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# Solid State Mechanochemical Simultaneous Activation of the Constituents of the *Silybum marianum* Phytocomplex with Crosslinked Polymers

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**ABSTRACT:** Simultaneous improvement of solubilization kinetics of main flavolignans of *Silybum marianum* extract was obtained cogrinding with two crosslinked polymers: micronized crospovidone, PVP-CL<sup>®</sup> and sodium carboxymethylcellulose, Ac-Di-Sol<sup>®</sup> in the 1:3 active-to-polymer weight ratio. By this process it was assessed that the main extract components lost its crystalline structure, and the powder surface area was increased by 2.1- and 1.7-fold in the coground products with Ac-Di-Sol<sup>®</sup> and PVP-CL<sup>®</sup>, respectively. This activated status of the dry extract remained stable over a period of 2 years. Solubilization kinetics resulted ameliorated both in terms of entire dry extract and in terms of single components. When the 1/3 coground systems with PVP-CL<sup>®</sup> and Ac-Di-Sol<sup>®</sup> were dissolved in saturated conditions they gave a concentration improvement compared to the native product of 8 and 31 times of silybin A, 7 and 27 times of silybin B, whereas in the case of silychristin a double concentration was obtained only using Ac-Di-Sol<sup>®</sup>. The *in vivo* studies on rats confirmed that this solubilization improvement corresponded to an effective oral bioavailability enhancement. The highest bioavailability improvement was obtained with Ac-Di-Sol<sup>®</sup>, with a relative bioavailability of 88.6, 17.96, and 16.4 compared to the extract for silybin A, silybine B, and silychristine, respectively. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:215–228, 2009

**Keywords:** *Silybum marianum* extract; solid state; mechanical activation; micronized crospovidone; crosslinked sodium carboxymethylcellulose; oral absorption; bioavailability; physical characterization; silybin A and B; silychristin

## INTRODUCTION

Standardized extracts from the fruit seeds of *Silybum marianum* L. Gaertn. (milk thistle, Asteraceae), are used in humans for the treatment

of liver diseases of different etiologies.<sup>1</sup> The interest in the potential benefits of *Silybum marianum* originates in antiquity and it is one of the first documented examples of plants used for maintenance of health and treatment of disease.<sup>2</sup> The group of active constituents, named silymarin, mainly consists of silybin (40–60%) and isosilybin (10–20%; each as pair of diastereoisomers A and B), silydianin and silychristin (20–45%).<sup>3</sup>

The therapeutic use of these flavonolignans is partly restricted by their insolubility in water. In particular, silybin, the main constituent, is

This paper is dedicated to Professor Fulvio Rubessa, who recently retired from his position as full professor at the Faculty of Pharmacy, University of Trieste, after more than 40 years of distinguished academic career.

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sparingly soluble in water and spontaneously tends to form nonabsorbable microcrystals. The oral bioavailability of this herbal medicine is therefore limited and it is strictly dependent on the galenical preparation, as shown for various silymarin products on the market.<sup>4</sup>

Several approaches to improve the oral bioavailability of silymarin have been attempted: its complexation with phosphatidylcholine,<sup>5,6</sup> the formation of an inclusion complex with  $\beta$ -cyclodextrins,<sup>7</sup> the creation of water-soluble matrices by spray drying or lyophilizing process,<sup>8</sup> solid dispersions with hydrophilic polymers such as PEG 6000<sup>9,10</sup> and, finally, the formulation of a self-microemulsifying systems.<sup>11</sup> With respect to these previous studies the purpose of the present research was to ameliorate the solubilization kinetics of each individual component of this herbal medicine. In fact, it is well known that the pharmacological activity of an herbal medicine depends on the overall activity of a variety of active compounds and synergistic action of these components is the basis of herbal recipes. For this purpose, the cogrinding process in a planetary mill was applied to increase simultaneously the *in vitro* dissolution performance of the main components of *S. marianum* extract by their incorporation in two crosslinked hydrophilic polymers.

The mechanical stress, given by this mechanochemical process, induces dislocation in the structures of both active principles and polymer, with consequent intra-particle embedding of the active crystallites and further active molecular dispersion in the carrier.<sup>12</sup> Micronized crospovidone PVP-CL<sup>®</sup> and crosslinked sodium carboxymethylcellulose, Ac-Di-Sol<sup>®</sup>, were chosen as polymeric carriers because both of them, through their different chemical affinity (the first is amphiphilic and the second is hydrophilic), have shown a good capability to improve the bioavailability of several poorly soluble drugs.<sup>13,14</sup>

After preparation of the binary systems, the effect of the process on the properties of the dry extract and on the main constituents was evaluated by studying their *in vitro* solubilization kinetics, their solid state and possible interaction between the components (XRD and DRIFT), their particle size and surface area in comparison with the starting raw materials and the simple physical mixtures (PMs). Finally, the *in vivo* oral absorption on rats of the 1:3 w/w active to Ac-Di-Sol<sup>®</sup> and PVP-CL<sup>®</sup> coground systems was tested.

## MATERIALS AND METHODS

### Materials

Silybin A and B, silychristin and *S. marianum* dry extract were provided by Indena S.p.A. (Milano, Italy). Naringenin pure standard was from Extrasynthese (Genay, France),  $\beta$ -glucuronidase/arylsulfatase (*Helix pomatia*) was from Boehringer Mannheim (Mannheim, Germany). Micronized crospovidone (PVP-CL<sup>®</sup> M) was purchased by BASF/Euphar (Milano, Italy) and crosslinked sodium carboxymethylcellulose (Ac-Di-Sol<sup>®</sup>) was provided by Shilling (Milan, Italy). All other chemicals, of analytical grade, and solvents HPLC grade, were provided by Carlo Erba (Milan, Italy).

The chemical structures of tested compounds are listed in Table 1.

### Preparation of Coground Mixtures

Dry extract alone and in combination of PVP-CL<sup>®</sup> or Ac-Di-Sol<sup>®</sup> were coground in 1:3 dry extract-to-polymer weight ratios in a planetary mill Fritsch P7 (Pulverisette, Contardi Fritsch s.r.l., Milan, Italy) at 360 rpm ( $V_P$ ; 12 Hz). For comparison purposes, also the 1:1 dry extract-to-polymer weight ratio was prepared. The planetary mill was equipped by four agate cylindrical grinding chambers with a capacity of 25 mL each containing 4 agate balls as grinding media, with a diameter of 12 mm. Fifty grams batches, previously blend in the suitable proportions with a stainless steel spatula, were simultaneously coground. The ball-to-powder mass ratio was 1.25. The grinding procedure was pursued for 1 h, stopping every 15 min for homogeneously mixing the mass with a stainless steel spatula.

