A Rapid and Simple Chromatographic Separation of Diastereomers of Silibinin and Their Oxidation to Produce 2,3-Dehydrosilybin Enantiomers in an Optically Pure Form

Giovanni Di Fabio, Valeria Romanucci, Cinzia Di Marino, Lorenzo De Napoli, Armando Zarrelli
Department of Chemical Sciences, University of Napoli 'Federico II', Napoli, Italy

Abstract

Silybin A and B were separated from commercial silibinin using the preparative HPLC method. The described method is rapid and effective in obtaining gram-scale amounts of two diastereoisomers with minimal effort. In our approach, silibinin was dissolved in THF (solubility greater than 100 mg/mL), an alternative solvent to H_2O or MeOH in which silibinin has a very low solubility (ca 0.05–1.5 mg/mL), and then separated into its two components using preparative RP-HPLC. By starting with purified diastereoisomers, it was possible to obtain the two enantiomers of 2,3-dehydrosilybin in good yields and optically pure using an efficient oxidation procedure. All of the purified products were fully characterised using NMR (^{1}H, ^{13}C), CD, $[\alpha]_D$, and ESI MS analyses. The purities of the products, which were evaluated using analytical HPLC, were greater than 98% in all cases.

Key words
prep-RP-HPLC · flavonolignans · *Silybum marianum* · Asteraceae · silibinin · silybin A · silybin B · 2,3-dehydrosilybin

Supporting information available online at
http://www.thieme-connect.de/ejournals/toc/plantamedica

Silibinin is the major flavonolignan found in silymarin, which is isolated from the fruits of *Silybum marianum* (L.) Gaertn. (*Carduus marianus* L., Asteraceae; milk thistle). It is an important and natural hepatoprotective drug (Flavobion™, Legalon™) that is widely used in the treatment of cirrhosis, chronic hepatitis, and liver diseases associated with excessive alcohol consumption and exposure to environmental toxins [1]. Previously, it was determined that silibinin exists in two diastereoisomeric forms, namely silybin A and B [2] (**1A** and **1B** in **Fig. 1**), in a ratio of approximately 1 : 1 with differing stereochemistries at positions C-7″ and C-8″ in the lignan moiety [3,4]. Silibinin is a natural compound with multiple biological activities operating at various cell levels. In the last decade, silibinin has received attention due to its alternative beneficial activities, which mostly include anticancer and chemopreventive actions, as well as cardioprotective, neuroprotective, and antiviral activities [1,5–11]. Milk thistle extract also contains 2,3-dehydrosilybin (**2A** and **2B** in **Fig. 1**), which has antioxidant activity superior to that of silibinin and a positive effect on some skin diseases (e.g., psoriasis, atopic eczema) [12–15]. Because of the low content of 2,3-dehydrosilybin in its natural state, this compound was practically neglected in studies on the biological activity of silibinin and silymarin; therefore, simple procedures were developed to obtain 2,3-dehydrosilybin [16,17] and its analogues [18,19].

A major problem hampering studies of optically pure silibinin is the extremely complicated separation of its diastereoisomers. This problem has been approached in past years by several research groups through the use of HPLC methods. Recently, a preparative HPLC method for the separation of silybin A, silybin B, and other silymarin congeners in larger quantities was described [20,21]. A multifaceted approach has been proposed by Křen et al. in which the chromatographic separation is performed on the mixture of the two diastereoisomers suitably derivatised by chemical or enzymatic methods [22–24]. Generally, these methods require special expertise in both organic synthesis and enzymatic kinetic resolutions. By careful analysis of the studies currently reported, it is the opinion of the authors that a rapid and less labourious method for the separation of silibinin is still required.

Here, we report a rapid and preparative separation (gram-scale amounts) of the diastereoisomers of silibinin using preparative HPLC (**Fig. 1**) and their oxidation to produce enantiomers of 2,3-dehydrosilybin in a very pure form using our optimised procedure [25]. Previous investigations have shown that the solubil-

