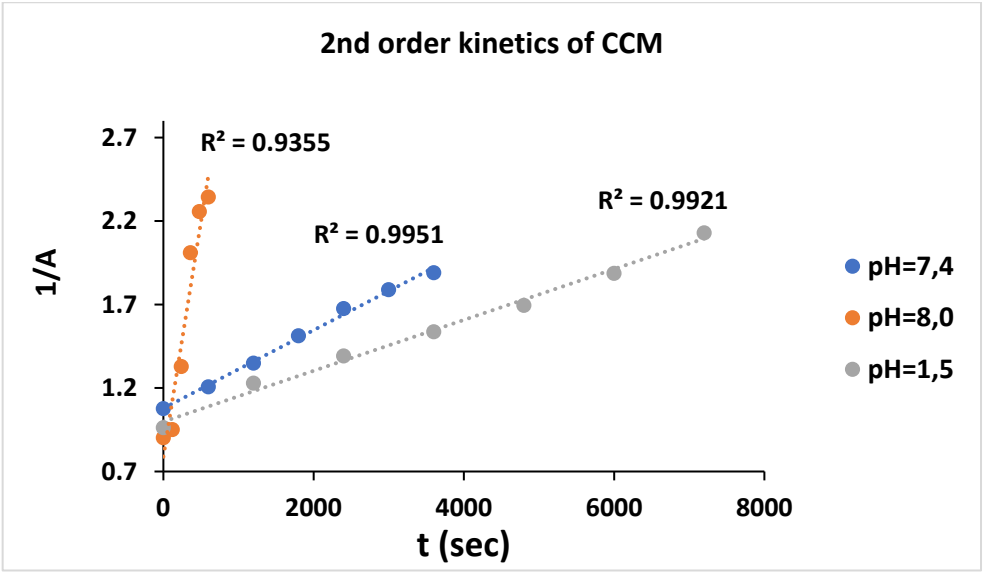

Figure 12 B. The second order degradation plot of CCM at different pH values and at 37°C.

Since correlations are better according to the 2nd order kinetics, in Table 3 the $t_{1/2}$ values were calculated assuming the process to follow this kinetic law.

Table 3. The half-life ($t_{1/2}$) time of CCM and its complexes by using of second order kinetics model.

Materials	$t_{1/2}$ (min)		
	pH 1.5	pH 7.4	pH 8.0
CCM	78±13.37	74±18.11	4±0.58
1CCM-1WPC_SD	397±33.42	1069±88.48	36±5.31
1CCM-0.5WPC_SD	237±9.11	742±102.36	17±0.22
1CCM-1WPC_FD	1656±211.61	784 ±125.3	22±2.65
1CCM-0.5WPC_FD	725±270.78	739±113.6	7±0.81

Under alkaline conditions (pH ≥8.0) a rapid decrease in absorbance was observed, and the half-life time of CCM was just 4 minutes (see in Table 3). The CCM solution has higher stability in acidic media than in alkaline ones, which has shown that curcumin is more chemically stable under acidic conditions [31]. The $t_{1/2}$ of CCM increased in all tested media by complexation of CCM with WPC. During the study first and second order kinetics model was fitted as shown in Fig. 12A and Fig. 12B, respectively. These results suggested that the second order kinetics described more exactly the degradation kinetics of CCM, thereby the degradation of CCM depends on the initial concentration.

Antioxidant activity determined by DPPH• free radical scavenging activity

Free radical scavenging activity of ascorbic acid, curcumin and its complexes were tested using the DPPH method. DPPH• method is highly reproducible and was applied on the study of antioxidant activity of food products [32].

The UV-vis spectra of the decrease of absorbance of DPPH by addition of different quantity of CCM solution, ascorbic acid (Vitamin C) solution, 1CCM-1WPC_SD solution, 1CCM-1WPC_FD solution, 1CCM-0.5WPC_SD solution, and 1CCM-0.5WPC_FD solution are shown in Figs. 13-18, respectively.