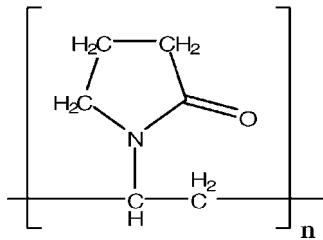
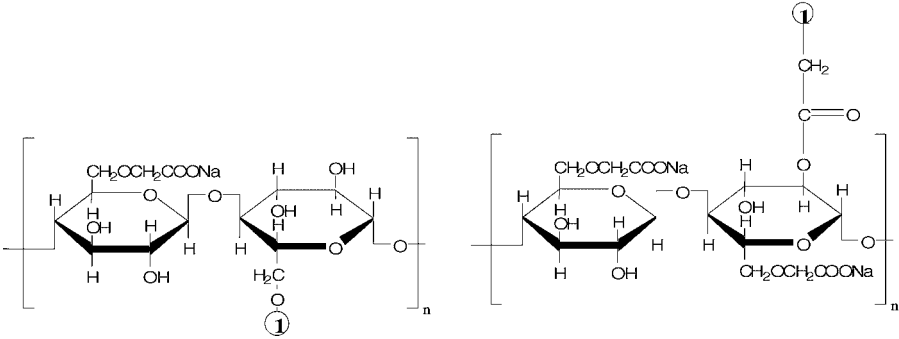
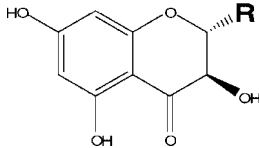
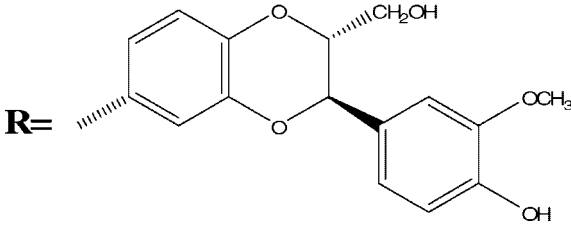
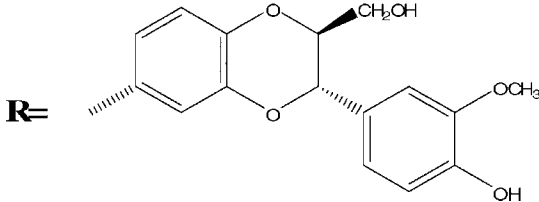
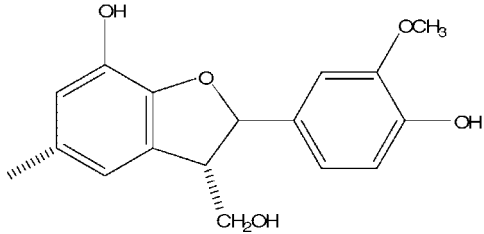
### Preparation of Physical Mixtures

For comparison purposes, PMs were prepared by manually mixing dry extract and each polymer using the same weight ratios as the coground systems.

### X-Ray Powder Diffraction Studies (XRD)

Solid state of the samples was studied by means of XRD technique using a STOE D500 (Siemens, Munich, Germany) diffractometer with Cu-K $\alpha$  radiation (1.5418 Å), monochromatized by a

**Table 1.** Chemical Structure of Carriers and Studied Flavonolignans

Compound	Abbreviation	Chemical Structure
Croscopovidone	PVP-CL <sup>®</sup>	
Croscarmellose sodium	Ac-Di-Sol <sup>®</sup>	
Flavonolignans		
Silybin A		
Silybin B		
Silychristin		

secondary flat graphite crystal. The current used was 20 mA and the voltage 40 kV. The scanning angle ranged from 5° to 30° of  $2\theta$ , steps were of 0.05° of  $2\theta$ , and the counting time was of 2 s/step.

### DRIFT Spectroscopy

Fourier transform-infrared spectra were obtained on a FT-IR spectrometer (FT-IR Perkin Elmer Spectrum One, Monza, Italy) using the diffuse reflectance method (DRIFT). The samples (dry extract, carriers and 1:3 (w/w) PM and coground system) were added to anhydrous KBr (FT-IR grade) in a 1:15 weight ratio (sample to KBr) and gently ground, thus avoiding solid transition possibly induced by extended grinding. The scanning range was 450–4000  $\text{cm}^{-1}$  and the resolution was 4  $\text{cm}^{-1}$ , scan number was 8 and scan speed 0.20  $\text{cm/s}$ .

### Porosity Measurements

The porosity of the samples has been determined with a mercury porosimeter (ThermoQuest Italia, Rodano, Italy) equipped by Macropore Pascal 140 low pressure porosimeter and Macropore Pascal 240 high pressure porosimeter (Carlo Erba Instruments, Milan, Italy). A dilatometer for powders with capillary diameter of 1.5 mm was loaded with 300 mg samples. Before measuring, a degasification procedure under vacuum pressure for 30 min was performed. The experiments were performed in triplicate. The volume of mercury intruded in function of the applied pressure was transformed in the porous distribution applying the model of cylindrical porosity of Washburn. The size distribution was calculated applying the Mayer and Stowe method,<sup>15</sup> whereas the specific surface area was calculated by using the method proposed by Rootare and Prenzlów.<sup>16</sup>

### Solubilization Kinetics

The solubilization kinetics of the coground systems was determined in nonsink conditions according to Nogami et al.<sup>17</sup> and compared to the corresponding PMs. In particular, an excess quantity of the samples was added to 900 mL of deionized water in a USP 28 dissolution vessel, at a temperature of  $37.0 \pm 0.5^\circ$  while stirring with a paddle at 200 rpm. The tested amount for each sample approximately corresponded to three

times its solubility in water. Samples (5 mL) were collected at predetermined time intervals, and filtered with Sartorius regenerated cellulose membrane 0.45  $\mu\text{m}$ . The dissolved dry extract was spectrophotometrically determined and absorbances were recorded at 287 nm.

Furthermore, the solubilization kinetics of three main components of the dry extract, silybin A and B, and silychristin from the coground systems and the starting extract was singularly determined, using the same conditions used for the determination of the entire dry-extract but quantifying the solubilized amount with a HPLC technique reported into details in the following paragraphs.

### In Vivo Absorption Studies

Experiments on animals complied with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC).

Animals used were male Sprague–Dawley rats (250–280 g weight) and were supplied by Centro Servizi Polivalenti di Ateneo (University of Trieste). Rats, with free access to water, were fasted overnight. Experimental formulations were administered by gastric gavage as aqueous suspensions to four rats. Each rat received a single dose of formulations: 50 mg/kg of *S. marianum* dry extract corresponding to 8.5, 8, and 10.5 mg/kg of silybin A, silybin B and silychristin, respectively, and 50 mg/kg of 1:3 w/w coground systems corresponding to 2.6, 2.25, 4.45 mg/kg of silybin A, silybin B and silychristin, respectively, as determined by HPLC.

Blood samples were collected from animal abdominal aorta in heparinized tubes at 0.5, 1, 2, and 4 h after administration. Blood samples were centrifuged at 2500 rpm for 15 min and plasma was separated and immediately frozen at  $-20^\circ\text{C}$ , and stored at this temperature till the analysis.

### Sample Preparation

For the determination of the flavolignans in the coground systems the procedure was the following: the suitable amount of dry extract as a powder or coground mixture was accurately weighted and transferred to a 25 mL volumetric flask and diluted with 20 mL of methanol. The mixture was sonicated for 15 min and then diluted to 25 mL

with methanol. The mixture was filtered through a 0.45  $\mu\text{m}$  nylon filter to remove any particle. The first 5  $\mu\text{L}$  of the filtrates were discarded and the subsequent were collected. Appropriate aliquots of filtrates were diluted with the mobile phase.