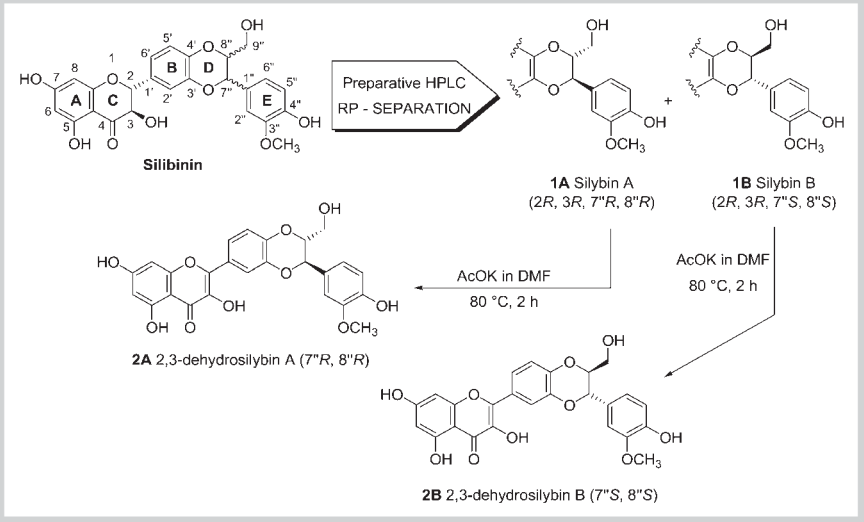

Fig. 1 Separation of two diastereoisomers of silibinin (**1A** and **1B**) and their oxidation to 2,3-dehydrosilybin A (**2A**) and 2,3-dehydrosilybin B (**2B**).

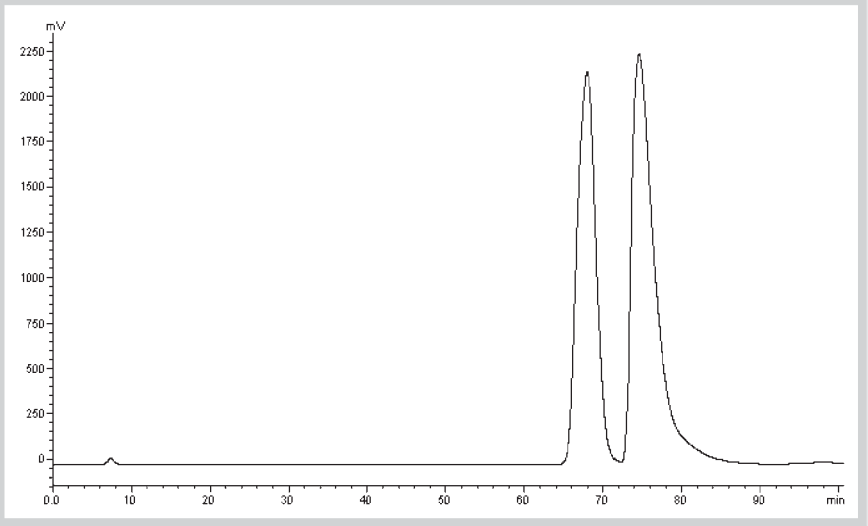

Fig. 2 Prep-HPLC profile of the silibinin. Silybin A (**1A**, t_R = 67.5 min) and silybin B (**1B**, t_R = 74.5 min). Conditions: Phenomenex Gemini C18-110A preparative column (10-µm particle size, 250 mm × 21.2 mm i. d.), eluted with H_2O/MeOH/MeCN (60 : 35 : 5, v/v/v) containing 0.1 % of TFA; flow rate 12 mL/min, monitored at 288 nm.

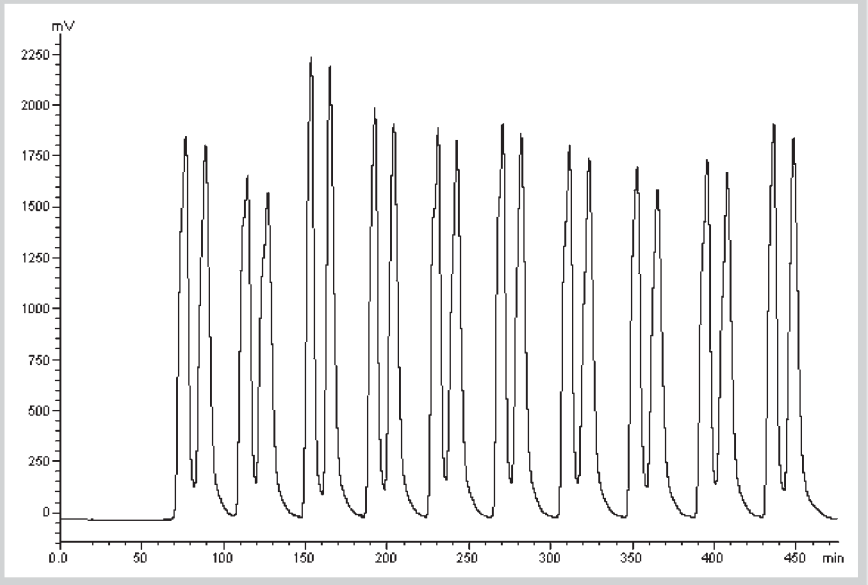

Fig. 3 HPLC profile of sequential injections.