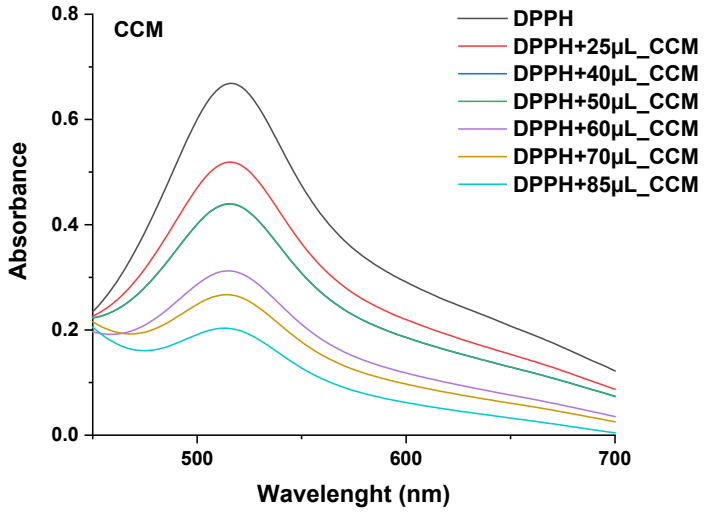

Figure 13. The UV-vis spectrum of the decrease of absorbance of DPPH by addition of different quantity of CCM solution

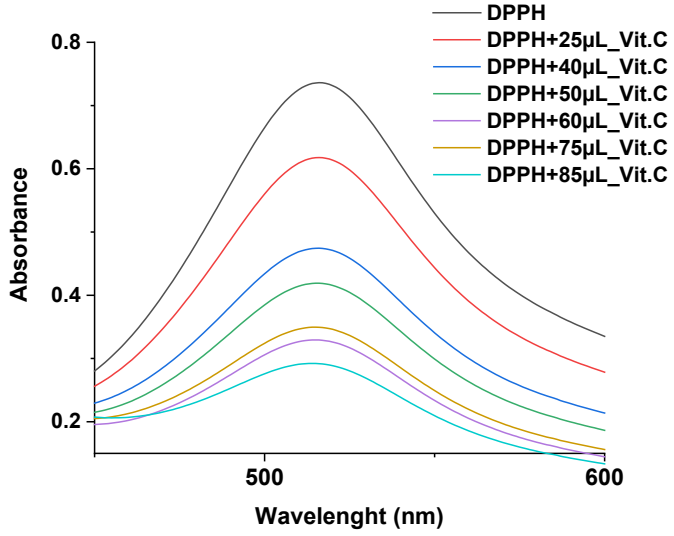

Figure 14. The UV-vis spectrum of the decrease of absorbance of DPPH by addition of different quantity of ascorbic acid (Vitamin C, Vit.C) solution

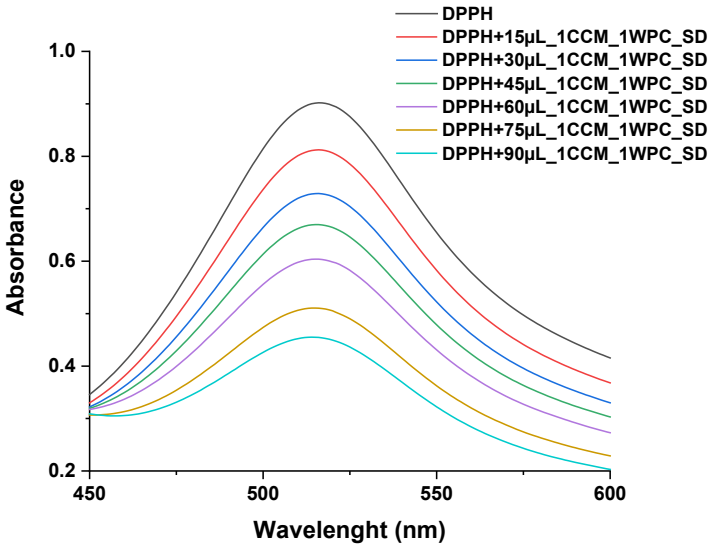

Figure 15. The UV-vis spectrum of the decrease of absorbance of DPPH by addition of different quantity of 1CCM-1WPC_SD solution