As for the determination of total flavolignans, silybin A and B, silychristin in plasma samples the following procedure was adopted. One milliliter serum sample containing the internal standard naringenin (50  $\mu\text{L}$  of an internal standard solution containing 2  $\mu\text{g}/\text{mL}$ ) was incubated with 1 mL of 1 M sodium acetate buffer pH 5.0 and 100  $\mu\text{L}$  of  $\beta$ -glucuronidase/arylsulfatase for 3 h at 37°C, while shaking at 55 rpm. After adding 2 mL of 0.5 M borate buffer solution pH 8.5, the flavonolignans and the internal standard naringenin were extracted into 5.5 mL diethyl ether by shaking at 55 rpm for 20 min. After centrifugation for 10 min at 2000g (12°C) the organic phase was transferred into 5 mL micro-reaction vessels and evaporated at 45°C under a stream of nitrogen. The residue was redissolved in 200  $\mu\text{L}$  of methanol, vortexed for 1 min, centrifuged and 50  $\mu\text{L}$  of the supernatant were used for HPLC analysis.

As for the chromatographic conditions, the column was a Hibar RT 250-4.6 Purosphere STAR RP-18 (5  $\mu\text{m}$ , 250 mm, 4.6 mm id, Merck, Darmstadt, Germany) maintained at 30°C. Chromatographic separation was carried out using an isocratic elution of water (pH 3.2 formic acid) and methanol (50–50%). The analysis was for a 30 min period at a flow rate of 1.0 mL/min. Injected volume of the sample was 20  $\mu\text{L}$ . Ultraviolet spectra were recorded in the range 200–600 nm and chromatograms were acquired at 220, 254, 270, 285, and 350 nm.

### Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated on the composite plasma curves. The area under the plasma concentration-time curve extrapolated to the last sampling time at which a quantifiable concentration is found ( $\text{AUC}_{\text{last}}$ ) was calculated using the log-linear trapezoidal method. Time and value of maximum concentration ( $t_{\text{max}}$  and  $C_{\text{max}}$ , respectively) were reported as observed. The relative bioavailability after oral administration ( $F_{\text{rel}}$ ) was calculated in Eq. (1):

$$F_{\text{rel}} = \frac{\text{AUC(PVP or Ac-Di-Sol)}/\text{Dose(PVP or Ac-Di-Sol)}}{\text{AUC(Extract)}/\text{Dose(Extract)}} \quad (1)$$

### Assay of Active Components by HPLC

Silybin A, B and silychristin were determined by a previously published HPLC method,<sup>18</sup> using a HPLC systems consisting of a HP 1090L instrument with a Diode array detector and managed by a HP 9000 workstation (Hewlett Packard, Palo Alto, CA). The HPLC system was interfaced with a HP1100 MSD API-electrospray (Hewlett Packard). The interface geometry, with an orthogonal position of the nebulizer, with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis. The same column, mobile phase and flow rate were used. Mass spectrometry operating conditions were optimized to achieve maximum sensitivity values: gas temperature 350°C at 11 L/min, nebulizer pressure 40 psi, quadrupole temperature 30°C and capillary voltage 3000 V. Full spectra from  $m/z$  100 to 1000 in negative ion mode were obtained.

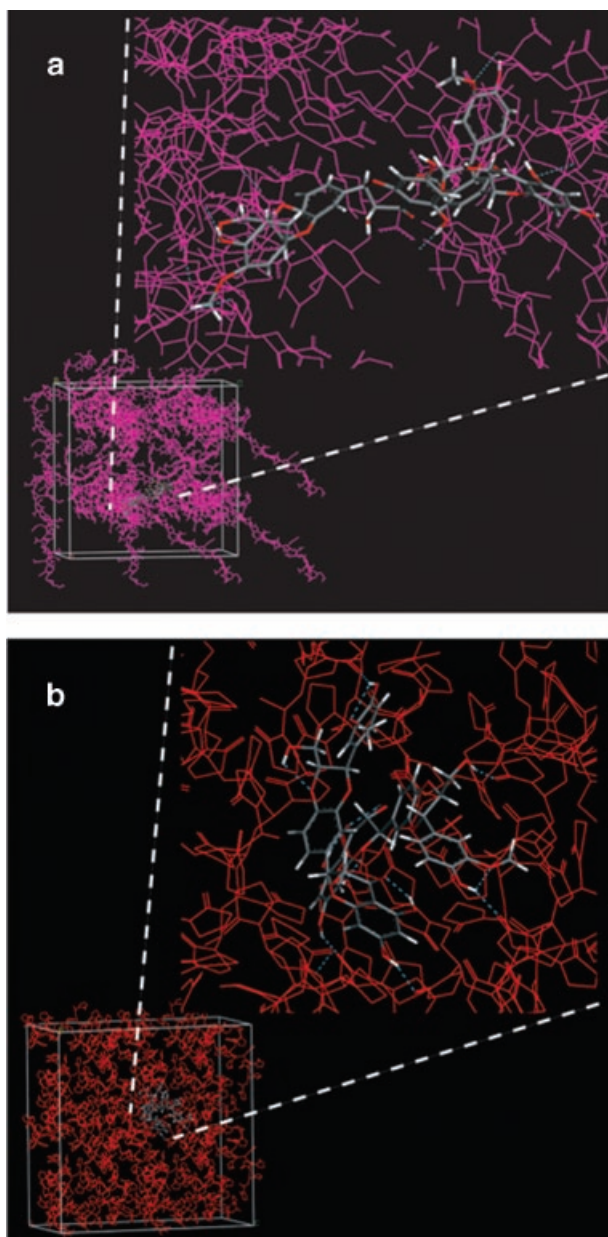
Pharmacokinetic analysis was performed using WinNonlin Version 2.1 (Pharsight Corporation, Mountain View, CA) software.

### RESULTS AND DISCUSSION

As previously stated, the aim of the work was to increase simultaneously the water solubility of the components of the phytocomplex by solid state mechanochemical activation with the use of hydrophilic crosslinked polymers as carriers and thus to possibly improve their oral bioavailability.

The solid state activation was performed in a planetary mill that is suitable for generating predominantly shearing interactions as a result of the rolling of steel ball elements on the wall of the grinding container. Briefly, the planetary mill generates a tangential mechanical strain, that promotes a plastic deformation of native

crystalline phases leading not only to a change in dimensions and shape of the particles but also to the accumulation of defects. Such defects can lead to nanocrystals and/or amorphization of the drug particles that get trapped in the amorphous polymer network. Both these phases represent an activated status of a drug that has a higher bioavailability and reduced response times.<sup>12</sup> Figure 1 is a schematic representation of possible interactions between active and each polymer.



**Figure 1.** Schematic representation of the composite structure and possible interaction between the flavonolignans and Ac-Di-Sol<sup>®</sup> (a) or PVP-CL<sup>®</sup> (b).