ity of silibinin in THF at 25 °C (greater than 140–150 mg/mL) is considerably greater than in H_2O or MeOH (0.05–1.5 mg/mL) [20], allowing for the injection of large quantities of analyte. The key point of our approach is dissolving the sample in THF, an alternative solvent to H_2O or MeOH in which silibinin has a very low solubility. Various types of HPLC columns and conditions (columns, flow, and mobile phases) were evaluated, and the optimum chromatographic conditions for the separation of the two diastereoisomers were determined by the use of a Phenomenex Gemini C18-110A preparative column (10-µm particle size, 250 mm × 21.2 mm i.d.) with a mobile phase consisting of H_2O/MeOH/MeCN (60 : 35 : 5, v/v/v) and 0.1 % TFA. In preliminary studies, we also used DMSO, which dissolves silibinin very well but resulted in poor resolution under all of the conditions tested. To attain the highest quantity of pure silybin A and silybin B and to avoid poor resolution due to the expansion of their chromatographic peaks, 500-µL injections containing 70 mg of silibinin were used. Better resolution was observed in all the tests performed when the sample was dissolved in THF and mixed with an equal volume of the eluent solution. As shown in **Fig. 2**, a good resolution was obtained when a 500-µL aliquot of 140 mg/mL of the silibinin solution was mixed with 500 µL of the eluent solution and then injected. The resolution of silybin A and silybin B was high enough to obtain pooled fractions containing practically pure single isomers. Using groups of 10 injections at a time, fractions of silybin A (t_R = 67.5 min) and silybin B (t_R = 74.5 min) were collected, and after removal of the mobile phase under reduced pressure, amorphous colourless powders of silybin A (300 mg) and silybin B (315 mg) were obtained by drying under vacuum. To make the procedure quicker and to maximise the functionality of our instruments, we carried out the injections in sequence every 40 min (**Fig. 3**). Thus, we were able to obtain approximately 1 g for each of the two diastereoisomers in a very pure form in less than a week. The purified products, whose HPLC purity was higher than 98 % (**Fig. 4**), were fully characterised by NMR (^{1}H, ^{13}C), CD (**Fig. 1S** in Supporting Information), ESI MS, and $[\alpha]_D$. The confirmation of two specific structures was also obtained by comparing the NMR and CD data with those published in the literature [3, 4].

Afterwards, diastereoisomers **1A** and **1B** were treated with potassium acetate in DMF at 80 °C to produce **2A** and **2B** (**Fig. 1**). In our procedure, a 0.1-M solution of pure silybin A (**1A**) or silybin B (**1B**) was treated with anhydrous AcOK in DMF at 80 °C for 2 h. The crude mixture was purified by chromatography on a silica gel