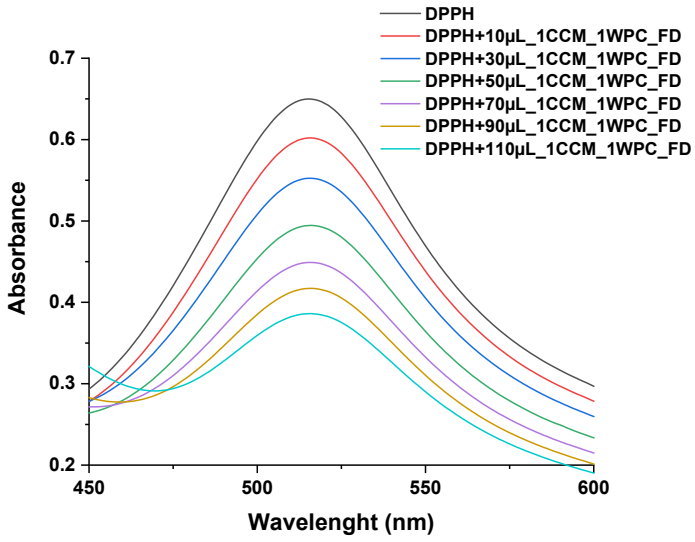

Figure 16. The UV-vis spectrum of the decrease of absorbance of DPPH by addition of different quantity of 1CCM-1WPC_FD solution

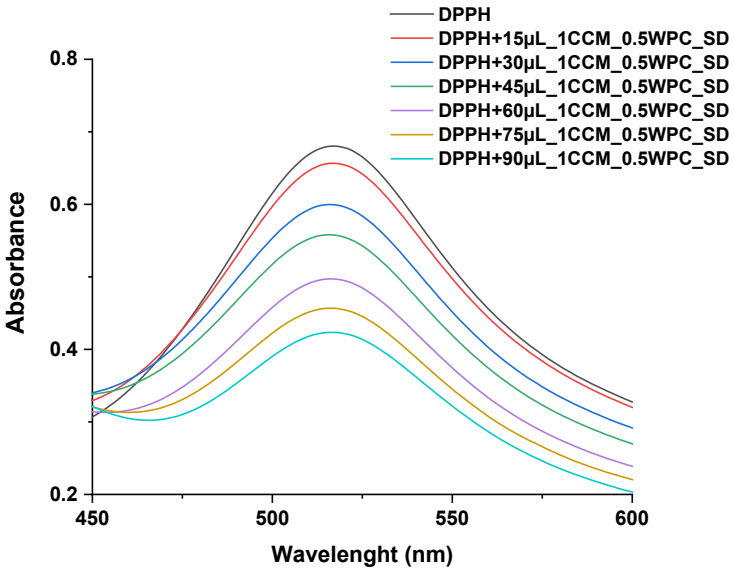

Figure 17. The UV-vis spectrum of the decrease of absorbance of DPPH by addition of different quantity of 1CCM-0.5WPC_SD solution

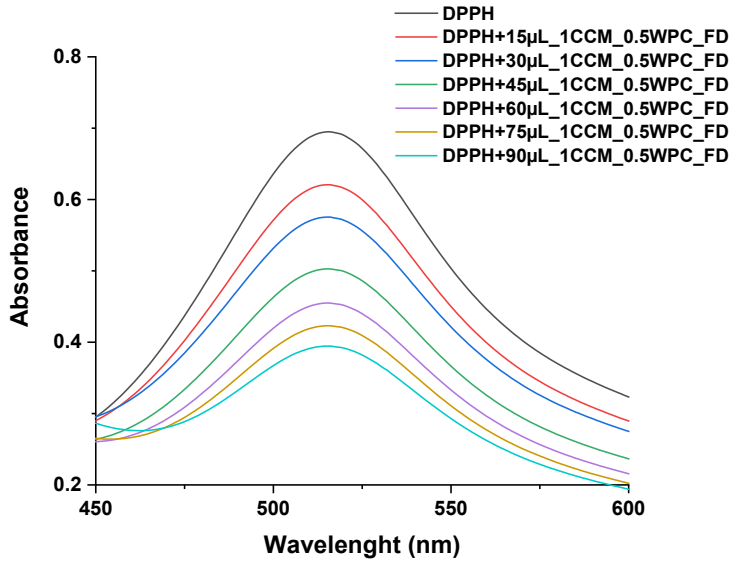

Figure 18. The UV-vis spectra of the decrease of absorbance of DPPH by addition of different quantity of 1CCM-0.5WPC_FD solution