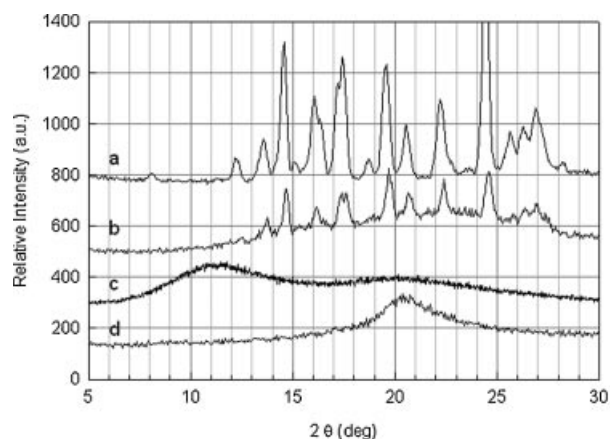
First of all, the extract alone was processed in the planetary mixer. However nonsatisfactory results were obtained. In fact the powder during the process adhered to the chamber walls and to the grinding balls forming a very hard layer, thus inhibiting the mechanochemical process itself. This inconvenient did not occur when the extract was diluted with the carriers. For this reason, all the subsequent trials were only conducted on binary mixtures. Simple PMs, prepared by manually mixing active and each polymer, were used as a matter of comparison of the coground systems.

To check possible solid state variations of the dry extract, XRD analysis was carried out. Figure 2 reports the diffractograms of the raw materials: the two carriers, PVP-CL<sup>®</sup> and Ac-Di-Sol<sup>®</sup>, *S. marianum* dry extract and its major active components, silybin A and B.

Both polymeric carriers show a complete lack of peaks and a halo pattern, typical of amorphous materials. In the pattern of PVP-CL<sup>®</sup> (Fig. 2c) two consecutive broad bands are evident in the range between 7° and 25° of 2 $\theta$ ; whereas only one hump is visible in the Ac-Di-Sol<sup>®</sup> pattern (Fig. 2d) between 17° and 23° of 2 $\theta$ .

Conversely, the diffraction pattern of silybin A + B (Fig. 2a) shows several very intense peaks, indicating the crystalline structure of the material. In particular, the highest signals are evident at 14.6°, 16.5°, 19.5°, 22.3°, and 24.5° of 2 $\theta$ .

The dry extract behaves at the XRD analysis as a semi-crystalline material, showing some peaks of medium intensity together with a strong underneath scattering phenomenon, due to its amorphous content (Fig. 2b). As for the crystalline



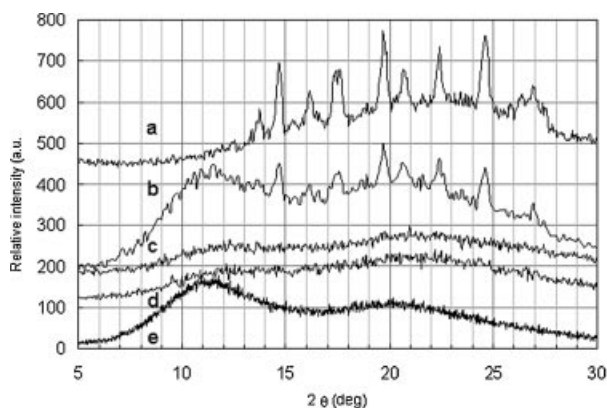
**Figure 2.** XRD patterns of the raw materials: silybin A + B (a), *Silybum marianum* dry extract (b), PVP-CL<sup>®</sup> (c), and Ac-Di-Sol<sup>®</sup> (d).

portion, it must be noticed that the main reflections of the extract major components, silybine A + B, are also present in the diffractogram of the dry extract with only a little upward shift phenomenon. Furthermore, the absence of signals different from those of silybin A and B in the diffractogram of the dry extract demonstrates that the other components are of amorphous nature or not detectable due to their little content in the extract. For example, the weight percentage of silydianin in the dry extract is about 6%, that is quite near to the sensitivity of this analytical method.<sup>19</sup> It can be concluded that the detectable crystalline component of the extract is almost totally represented by silybine A and B.

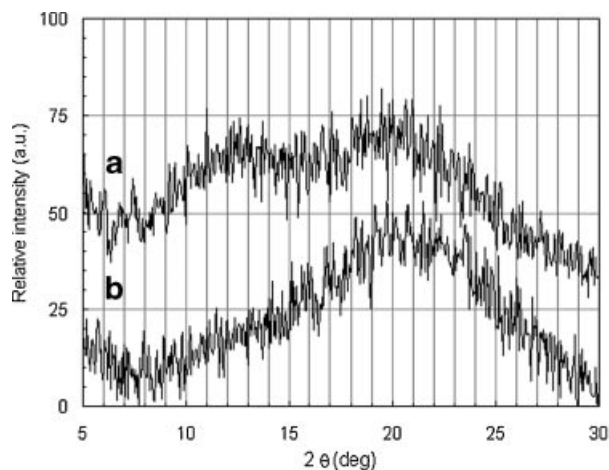
In Figure 3 the diffractograms of the dry extract/PVP-CL<sup>®</sup> binary systems are presented and compared to the single components. The XRD patterns of the coground systems of the 1:1 and 1:3 active to polymer weight ratios are almost superimposable (Fig. 3c and d, respectively). Both of them are typical of completely amorphous materials, with a lack of peaks and an evident scattering phenomenon. Two large humps can be recognized: the first one, at about 12° of 2θ, probably attributable to the polymer that shows his apex at this angle (see Fig. 3e), and the second one, with a maximum at about 22° 2θ, probably due to the transition into an amorphous state of the dry extract, that reveals in its semicrystalline original form its major peaks in this range. This fact is more significant when considering the 1:1 w/w coground system (prepared only for comparison purposes), where the absence of signals cannot be

ascribed to the low content of silybin A + B (that actually represents about 16.5% w/w of the mixture) but to the amorphization of the crystalline component. To check whether this phenomenon is due to the presence of the polymer or to the mechanochemical activation process, the XRD patterns of the coground systems were compared to those of the corresponding PMs, containing the same dry extract to polymer weight ratio as the coground systems. For the sake of brevity, only the 1:3 w/w PM is depicted in Figure 3b. This pattern still shows a certain degree of residual crystallinity of silybine A + B as indicated by the peaks in its typical angles (e.g., 14.6°, 16.5°, 19.5°, 22.3°, and 24.5° of 2θ), in contrast to the corresponding coground system. This means that the mechanochemical activation is responsible for the achievement of a dry extract of amorphous character in all his components. Moreover, the energetic contribution given by the mechanochemical activation, favors the interaction between dry extract amorphous components and the polymeric carrier. Moreover, this interaction results in the stabilization of the highly reactive amorphous state. In fact, repeating the XRD analysis on aging, the amorphous character of the 1:3 w/w coground systems with PVP-CL<sup>®</sup> was maintained also after 2 years (Fig. 4a).

