Peer-reviewed version available at *Antioxidants* **2019**, *8*, 304; doi:10.3390/antiox8080304

Article

A Green Ultrasound-Assisted Extraction Optimization of the Natural Antioxidant and Anti-aging Flavonolignans from Milk Thistle *Silybum marianum* (L.) Gaertn. Fruits for Cosmetic Applications

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Abstract: *Silybum marianum* (L.) Gaertn. (aka milk thistle) constitute the almost exclusive source of silymarin, a mixture of different flavonolignans, and is thus considered as a unique model for their extraction. The present research deals with ultrasound-assisted extraction (UAE) of *S. marianum* flavonolignans and their quantification using LC system. The optimal conditions for UAE were: aqueous EtOH 54.5% (v/v) as solvent, applying an ultrasound frequency of 36.6 kHz during an extraction time of 60 min at 45°C with a liquid to solid ratio of 25:1 ml/g DW. Following optimization, the extraction method was validated according to international standards of the association of analytical communities (AOAC) in order to ensure its precision and accuracy for the quantitation of the individual silymarin components. The efficiency of UAE was compared with maceration protocol of the same duration. The optimized and validated conditions allowed highest extraction yields of flavonolignans in comparison to maceration. The antioxidant capacity of the extracts was confirmed by the CUPRAC assays and inhibition of advanced glycation end products. The skin anti-aging action was also confirmed toward the strong *in vitro* inhibition capacity of the obtained extract against collagenase and elastase enzymes. The procedure presented here allows a green efficient extraction and quantification of the main flavonolignans from the fruits of *S. marianum* with attractive antioxidant and anti-aging activities for future cosmetic applications.

Keywords: *Silybum marianum*; silymarin; flavonolignans; ultrasound-assisted extraction; design of experiement; antioxidant; anti-aging

1. Introduction

Silybum marianum (L.) Gaertn. aka Milk thistle (Asteraceae family) is one of the oldest of all known herbal medicine. This plant is grown as an annual, winter annual and biennial herb depending on climate [1]. In its seed it accumulates high levels of taxifolin-derived flavonolignans such as silydianin (SILD), silychristin (SILC), silybins A (SILA) and B (SILB) and isosilybins A (ISILA) and B (ISILB) (Figure 1) which mixture is named silymarin (SILM) [2,3]. These compounds result from the oxidative coupling of a flavonoid part with coniferyl alcohol, the lignan precursor in plants [4]. *S.*

marianum constitute the almost unique source of these flavonolignans deriving from taxifolin and is thus considered as the model plant for the study of their biosynthesis [5] as well as an attractive resource in order to valorise these compounds for industrial applications, in particular as cosmeceuticals.

