SYSTEMATIC IDENTIFICATION OF ACTIVE INGREDIENTS OF SILYBUM MARIANUM SEED

Lajos Nagy, Ákos Kuki, György Deák, Bernadett Biri, Miklós Nagy, Miklós Zsuga, Sándor Kéki*
Department of Applied Chemistry, University of Debrecen

ABSTRACT
Silybum Marianum seed and its extract Silymarin are widely used to treat and prevent many liver diseases such as cirrhosis. Due to other biological effects (antitumor, antioxidant, cytotoxic activities) numerous Silymarin content drugs (Silegon, Legalon etc.) are commercially available worldwide. Thus the development of applicable extraction and separation methods has become sorely needed as well as the improvement of analytical methods (HPLC, UPLC, HPLC-, UPLC-MS and MS/MS) for these flavonolignans in order to realize a unified quality control. This paper reviews some extraction and separation methods as well as the fragmentation patterns of different active components of Silymarin under MS/MS conditions.

INTRODUCTION
Nowadays, the hepatoprotective and/or antitumor preparations have main role in the pharmaceutical research. More than a half thousand additives can be found in our food, cosmetics and other everyday products. The effects of a fraction of these materials are not clear for the human health. The family of flavanolignans is one of the well-known antitumor agents [1-4] and hepatoprotective [5, 6]; these materials can be isolated from Silymarin extracted from milk thistle also known as Silybum Marianum in its scientific name. The main active components of this extract which is a mixture of different flavonolignans are shown in Fig. 1. Beside the antitumor and hepatoprotective effects of the flavonolignan constituents of Silymarin their cytotoxic [7, 8] and antioxidant activities [9, 10] are also known. Owing to the above mentioned pharmacologically important effects these components are applied in preventing and treating of liver disorders such as liver cirrhosis, hepatitis and nonalcoholic fatty liver [11-13]. Furthermore it turned out that the flavonolignans also had a preventing effect against cardiovascular diseases [14] and cancers [15, 16]. It was shown that the antioxidants in Silymarin could reduce low-density lipoprotein (LDL) contributing to prevent cardiovascular diseases [17]. In some *in vivo* animal models it was found that these flavonolignans could decrease the risk of skin cancer [18] due to its biological effects as mentioned above.

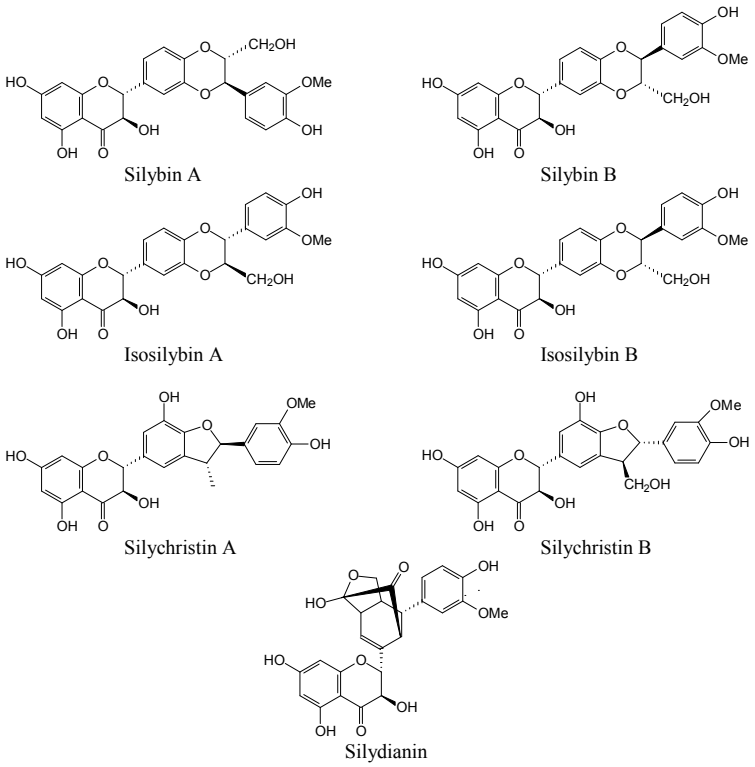

Figure 1. *Structure of the main Silymarin components*

Extraction

Owing to its numerous biological effects several methods were developed for extracting Silymarin from Silybum Marianum seeds. The main active compounds were extracted from the Silymarin by Wagner et al. in 1974 [19]. In this method methanol was used as extraction solvent and the presence of active flavonolignans was detected by thin layer chromatography (TLC). Later many articles were reported in the extraction topic such as Chen et al. where different solvent were compared to extract Silymarin from Silybum Marianum, or Wallace et al. where a single-stage extraction of whole and defatted seeds was studied by different solvents [20, 21]. Some patents were also reported in this topic. One of them was achieved by Kahol et al. which was tested by us resulted a powdered Silymarin and seed oil. In this method the seeds are precooled and then powdered in a hammer mill and are defatted by hexane extraction thereafter. Finally the Silymarin fraction is extracted by acetonitrile from the defatted seeds [22].