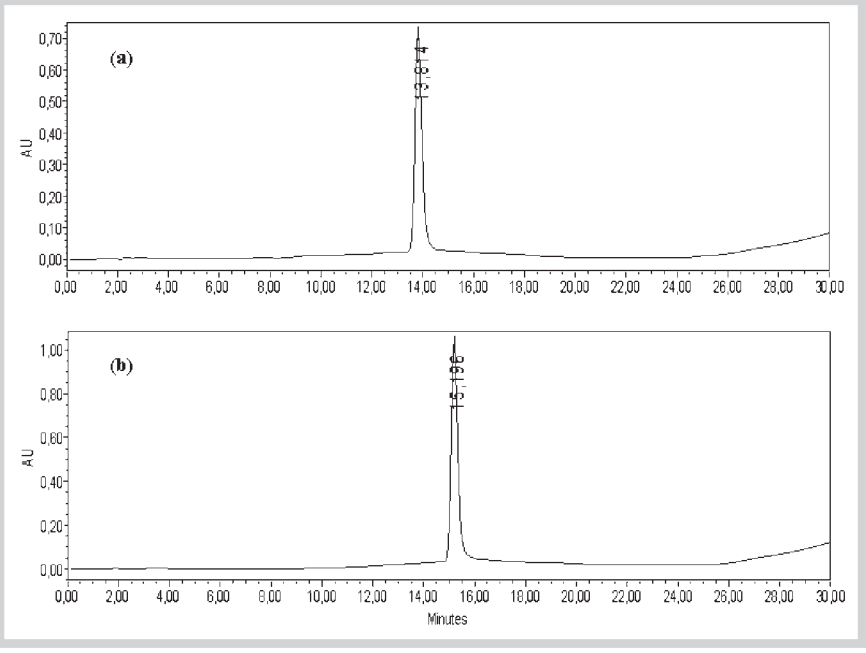

Fig. 4 Analysis of silybin A (**a**) and silybin B (**b**) by analytical HPLC: RP18 column Phenomenex LUNA (5-µm particle size, 10.0 mm × 250 mm i. d.), eluted with H_2O/MeOH containing 0.01% acetic acid (50 : 50, v/v), flow rate 0.8 mL/min, and monitored at 288 nm.

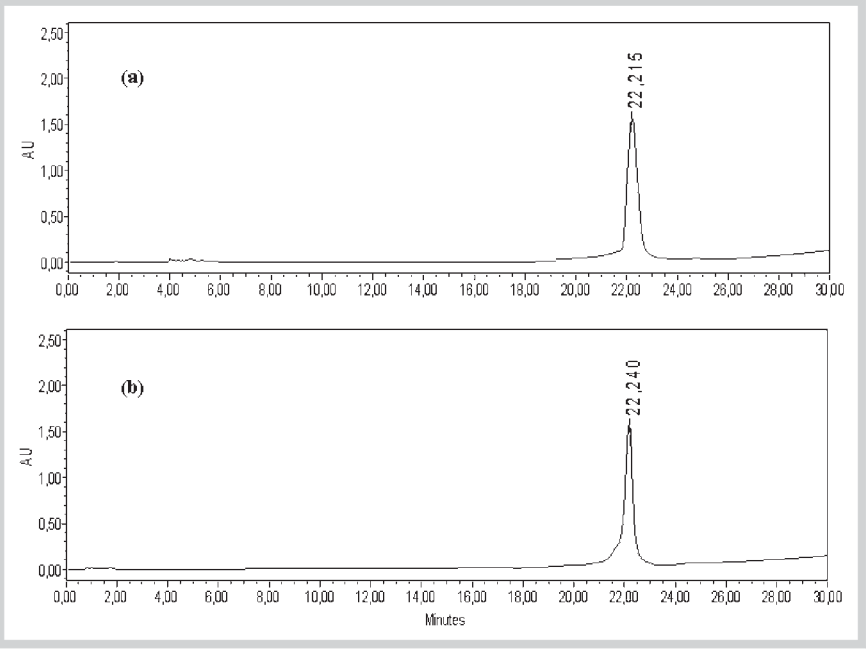

Fig. 5 Analysis of 2,3-dihydrosilybin A (**a**) and 2,3-dihydrosilybin B (**b**) by analytical HPLC: RP18 column Phenomenex LUNA (5-µm particle size, 10.0 mm × 250 mm i. d.), eluted with H_2O/MeOH containing 0.01% acetic acid (50 : 50, v/v), flow rate 0.8 mL/min, and monitored at 288 nm.

column (eluent: $CHCl_3$/acetone, 80 : 20, v/v) to yield **2A** and **2B** in good yields, and the HPLC purity was greater than 98% (**Fig. 5**). The purified products were fully characterised by NMR (^1H, ^{13}C), CD, ESI MS, and $[\alpha]_D$. The obtained data were compared with those published in the literature [12].

In conclusion, we have reported a simple and robust purification method to obtain two diastereoisomers of silibinin in a very pure form. Moreover, by starting with two separated diastereoisomers, we have produced two enantiomers of 2,3-dehydrosilybin in an optically pure form using our optimised oxidation procedure. The described preparative HPLC method is effective in obtaining gram-scale amounts of the standards silybin A and silybin B in a short period of time. All purified products were fully characterised by NMR (^1H, ^{13}C), CD, $[\alpha]_D$, and ESI MS analyses. From the HPLC analysis, the purity of the analogues obtained was determined to be greater than 98% on average. Notably, this method provides the opportunity to obtain large amounts of pure diastereoisomers of silybin and enantiomers of 2,3-dehydrosilybin with minimal effort, which opens up the possibility of conducting further experiments to clarify the many biological properties of these metabolites.