In Figure 5 the diffractograms of the dry extract/Ac-Di-Sol<sup>®</sup> binary systems are presented and compared to the single components. Also in this case, the XRD patterns of the 1:1 and 1:3 w/w coground systems are almost superimposable (Fig. 5c and d), both showing a strong scattering

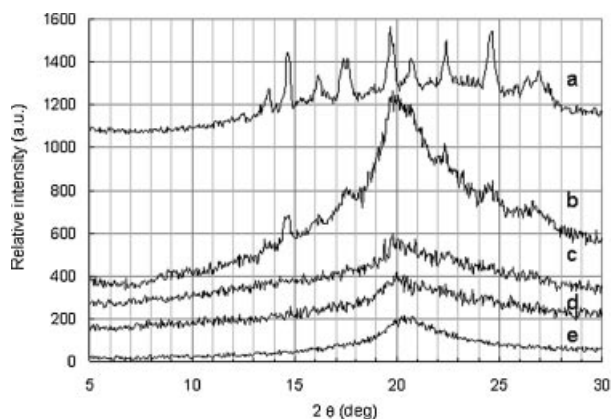


**Figure 3.** XRD patterns of the binary mixtures with PVP-CL<sup>®</sup>: 1:3 w/w PM (b), 1:1 w/w coground system (c) and 1:3 w/w coground system (d), compared to pure *Silybum marianum* dry extract (a) and pure PVP-CL<sup>®</sup> (e).



**Figure 4.** XRD patterns of the 1:3 w/w coground systems with Ac-Di-Sol (b), and PVP-CL<sup>®</sup> (a) after 2 years of ageing.



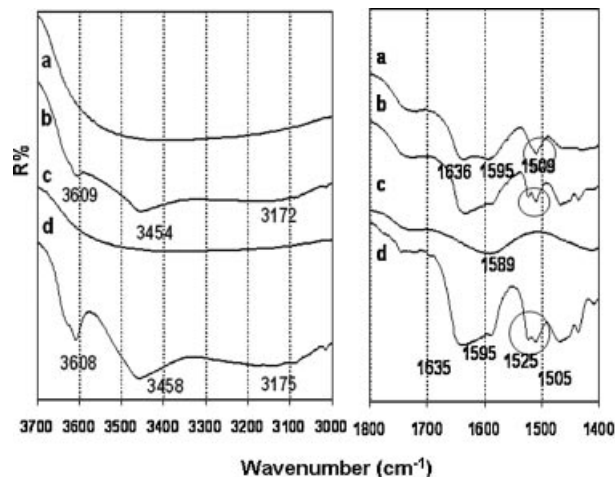


**Figure 5.** XRD patterns of the binary mixtures with Ac-Di-Sol<sup>®</sup>:1:3 w/w PM (b), 1:1 w/w coground system (c), and 1:3 w/w coground system (d), compared to pure *Silybum marianum* dry extract (a) and pure Ac-Di-Sol<sup>®</sup> (e).

phenomenon and a band in correspondence of the apex of the polymer. In the 1:3 w/w PM (Fig. 5b), in addition to the band of Ac-Di-Sol<sup>®</sup>, a reduced scattering phenomenon can be noticed together with some signals of low intensity, in correspondence to the most intense signals of the dry extract (14.5°, 17.5°, 22.5°, and 24.5° of 2θ). Also in this case the XRD analysis was repeated on aging on the 1:3 w/w coground systems, attesting the absence of crystalline lattice after 2 years from the preparation (Fig. 4b). This means that cogrinding the dry extract with both PVP-CL<sup>®</sup> and Ac-Di-Sol<sup>®</sup>, in 1:1 and 1:3 weight ratios, a completely amorphous and stable product is obtained.

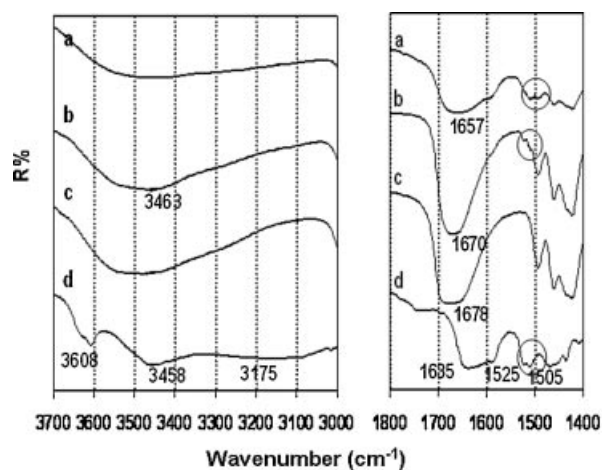
It is well known that vibrational changes can serve as probes of intermolecular interactions in solid materials. Amongst the different IR techniques DRIFT is the best choice because minimal sample manipulation is required,<sup>20</sup> and for this reason it has been employed to verify previous interactions hypotheses. The possible interaction would occur between the –OH, C=O, and –O– groups of flavonolignans,<sup>21,22</sup> and –OH, –O–C=O, C=O, –O– groups of Ac-Di-Sol<sup>®</sup><sup>23,24</sup> or C=O and –NH of PVP-CL<sup>®</sup>.<sup>25,26</sup> Any interaction would be reflected by shifts in the vibrations of these groups.

In Figures 6 and 7 the DRIFT spectra of the –OH stretching and carbonyl stretching region are reported: in particular each figure compares the features of 1:3 (w/w) coground system (a), corresponding PM (b), each carrier (c), the dry extract (d).



**Figure 6.** DRIFT spectra of the –OH stretching and carbonyl stretching region: 1:3 (w/w) coground system (a), corresponding PM (b), Ac-Di-Sol<sup>®</sup> (c), the dry extract (d).

In agreement with previous physical characterizations, this analysis reveals that the PM with Ac-Di-Sol (Fig. 6) can be simply regarded as the superimposition of those of dry extract and carrier in the whole spectrum, suggesting the absence of significant chemical interactions, whilst in the coground system some peaks are missing and others are remarkably decreased. In particular, the band at 3608 cm<sup>-1</sup> assigned to the free –OH, the band at 3458 cm<sup>-1</sup> and the shoulder at 3175 cm<sup>-1</sup>, attributable to the –OH intermolecular association<sup>21</sup> are still present in the PM. On



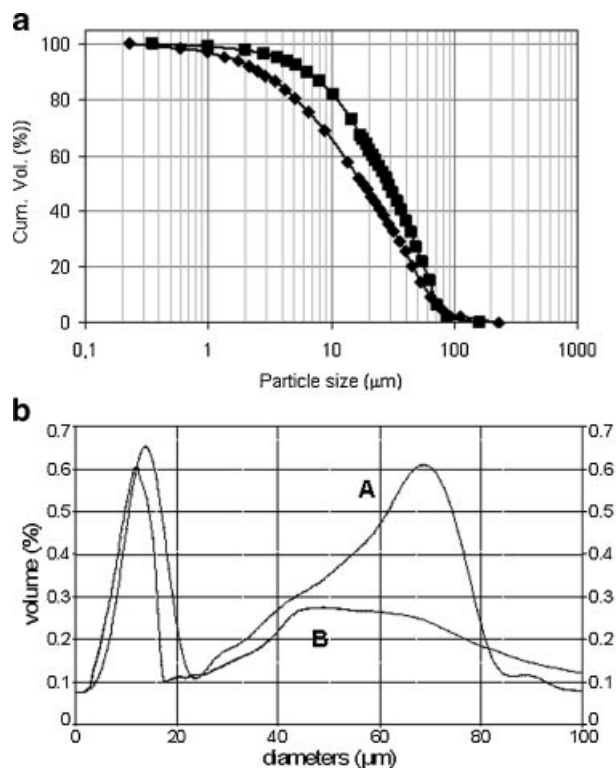
**Figure 7.** DRIFT spectra of the –OH stretching and carbonyl stretching region: 1:3 (w/w) coground system (a), corresponding PM (b), PVP-CL<sup>®</sup> (c), the dry extract (d).

the contrary, these features are missing in the coground system and replaced by a unique broad band. As for the carbonyl stretching region, the PM substantially corresponded to the superimposition of the peaks of the 2 components; whereas in the coground no difference was observed in the position of the bands, but the percentage absorption of the  $1635\text{ cm}^{-1}$  peak is remarkably reduced while that of  $1595\text{ cm}^{-1}$  is increased; further the peak at  $1525\text{ cm}^{-1}$  is absent. It seems that when the dry extract is coground with Ac-Di-Sol<sup>®</sup>, the hydrogen bonding is decreased but no indication of interaction between drug and carrier was observed.