Separation

The separation of active components of Silymarin was performed by high performance liquid chromatography (HPLC) [23, 24], ultra-performance liquid chromatography (UPLC) [25] and capillary zone electrophoresis (CZE) [26]. The development of these methods was necessary to establish a standard quality control as these Silymarin extract containing commercial products such as Silegon, Legalon are available in the market. The different HPLC methods work with reverse phase C18 column and mainly water-methanol based mixture mobile phase. Some of these methods are summarized in Table 1. This way the main Silymarin components can be separated completely.

Table 1
Summary of different HPLC methods

Method	Mobile phase	Column	Detection wavelength (reference)
1	85 % phosphoric acid-methanol-water 0.5:46:64 v/v (1 mL/min	Purospher RP18 (150 mm x 4 mm, 5 µm)	288 nm [26]
2	Eluent A: 5 mM ammonium acetate pH=4, eluent B: methanol/water/ formic acid (95:5:0.1, v/v/v), 0.2 mL/ min (gradient method)	YMC ODS-AQ C18 (100 mm x 2.1 mm, 5 µm)	There was no UV detection. [27]
3	methanol-water 45:55	Prodigy C18-A (250 mm x 4.6 mm, 5 µm)	288 nm [28]
4	Eluent A: methanol, eluent B: water-dioxane 9:1 (gradient method)	Shim-pack VP-ODS (150×4.6mm i.d. 5µm) Pre-column (10×4.6 mm i.d. 5µm)	288 nm [29]
5	Methanol-acetonitrile-ammonium dihydrogen phosphate (24:24:50, pH=5)	Hypersil C18 (250 mm x 4.6 mm, 5 µm)	287 nm [30]

In 2003 Lee et al. were the first who could isolate Silybin A, Silybin B, isosilybin A and isosilybin B isomers. In this work X-ray crystallographic analysis, (1)H and (13)C NMR as well as two-dimensional NMR (COSY, HMQC, HMBC) measurements were applied for the unambiguous structure determination of these diastereomers [31].

Fragmentation of Silymarin components

Due to its important biological effects it was strong need to study the fragmentation of Silymarin compounds in order to get a better insight in the structure of these active flavonolignans. The fragmentation behavior of silybin was studied under electron ionization (EI) [32, 33], electrospray [34] and atmospheric pressure chemical ionization [35] conditions. The isolation of each active Silymarin component is difficult and expensive process thus HPLC-MS and UPLC-MS techniques were preferable for studying the fragmentation of different active constituents. Lee et al. developed an HPLC-ESI-MS/MS method which was suitable to separate and characterize silybin, isosilybin, silydianin and silychristin. In this report these were fragmented by HPLC-MS/MS using different collision energies [27]. The fragmentation behavior and retention time of the given compound were compared to its reference standard. Fragmentation pathways were proposed and determined the main product ions in case of six main constituents in negative ion mode. It was found that four main fragments appeared in the MS/MS spectra from the Silymarin

without separation (m/z 453, 301, 152 and 125). Using HPLC separation the MS/MS measurements of each pure compound could be performed. Based on the MS/MS measurements it turned out that m/z 301 appeared in the MS/MS spectra of silydianin, silybins and isosilybins but neglected in the case of silychristin. The other three mentioned peaks could be detected in all cases. The m/z 453 was formed by losing a CO from the precursor ion (m/z 481 in negative ion mode) and the formation of m/z 152 and 125 ions could be explained by the cleavage of the chromanone ring. An intensive m/z 301 ion appeared in the spectra of silybin A and B the relative abundance of this ion was observed much higher than in case of isosilybins. This peak came from the cleavage of 1,4-benzodioxane ring. Other important finding is that m/z 419 only in the silychristin spectra, whereas the m/z 169 only in the silydianin spectra was noticed. The spectra of silybins and isosilybins were similar but m/z 451 and m/z 355 ions were typical of silybins spectra and could not be detected in case of isosilybins. The structure of some mentioned product ions proposed by Lee et al. are shown in Fig. 2.