Material and Methods

The preparative HPLC method was performed with a Shimadzu LC-8A PLC system equipped with a Shimadzu SCL-10A VP system control and Shimadzu SPD-10A VP UV-VIS detector. A Phenomenex Gemini C18-110A preparative column (10-µm particle size, 250 mm × 21.2 mm i. d.) was used, and the mobile phase of H_2O/MeOH/MeCN (60 : 35 : 5, v/v/v), containing 0.1% of TFA, was delivered isocratically at 12 mL/min. The chromatograms were monitored at 288 nm. The HPLC system was controlled by LC Real-

Time Analysis software (Shimadzu Corporation). The silibinin solution was prepared by dissolving and sonicating the accurately weighed compound in THF. The obtained solution (ca. 140 mg/mL) was then applied to Nylon filtres (pore size = 0.45 µm). A 500-µL volume of the silibinin solution was mixed with 500 µL of the mobile phase and then applied to the chromatographic system. Silybin A and silybin B peaks were collected manually.

The analysis was performed with a Waters 1525 HPLC equipped with a binary gradient pump and a Waters 2996 Photodiode Array Detector using an RP18 column Phenomenex LUNA (5-µm particle size, 10.0 mm × 250 mm i.d.). The mobile phase of H_2O/MeOH, containing 0.01% acetic acid (50 : 50, v/v), was delivered isocratically at 0.8 mL/min. The chromatograms were monitored at 288 nm. Standard solutions of silybin A, silybin B, 2,3-dehydrosilybin A, and 2,3-dehydrosilybin B were prepared by dissolving the accurately weighed standard compounds in THF to yield final concentrations of 5 mg/mL. A 25-µL volume of each analyte was applied to the chromatographic system for the determination of purity.

Supporting information

Details on the general experimental procedures, the physicochemical data of compounds **1A, 1B, 2A**, and **2B**, the NMR data of **1A, 1B,** and **2** as well as the CD spectra of compounds **1A, 1B, 2A**, and **2B** are available as Supporting Information.

Acknowledgements

Financial support by AIPRAS *Onlus* (Associazione Italiana per la Promozione delle Ricerche sull'Ambiente e la Saluta umana) is acknowledged. We also thank Tecno Bios® for the HPLC facilities.

Conflict of Interest

There are no conflicts of interest of all authors with respect to this work.