As for the PVP-CL<sup>®</sup> systems (Fig. 7) the band at  $3608\text{ cm}^{-1}$  and at  $3458\text{ cm}^{-1}$  and the shoulder at  $3175\text{ cm}^{-1}$  are completely absent in the coground system, whilst in the PM only one peak is recognizable: modest and shifted at  $3463\text{ cm}^{-1}$ . Looking at the carbonyl stretching region, it can be noticed that the band most likely assigned to the vibration of the C=O of the polymer<sup>25</sup> remarkably shifted from  $1678\text{ cm}^{-1}$  toward lower frequencies (to  $1670\text{ cm}^{-1}$  in the PM and  $1657\text{ cm}^{-1}$  in the coground). All these facts suggest the existence of moderate interactions, probably consisting in hydrogen bonding, in the PM and severe in the coground system between the flavonolignans and PVP-CL<sup>®</sup>. This is not surprising since it is known that PVP-CL<sup>®</sup> which is effective in binding polyphenols.<sup>26,27</sup>

An additional important information that can be get from the spectra of the coground systems is the confirmation of the disruption of the crystal lattice after mechanochemical activation. In fact, the splitting phenomenon between  $1525$  and  $1505\text{ cm}^{-1}$ , typical feature of a crystal lattice in the correlation field, is present in the active, is still present in the PMs but is absent in both coground systems, where is replaced by a unique peak at about  $1509\text{ cm}^{-1}$  (see circles in Figs. 6 and 7).

The next step was to study the influence of the mechanochemical process on the particle size and the specific surface area of the produced particles. In Figure 6a the oversize cumulative distribution of the 1:3 w/w coground systems with Ac-Di-Sol<sup>®</sup> are depicted and compared to the simple PMs. The 1:3 w/w coground system shows in all the dimensional range a remarkable reduction of the particle size with respect to the untreated mixture of powders (Fig. 8a). When plotting the volume percentage as a function of the diameter (Fig. 8b), it appears that the distribution is bimodal for both coground and PM, with the



**Figure 8.** Cumulative size distribution of the particles the binary mixtures with Ac-Di-Sol<sup>®</sup>: 1:3 w/w PM (■), 1:3 w/w coground system (◆) (a); differential plot of the particle size in the 1:3 w/w PM (curve A) and coground system with Ac-Di-Sol<sup>®</sup> (curve B) (b).

first narrow curve in the 2–22  $\mu\text{m}$  size range and the second broad curve representing the larger particles with a diameter between 22 and 100  $\mu\text{m}$ . After mechanochemical activation a dramatic reduction of the frequency of the particles having dimensions between 22 and 100  $\mu\text{m}$  is attested (curve B). As showed in Table 2, this particle size reduction corresponds to a remarkable increase (210%) of the specific surface area with respect to the simple PM. In the case of the 1:3 w/w binary systems with PVP-CL<sup>®</sup> (Fig. 9), a different effect of the cogrinding process was noticed: the cumulative oversize distribution (Fig. 9a) showed an increase of the percentage of particles smaller than 10  $\mu\text{m}$  and a diminution of the percentage of particles with superior size. Looking at the differential plot of the particle size of the same samples (Fig. 9b) the following features can be noticed: in both diagrams a sharp peak followed by a large broad band is showed, attesting a bimodal distribution of particle size. Observing the first peak, representing the fines, it can be noticed that the size dimension is reduced after cogrinding

**Table 2.** Specific Surface Area Calculated by the Hg Porosimetry Analysis

Sample	Specific Surface Area (m <sup>2</sup> /g)
1:3 w/w dry extract/Ac-Di-Sol <sup>®</sup> PM	0.77
1:3 w/w dry extract/Ac-Di-Sol <sup>®</sup> coground system	1.62
1:3 w/w dry extract/PVP-CL <sup>®</sup> PM	0.83
1:3 w/w dry extract/PVP-CL <sup>®</sup> coground system	1.41

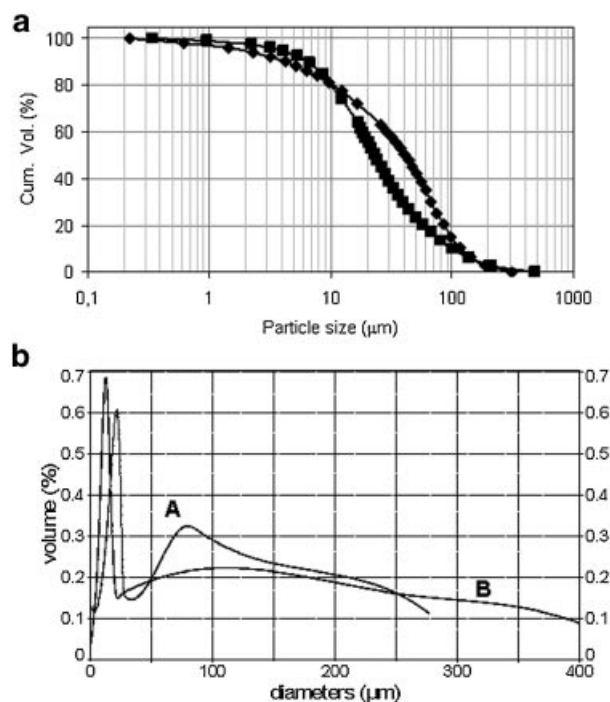
(curve B) and its percentage is higher than in the PM (curve A). As for the large particles, expressed by the second broad band, in the PM they had a maximum at about 50  $\mu\text{m}$  and were not superior to 270  $\mu\text{m}$  whilst in the coground system the band is broader since the particles were comprised between 50 and 400  $\mu\text{m}$  with a maximum ranging about 120  $\mu\text{m}$ . The overall result is a widening of the particles size distribution: some particles are reduced while some are increased, resulting in an increase of fines and a presence of large particles of different sizes, including a little percentage of large agglomerates superior to 270  $\mu\text{m}$ . While the reduction of particles diameter is easily predictable after cogrinding, the presence of large

agglomerate is not common but it is anyway not totally new, especially when using crospovidone. In fact a similar undesired particle growth was noticed in some previous mechanochemical activation experiences,<sup>28</sup> when large and hard agglomerates were obtained. These authors have found that the phenomenon can be related to formulation variables, such as the polymeric material, the drug-carrier combination but also to process variables, for example, the type of vibrational mill, the grinding time, the frequency used. Since in this case the process variables were kept constant for the preparation of the samples, the formulation variables seem to play a prevalent role in this phenomenon: in particular the presence of crospovidone and the nonnegligible quantity of water trapped in its matrix may be responsible for the formation of large agglomerates, with a mechanism probably similar to a wet granulation process.