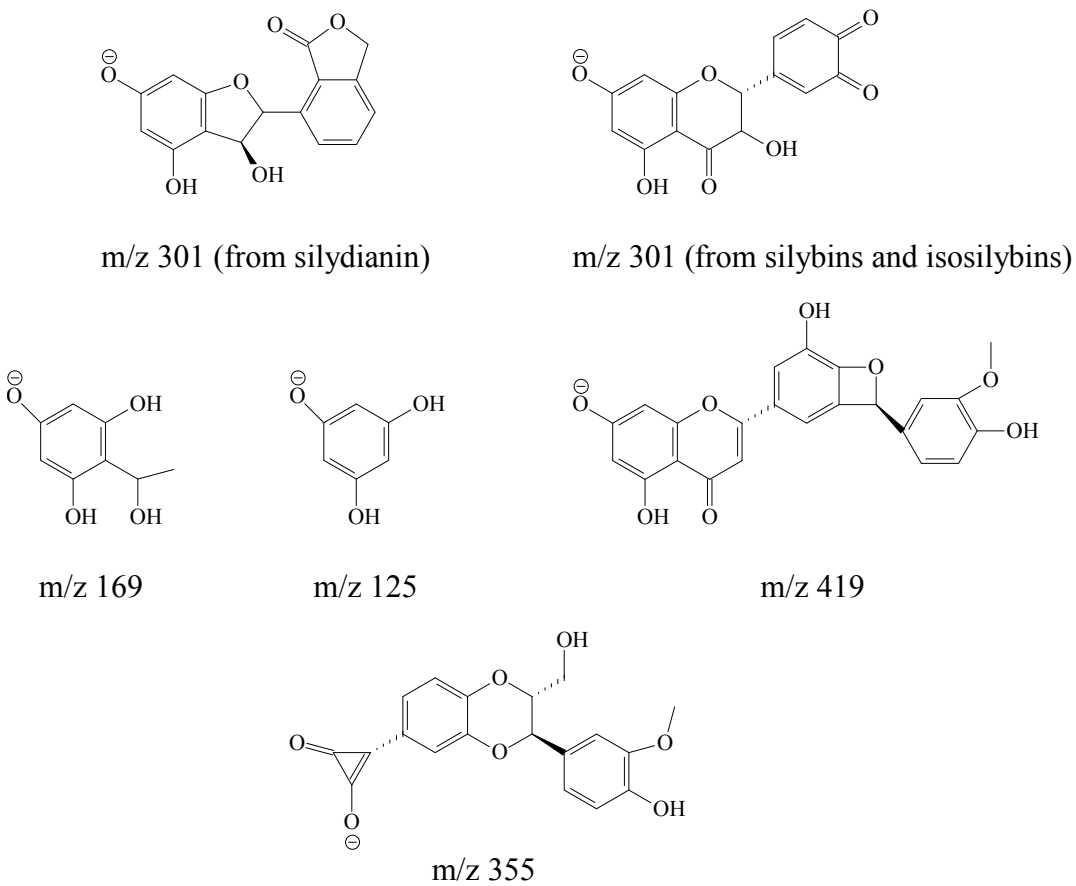

Figure 2.
Structure of different Silymarin product ions formed under MS/MS conditions

Based on these findings the main Silymarin components can be unambiguously identified by MS/MS measurements.

Knowing its fragmentation pattern and using UPLC which is a more effective separation method than HPLC a rapid separation and characterization can be achieved. In Wang et al. work seven active flavonolignans were separated and studied by UPLC-ESI-MS^n method [25]. The separated compounds were fragmented under collision-induced dissociation-MS/MS (CID-MS/MS) conditions and fragmentation pathways studied by CID-MS^3 and CID-MS^4 experiments. In CID-MS^n measurements some product ions (m/z 463, 453, 355, 301) were selected which were important to discriminate between Silymarin compounds. The m/z 463 was formed from precursor ion by losing a water molecule and the structure of other three product ions can be seen in Fig. 3. Based on these experiments the fragmentation pathways were proposed for silychristin A and B.

The analysis time was less than 8 minutes so the UPLC-ESI-MS^n was an effective, fast method to separate and identify active Silymarin components.

Prospects in Silymarin research

The active Silymarin flavonolignans have numerous useful biological effects so they are widely used to protect the liver and for the treatment of cancers. The

low water solubility of these compounds results in its low bioavailability so it is essential to develop a Silymarin product with good or much better water solubility. In some articles it was reported that the isolated active flavonolignans had differential effects in cancer preventing and treatment [36]. So by accomplishing a fast and economical method to separate and purify the most active isomer for the given disease a more effective drug can be made. Furthermore when its antioxidant properties were studied it turned out that it was decreased by increasing purity of Silymarin [26]. Thus it is possible that Silymarin contains other unknown important compounds with high antioxidant properties.

Acknowledgements

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