The use of DPPH• method provides an easy, quick path to assess the *antioxidant properties of curcumi*n [33, 34]. The antioxidant effect of CCM was measured by the discoloration of a purple-colored methanol solution of the stable DPPH• radical.

The %DPPH radical scavenging activity = $\{(A_0-A_t)/A_0\} * 100$

where A_0 is the absorbance of DPPH solution without antioxidant, and A_t is absorbance of DPPH with different (test, t) concentrations of antioxidant.

By plotting of %DPPH radical scavenging activity in function of antioxidant concentrations (e.g., Fig. 19 and Fig. 20), and from the characteristic regression line equation, the value of IC_{50} can be determined:

IC_{50} = (50-y intercept) / slope

IC_{50} is the concentration, which is effective in producing 50% of the maximal effect of DPPH solution, without antioxidant.

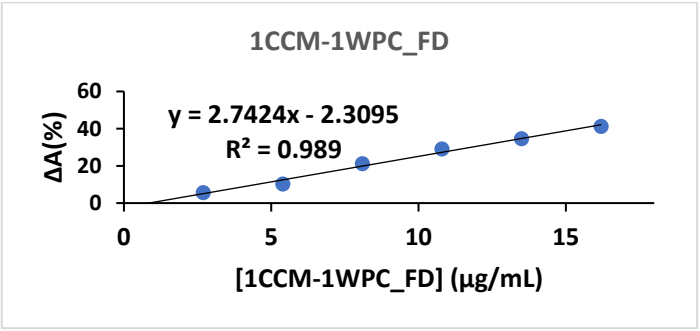

Figure 19. The plot of %DPPH radical scavenging activity in function of 1CCM-1WPC_FD concentration (µg/mL)

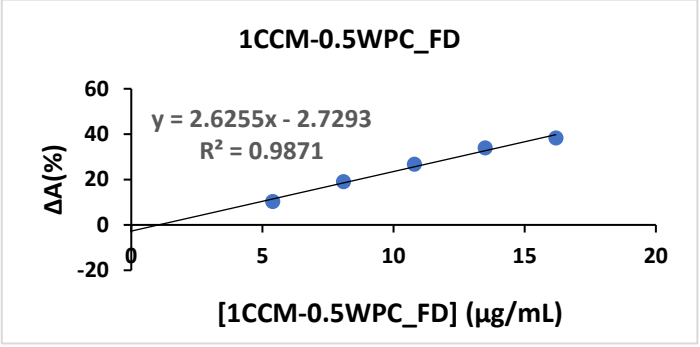

Figure 20. The plot of %DPPH radical scavenging activity in function of 1CCM-0.5WPC_FD concentration (µg/mL)

The result IC_{50} was expressed in molar concentration, M, of consumed sample per gram of DPPH• free radical.

The scavenging effect of complexed curcumin and standards on the DPPH radical decreased in the order of 1CCM:1WPC_SD ≥ 1CCM:1WPC_FD ≥ 1CCM:0.5WPC_SD ≥ 1CCM:0.5WPC_FD ≥ CCM ≥ ascorbic acid (Table 4).