It should be noticed that the finding of the large agglomerates cannot be an artifact of the analysis conditions, since it is well known that the mercury porosimeter, in contrast to other particle size analyzers, permits to overcome problems related to the undesired aggregation of the sample, through an appropriate study of the hysteresis in the Hg intrusion–extrusion curve of the considered sample.<sup>29</sup>

Despite the results of particle size distribution, a pronounced increase of the specific surface area of the coground systems with crospovidone was noticed with respect to the PM: the surface area of the coground product is about twice than that of the PM, as reported in Table 2, testifying that the cogrinding procedure lead to a system theoretically more prone to the dissolution.

The subsequent step consisted in the evaluation of the *in vitro* dissolution performances of the coground systems using the solubilization kinetics method in comparison to the PMs and to the starting dry extract. Within this aim, on the one hand the release of the entire dry extract was analyzed, and on the other hand the release of



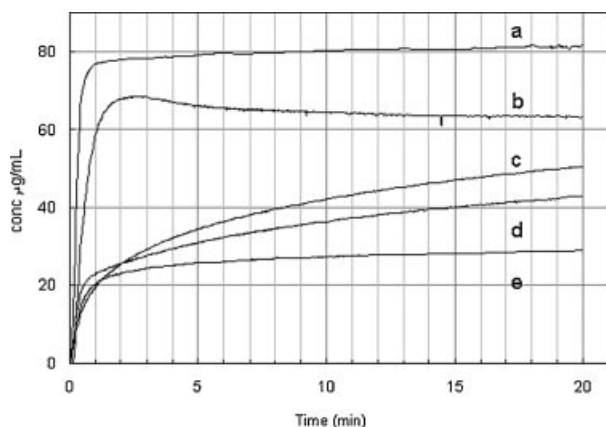
**Figure 9.** Cumulative size distribution of the particles the binary mixtures with PVP-CL<sup>®</sup>: 1:3 w/w PM (■), 1:3 w/w coground system (◆) (a); differential plot of the particle size in the 1:3 PM (curve A) and coground system with PVP-CL<sup>®</sup> (curve B) (b).

three main components of the dry extract (silybin A and B, and silychristin, all together representing about the 54% of flavolignans) was singularly tested determining the solubilized amount of each component.

As reported in Figure 10, the better performance of both coground systems compared to the dry extract is evident, both in terms of rate and extent of dissolution, testifying the mechanochemical process led to an activated status easier to be dissolved, thanks to the increased specific surface area and to the absence of crystalline lattice in the active components.

Amongst the samples, the best profile can be achieved with the 1:3 w/w coground with Ac-Di-Sol<sup>®</sup>, being able to maintain the highest solubilized amount of active (81.30  $\mu\text{g}/\text{mL}$ ) for the entire duration of the analysis. In the case of the PVP-CL<sup>®</sup> a supersaturation phenomenon was noticed after 3 min from the beginning of the test, that led to a little decrease of the components concentrations. After that the solubilized amount of actives is kept almost stable at about 63  $\mu\text{g}/\text{mL}$ , thanks to the presence of the remarkable chemical interactions between PVP and dry extract components.

As a confirmation that the mechanochemical process positively influences the solubilization kinetics of the flavonolignans, a very different behavior was observed with the PMs, where no solubilization improvement was attested. As expected, the PM containing Ac-Di-Sol<sup>®</sup>, simply consisting of a mixture of the components without any significant interaction, displayed a solubilization kinetics not very different from that of the



**Figure 10.** Solubilization kinetics of the entire dry extract in 1:3 w/w coground system with Ac-Di-Sol (a), 1:3 w/w coground system with PVP-CL<sup>®</sup> (b), pure *Silybum marianum* dry extract (c), 1:3 w/w PM with Ac-Di-Sol<sup>®</sup> (d), 1:3 w/w PM with PVP-CL<sup>®</sup> (e).

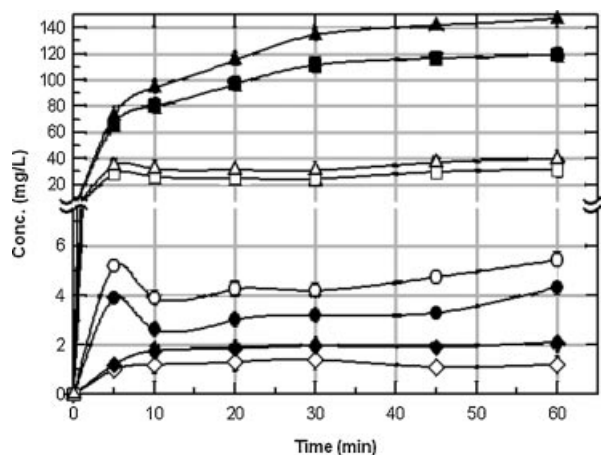
pure dry extract. Conversely, in the case of the PM with PVP-CL<sup>®</sup>, where modest chemical interactions have been found by DRIFT analysis, in accordance to literature data,<sup>26,30</sup> a release profile inferior to the native extract was obtained.

As for the release of the single components of the dry extract, the profiles are shown in Figure 11.

The dissolutions of both silybin A and silybin B resulted to be dramatically enhanced when coground in presence of PVP-CL<sup>®</sup> and Ac-Di-Sol<sup>®</sup>. The release of silybin A and B from the coground systems with crospovidone led respectively to eight- and sevenfold higher concentration level compared to the native extract. Once again a supersaturation phenomenon could be noticed after 5 min from the beginning of the test. After about 30 min of release, the concentration of the active components tended to increase again probably because at this time the complete disintegration of the above-mentioned large aggregates was reached and hence the dissolution process was boosted.

Analogously, the highest solubility value at the equilibrium was reached from the coground system prepared with Ac-Di-Sol<sup>®</sup> obtaining a concentration increase of about 31- and 27-fold for silybin A and B, respectively.

As for the third active component, silychristin, the presence of Ac-Di-Sol<sup>®</sup> doubled its release



**Figure 11.** Solubilization kinetics of the single components of the dry extract: Silybin B (▲) and Silybin A (■) in 1:3 w/w coground system with Ac-Di-Sol<sup>®</sup>, Silybin B (△) and Silybin A (□) in the 1:3 w/w coground system with PVP-CL<sup>®</sup>, Silybin B (●) and Silybin A (○) in pure *Silybum marianum* dry extract, Silychristin in 1:3 w/w coground system with Ac-Di-Sol<sup>®</sup> (◆) and in the pure *Silybum marianum* dry extract (◇).

with respect to the dry extract. Conversely, the concentration of silychristin released from PVP-CL<sup>®</sup> was too little and not sufficient to be detected by HPLC, probably due to the above mentioned chemical interactions. Since the solid state of silychristin is unchanged after cogrinding with Ac-Di-Sol<sup>®</sup>, its solubilization enhancement seems to be attributable to the favorable presence of the hydrophilic carrier and to the increased surface area after mechanochemical process.