References

1 *Gažák R, Walterová D, Křen V.* Silybin and silymarin: new and emerging applications in medicine. Curr Med Chem 2007; 14: 315–338
2 *Kroll JD, Shaw HS, Oberlies NH.* Milk thistle nomenclature: why it matters in cancer research and pharmacokinetic studies. Integr Cancer Ther 2007; 6: 110–119
3 *Napolitano JG, Lankin DC, Graf TN, Friesen JB, Chen SN, McAlpine JB, Oberlies NH, Pauli GF.* HiFSA fingerprinting applied to isomers with near-identical NMR spectra: the silybin/isosilybin case. J Org Chem 2013; 78: 2827–2839
4 *Kim NC, Graf TN, Sparacino CM, Wani MC, Wall ME.* Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). Org Biomol Chem 2003; 1: 1684–1689
5 *Davis-Searles PR, Nakanishi Y, Kim NC, Graf TN, Oberlies NH, Wani MC, Wall ME, Agarwal R, Kroll DJ.* Milk thistle and prostate cancer: differential effects of pure flavonolignans from *Silybum marianum* on antiproliferative end points in human prostate carcinoma cells. Cancer Res 2005; 65: 4448–4457
6 *Thelen P, Jarry H, Ringert RH, Wuttke W.* Silibinin down-regulates prostate epithelium-derived Ets transcription factor in LNCaP prostate cancer cells. Planta Med 2004; 05: 397–400
7 *Faezizadeh Z, Mesbah-Namin SA, Allameh A.* The effect of silymarin on telomerase activity in the human leukemia cell line K562. Planta Med 2012; 9: 899–902
8 *Varghese L, Agarwal C, Tyagi A, Singh RP, Agarwal R.* Silibinin efficacy against human hepatocellular carcinoma. Clin Cancer Res 2005; 11: 8441–8448
9 *Singh RP, Gu M, Agarwal R.* Silibinin inhibits colorectal cancer growth by inhibiting tumor cell proliferation and angiogenesis. Cancer Res 2008; 68: 2043–2050
10 *García-Maceira P, Mateo J.* Silibinin inhibits hypoxia-inducible factor-1alpha and mTOR/p70S6K/4E-BP1 signalling pathway in human cervical and hepatoma cancer cells: implications for anticancer therapy. Oncogene 2009; 28: 313–324
11 *Payer BA, Reiberger T, Rutter K, Beinhardt S, Staettermayer AF, Peck-Radosavljevic M, Ferenci P.* Successful HCV eradication and inhibition of HIV replication by intravenous silibinin in an HIV-HCV coinfected patient. J Clin Virol 2010; 49: 131–133
12 *Gažák R, Svobodová A, Psotová J, Sedmera P, Přikrylová V, Walterová D, Křen V.* Oxidised derivatives of silybin and their antiradical and antioxidant activity. Bioorg Med Chem 2004; 12: 5677–5687
13 *Dvořák Z, Vrzal R, Ulrichová J.* Silybin and dehydrosilybin inhibit cytochrome P450 1A1 catalytic activity: a study in human keratinocytes and human hepatoma cells. Cell Biol Toxicol 2006; 22: 81–90
14 *Huber A, Thongphasuk P, Erben G, Lehmann WD, Tuma S, Stremmel W, Chamulitrat W.* Significantly greater antioxidant anticancer activities of 2,3-dehydrosilybin than silybin. Biochim Biophys Acta 2008; 1780: 837–847
15 *Zhan T, Digel M, Küch EM, Stremmel W, Füllekrug J.* Silybin and dehydrosilybin decrease glucose uptake by inhibiting GLUT proteins. J Cell Biochem 2011; 112: 849–859
16 *Halbach G, Trost W.* Chemistry and pharmacology of silymarin. Studies on various metabolites of silybin. Arzneimittelforschung 1974; 24: 866–867
17 *Pelter A, Hänsel R.* Struktur des Silybins: I. Abbauversuche. Chem Berl 1975; 108: 790–802
18 *Maitrejean M, Comte G, Barron D, El Kirat K, Conseil G, Di Pietro A.* The flavanolignan silybin and its hemisynthetic derivatives, a novel series of potential modulators of P-glycoprotein. Bioorg Med Chem Lett 2000; 10: 157–160
19 *Džubák P, Hajdúch M, Gažák R, Svobodová A, Psotová J, Walterová D, Sedmera P, Křen V.* New derivatives of silybin and 2,3-dehydrosilybin and their cytotoxic and P-glycoprotein modulatory activity. Bioorg Med Chem 2006; 14: 3793–3810
20 *Li W, Han JP, Li ZW, Li XX, Zhou SP, Liu CX.* Preparative chromatographic purification of diastereomers of silybin and their quantification in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B 2008; 862: 51–57
21 *Graf TN, Wani MC, Agarwal R, Kroll DJ, Oberlies NH.* Gram-scale purification of flavonolignan diastereoisomers from *Silybum marianum* (milk thistle) extract in support of preclinical *in vivo* studies for prostate cancer chemoprevention. Planta Med 2007; 73: 1495–1501
22 *Křen V, Sedmera P, Kubisch J, Halada P, Přikrylová V, Jegorov A, Cvak L, Gebhardt R, Ulrichová J, Šimánek V.* Glycosylation of silybin. J Chem Soc [Perkin I] 1997: 2467–2474
23 *Monti D, Gažák R, Marhol P, Biedermann D, Purchartová K, Fedrigo M, Riva S, Křen V.* Enzymatic kinetic resolution of silybin diastereoisomers. J Nat Prod 2010; 73: 613–619
24 *Gažák R, Marhol P, Purchartová K, Monti D, Biedermann D, Riva S, Cvak L, Křen V.* Large-scale separation of silybin diastereoisomers using lipases. Process Biochem 2010; 45: 1657–1663
25 *Zarrelli A, Sgambato A, Petito V, De Napoli L, Previtera L, Di Fabio G.* New C-23 modified of silybin and 2,3-dehydrosilybin: synthesis and preliminary evaluation of antioxidant properties. Bioorg Med Chem Lett 2011; 21: 4389–4392

received February 11, 2013
revised May 23, 2013
accepted May 24, 2013

Bibliography
DOI http://dx.doi.org/10.1055/s-0032-1328703
Published online June 27, 2013
Planta Med 2013; 79: 1077–1080

ISSN 0032-0943

Correspondence
Prof. Armando Zarrelli
Department of Chemical Sciences
University of Napoli 'Federico II'
Via Cintia 4
80126 Napoli
Italy
Phone: + 39 081 67 44 72
Fax: + 39 081 67 43 93
zarrelli@unina.it