Table 4. Half-maximal inhibitory concentrations (IC_{50}) of ascorbic acid, CCM, and obtained CCM-WPC complexes

Materials	IC_{50} (M)	Materials	IC_{50} (M)
Ascorbic acid	$3.71x10^{-5}$	CCM	$2.95x10^{-5}$
1CCM:1WPC_SD	$8.48\ x10^{-7}$	1CCM:0.5WPC_SD	$1.83x10^{-6}$
1CCM:1WPC_FD	$1.04x10^{-6}$	1CCM:0.5WPC_FD	$1.88x10^{-6}$

Comparing the used molar ratios of CCM and WPC within their complexes, the 1CCM-1WPC (1:1 mole ratio) complexes had higher antioxidant effect (Table 4) than the 1CCM-0.5 WPC (1:0.5 mole ratio) complexes. On the other hand, the 1CCM-0.5WPC (1:0.5 mole ratio) complexes were not affected by the used preparation spray drying, SD, or freeze drying, FD, method. However, the 1CCM:1WPC_SD complex had the highest antioxidant effect (about 35x improvement, compared to CCM antioxidant effect). Certainly, the encapsulation of CCM in whey protein leads to an enhanced antioxidant effect of CCM, and thus, these complexes are potential carriers for CCM in vitro and in vivo.

CONCLUSIONS

During our study the 1CCM-1WPC and 1CCM-0.5WPC complexes were prepared by spray drying and lyophilization methods. The resulted complexes and the raw materials were analyzed by TG, XRD and SEM techniques and their solubility and stability were comparatively examined. Moreover, the antioxidant properties of CCM and its protein complexes are evaluated and discussed in comparison with the antioxidant activity of vitamin C. The pure curcumin has a crystalline structure, which by complexation becomes a compound (material) which is amorphous, and this amorphous nature of complexes also contributes to the significant increase of CCM solubility. The morphology of CCM, WPC and their complexes is different depending on the used drying techniques. The antioxidant properties, solubility and stability of curcumin increased by complexation with whey protein

concentrate. Therefore, the complexes of curcumin-whey protein concentrate stabilized curcumin from degradation improving the solubility and stability as well as the anti-oxidant ability of curcumin.

EXPERIMENTAL SECTION

Materials and methods

Curcumin of high purity (95%) and ethanol were purchased from Sigma-Aldrich (Darmstadt, Germany), while whey protein concentrate (80%) was bought from Foodcom (Warsaw, Poland), disodium hydrogen phosphate (Na_2HPO_4) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Merck (Darmstadt, Germany), and the methanol from VWR chemicals (Fontenay-sous-Bois, France). Potassium dihydrogen phosphate, NaCl and HCl (36%) were obtained from Sigma-Aldrich (Darmstadt, Germany). During the experiment ultrapure water was used, which was purified with Milli-Q® IQ 7003. All compounds were used without further purification.

Preparation of samples

During our research spray drying and lyophilization methods were applied as preparation of the samples [8]. In the two cases mol ratios were 1CCM:1WPC and 1CCM:0.5WPC. CCM in ethanol and WPC in purified water were dissolved according to the molar ratios. After mixing of CCM and WPC solutions, the obtained solution was stirred for 4 hours in dark conditions and room temperature. In the final solutions the ethanol portion does not exceed 5% of the protein solution [35]. After 4 hours stirring the solutions were dried with two different methods:

- Spray drying (Pilotech, YC-018A) parameters were: T_{inlet} =165ºC, T_{outlet}= 49ºC, ventilator frequency 58 Hz, solution flow rate 5 mL/min. In the end a fine, homogeneous yellow-orange, powder was obtained (1CCM-1WPC_SD and 1CCM-0.5WPC_SD).
- Freeze drying (Alpha 1-2 LD plus) parameters were: -55ºC temperature, 0,06 mbar pressure, duration of the operation was 24 h (1CCM-1WPC_FD and 1CCM-0.5WPC_FD)

Solubility in water

Samples equivalent to 0.53 mg of CCM were added to screw-capped vessels containing 5 mL distilled water and were treated by sonication (Elmasonic S300/H) at 36ºC for 2 hours. The solutions were filtered with a

0.45 μm disc filter. Filtered 200 μl solution was diluted with 1800 μl ethanol and the absorption of CCM was measured by an UV-Vis spectrometer (*Jasco 670*) at 425 nm.