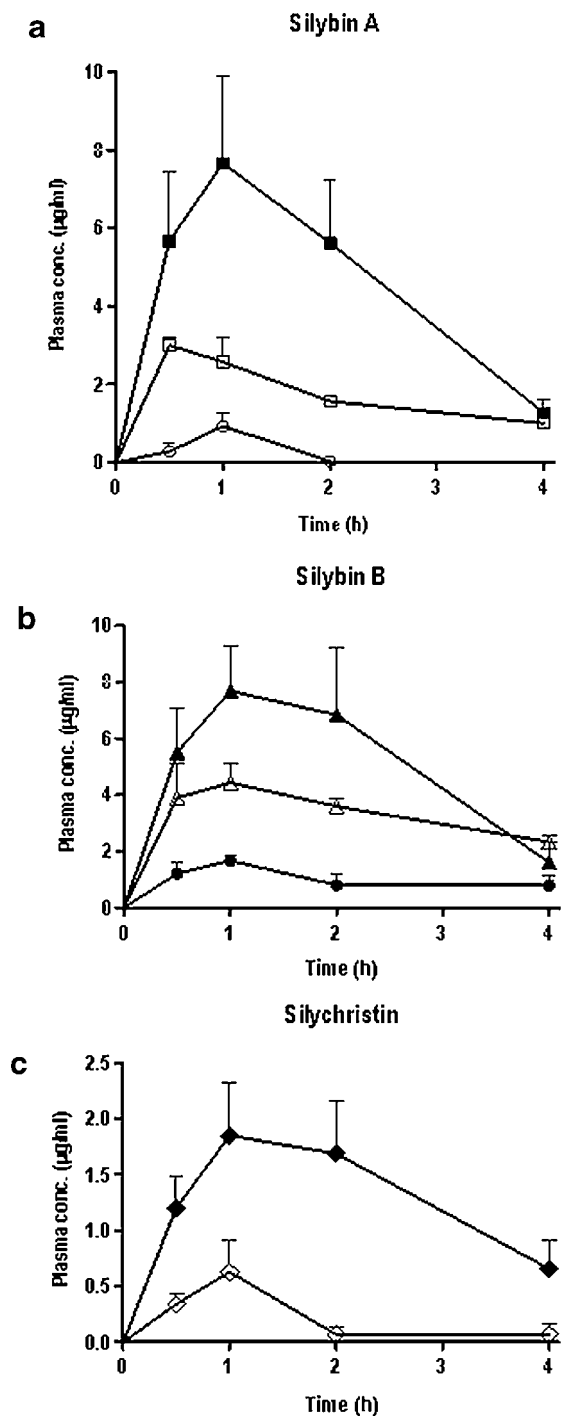
As a completion of this research, the *in vivo* bioavailability of the main *S. marianum* components after oral administration in rats of the two coground systems was assessed in comparison with the pure dry extract. In Figure 12 the plasma concentration profiles are presented whilst the pharmacokinetic parameters are listed in Table 3.

As regards to silybine A, a remarkable increase of its bioavailability after mechanochemical activation with both crosslinked polymer was obtained with respect to the pure dry-extract. In particular, Ac-Di-Sol<sup>®</sup> and PVP-CL<sup>®</sup> led to an oral relative bioavailability with respect to the extract of 88.6 and 34.5, respectively. Whereas for silybine B, its bioavailability was 17.96- and 12.1-fold higher than that associated with intake of the dry extract, when using Ac-Di-Sol<sup>®</sup> and PVP-CL<sup>®</sup>, respectively. The relative bioavailability of silychristine with Ac-Di-Sol<sup>®</sup> obtained an increase of 16.4-fold. As for the coground system with PVP-CL<sup>®</sup>, analogously to the behavior noticed during the *in vitro* analyses, the chemical interactions are responsible for the lack of an appropriate release of the active compound from the composite. From this experimental evidence, the improvement of the bioavailability of all tested flavolignans is higher when using as a carrier Ac-Di-Sol<sup>®</sup>.

## CONCLUSIONS

The main components of *S. marianum* dry extract were all contemporarily activated through the mechanochemical activation with polymeric crosslinked carriers, PVP-CL<sup>®</sup> and Ac-Di-Sol<sup>®</sup>. The activation was confirmed by the structural change of main crystalline components, the change in the size and surface area of the powders and by the release performances of each single component in water. This activated status remained stable over a period of 2 years.

As a confirmation that the mechanochemical process positively influences the solubilization



**Figure 12.** Mean plasma levels of the main flavolignans of *Silybum marianum*, obtained after single oral dose (50 mg/kg): (a) silybin A in 1:3 w/w coground system with Ac-Di-Sol<sup>®</sup> (■), with PVP-CL<sup>®</sup> (□), and in *Silybum marianum* dry extract (○); (b) silybin B in 1:3 w/w coground system with Ac-Di-Sol<sup>®</sup> (▲), with PVP-CL<sup>®</sup> (△), and in *Silybum marianum* dry extract (●); (c) silychristin in 1:3 w/w coground system with Ac-Di-Sol<sup>®</sup> (◆) and in the *Silybum marianum* dry extract (◇).

**Table 3.** Pharmacokinetic Parameters Relative to Main Components of *Silybum marianum* after Oral Administration in Rats of the Dry Extract and Coground Systems

	Silybine A			Silybine B			Silychristine	
	Extract	PVP-CL <sup>®</sup>	Ac-Di-Sol <sup>®</sup>	Extract	PVP-CL <sup>®</sup>	Ac-Di-Sol <sup>®</sup>	Extract	Ac-Di-Sol <sup>®</sup>
Dose (mg/kg)	8.5	2.6	2.6	8	2.25	2.25	10.5	4.45
$t_{max}$ (h)	1.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00
$C_{max}$ ( $\mu\text{g/mL}$ )	0.93	3.00	7.66	1.67	4.43	7.69	0.63	1.85
$AUC_{last}$ ( $\text{h} \times \mu\text{g/mL}$ )	0.63	6.70	17.20	3.79	12.90	19.15	0.72	5.02
Dose-normalized parameters								
$C_{max}$ ( $\mu\text{g/mL}$ )	0.11	1.15	2.95	0.21	1.97	3.42	0.06	0.42
$AUC_{last}$ ( $\text{h} \times \mu\text{g/mL}$ )	0.07	2.58	6.61	0.47	5.73	8.51	0.07	1.13
$F_{rel}$ (PVP-CL <sup>®</sup> or Ac-Di-Sol <sup>®</sup> vs. Extract)	1	34.5	88.6	1	12.1	17.96	1	16.4

kinetics of the flavonolignans, a very different behavior was observed with the PMs, where no solubilization improvement was attested. The solubilization kinetics resulted ameliorated both in terms of entire dry extract and in terms of single components, in comparison to the PMs and to the pure extract, achieving the best *in vitro* profile with the 1:3 w/w coground with Ac-Di-Sol<sup>®</sup>. These findings were confirmed by the *in vivo* absorption studies in rats that revealed a very remarkable improvement of the bioavailability of all the tested flavolignans when coground with Ac-Di-Sol<sup>®</sup>.

It can be concluded that the solid state mechanochemical process is a viable means to increase the *in vitro* solubilization of various active components and therefore to improve their bioavailability when given by oral route.

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