Stability measurement of CCM and its complexes at different pH values

For determination of CCM stability, a CCM was dissolved in ethanol ($2x10^{-3}$ M stock solution) and dropped in buffer solutions (1.5; 7.4 and 8.0 pH), which are given in Table 5, at 37 ºC and the variation of the absorbance was measured around 425 nm at different time intervals with UV-Vis spectrophotometer, *Jasco V-670*. The constant temperature was provided by a heating circulator, *Julabo MA 4*. In case of complexes, the powders were dissolved in buffers and the decrease of absorbance was measured as in case of CCM.

Table 5. The recipe of used buffer solutions

BUFFER SOLUTION	USED MATERIALS	Used quantity for 1 L	M (g/mol)	Reference
pH 7.4	Na_2HPO_4	17.9 g	141.96	[37]
	KH_2PO_4	3.6 g	136.086	
	NaCl	4.3 g	58.44	
pH 1.5	HCl (2N)	50 mL	36.46	
pH 8.0	Na_2HPO_4	14.2 g	141.96	
	HCl (0.1M)	44.9 mL	36.46	

The used buffers were prepared based on the recipe in Table 5 using purified water as solvent.

The daylight and artificial lighting can affect remarkably the degradation of CCM [36], as the measurements were carried out in the absence of light.

DPPH• free radical scavenging activity

The DPPH method (2.2-diphenyl-1-picryl hydrazyl) is based on the catching of the DPPH free radical by antioxidants, causing a decrease in absorbance at 517 nm wavelength [38]. The course of the reaction can also be seen with the naked eye, as the dark purple radical loses its color when reacting with antioxidants.

Table 6. The used stock solutions for antioxidant activity measurements

Stock solution	Solvent	Concentration (mol/L)
DPPH	Methanol	$1.01x10^{-7}$
Ascorbic acid	Purified water	$1.13x10^{-7}$
CCM	Methanol	$9.77x10^{-7}$
1CCM-1WPC_SD	Methanol	$4.78x10^{-6}$
1CCM-1WPC_FD	Methanol	$4.78x10^{-6}$
1CCM-0.5WPC_FD	Methanol	$2.43x10^{-6}$
1CCM-0.5WPC_SD	Methanol	$2.43x10^{-6}$

A series of solutions was prepared by adding different concentrations of antioxidant (0-90 µL) solution to 3 mL of DPPH stock solution and diluting it with methanol to a final volume of 5 mL. The reference solution was without antioxidants (Table 6). Ascorbic acid was used as standard. The solutions must be stored in the dark for half an hour before the measurement. The measurements were made by a UV-Vis spectrophotometer, Jasco V-670, at 517 nm.

X-ray powder diffraction (XRD)

The crystallographic structure of curcumin and its complexes were studied by XRD analysis as presented elsewhere [39, 40]. The patterns of WPC, CCM, 1CCM:1WPC_SD, 1CCM:0.5WPC_SD, 1CCM:1WPC_FD and 1CCM:0.5WPC_FD after freeze drying or spray drying were obtained by a Bruker D8 Advance diffractometer equipped with a goniometer and graphite bent crystal monochromator (CuKα1 radiation). The measurements were done at a filament current of 40 mA and operating voltage of 40 kV using Ge 111 monochromator. The scanned angle was set from 2θ of 5°–39° and the scan rate was 1° s^{-1}.

Scanning electron microscopy (SEM)

The samples were spread over the double-sided conductive tape (12 mm) fixed on aluminum stubs and coated with a 10 nm layer of gold. SEM images were done on a Hitachi SU8230 High Resolution Scanning Electron Microscope equipped with a cold field emission gun and an 80 X-Max system from Oxford Ins. for EDS analysis.

For this analysis the microscope was operated at 30 kV in high mag. mode. Approximately 85% of the carbon disk was scanned to give a realistic overview of the sample and only a few representative areas were captured.

Thermogravimetric analysis (TG)

Thermogravimetric analysis was performed with SDT Q600 TA Instruments thermogravimeter, in air (with 12% oxygen) flow of 20 mL min^{-1}, heating rate 10 °C min^{-1} and temperature range of 30–600 °C. The sample mass used was 8.0 ± 1.0 mg in an alumina cell.

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