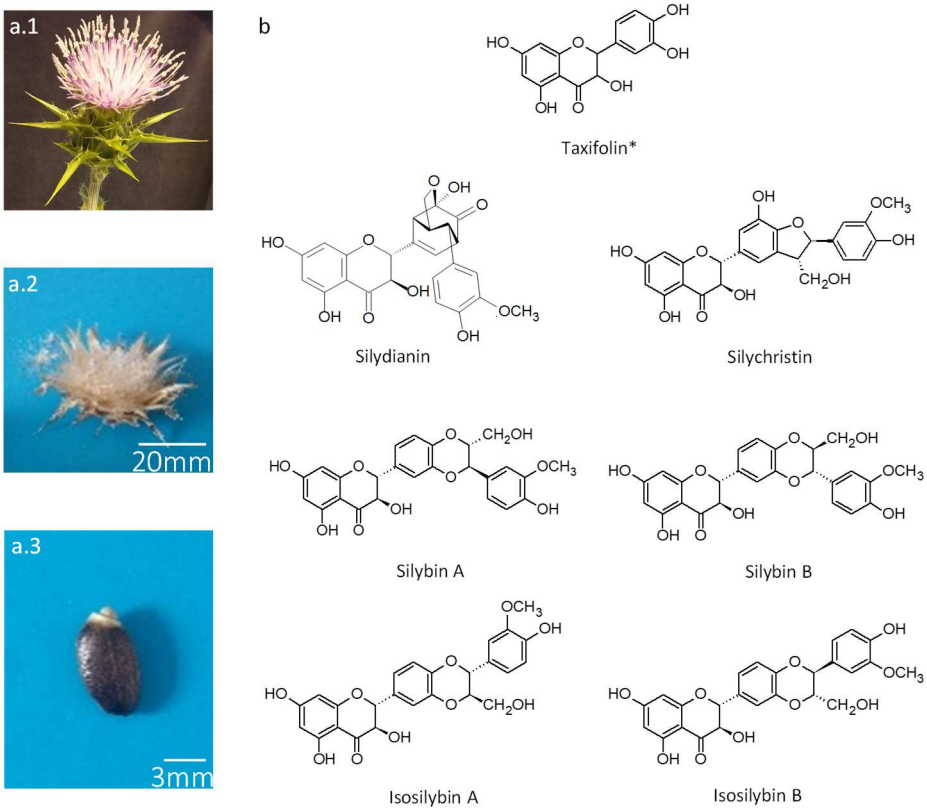

Figure 1. a.1. Representative picture of a flowering capitulum of milk thistle (*Silybum marianum* (L.) Gaertn.); a.2. Representative picture of an open mature capitulum bearing mature fruit (achenes) of milk thistle (*Silybum marianum* (L.) Gaertn.); a.3. Representative picture of a mature fruit (achene) of milk thistle (*Silybum marianum* (L.) Gaertn.); b. Structures of the six flavonolignans (silychristin (SILC), silydianin (SILD), silybin A (SILA), silybin B (SILB), isosilybin A (ISILA) and isosilybin B (ISILB)) and one flavonoid (*, taxifolin, TAX)) from the silymarin (SILM) mixture extracted from milk thistle (*Silybum marianum* (L.) Gaertn.) mature fruit (achene).

Milk thistle is one of oldest medicinal plants, used for centuries to cure various ailments and traditionally used in the European pharmacopoeia as liver detoxifier [6-8] as well as a unique remedy against *Amanita phalloides* intoxication [9,10]. For many decades, milk thistle grew in fields as a food crop and for the cure of hepatobiliary diseases [11], and some established commercial cultivars are available in Europe. Since that, multiple other biological activities of flavonolignans have been described and investigated for numerous pharmacological action which could benefit to human health for ovarian cancer [12] or breast cancer [13] for example. More recently, SILM and its flavonolignans have received a growing interest for their potent antioxidant and anti-aging activities relevant to cosmetic [14–19]. In particular, silybins are the most active compounds in SILM and display a wide range of biological activities, such as antioxidant and skin anti-inflammatory [20]. In cosmetic, the anti-aging activities of plant extracts have been ascribed to their capacity to decrease the damages to the skin caused by reactive oxygen and/nitrogen species (ROS/RNS), along with their aptitude to control the activity of various enzymes involved in skin aging progression. For example, their capacity to inhibit elastase or collagenase involved in the cleavage of extracellular matrix components. For instance, Vostalova et al. [15] have reported on the inhibitory actions of SILM, its flavonolignans and some derivatives toward the collagenase and elastase and evidenced diverse

affinities against these enzymes. Moreover, SILM also confers UV-B protection [21] which could result in an effective skin protection against sunburn or skin cancers [22]. All these biological activities trigger the necessity to develop efficient green extraction protocols for SILM.

Many green extraction methods of plant natural products have already been published using microwave-assisted extraction [23,24], pressured liquid extraction [25], cellulase-assisted [26] or ultrasound-assisted extraction [27] for example. In the present study, we focused on ultrasound-assisted extraction, which is one of the most simple and economic method for improving the extraction yield of plant [28]. It has a short extraction time with a reduced amount of solvent making it a green extraction procedure and can be rapidly upscaled for industrial purposes [29]. Ultrasounds allow mass transfer intensification, cell disruption, better penetration of the solvent improving the extraction and also capillary effects, limit the degradation of the herb constituents even with high frequency ultrasound [30].

To obtain the optimal conditions for extraction yields, a response surface methodology (RSM) (mathematical and statistical technique) has been used. The objective is to optimize a response (extraction yield) influenced by several independent variables that are the extraction time, concentration of EtOH and ultrasound power. Bioassays were performed to evaluate the antioxidant and anti-aging activities of the extracts. These biological activities were correlated to the phytochemical composition of the corresponding extracts.

2. Materials and Methods

2.1. Plant material

All milk thistle (*Silybum marianum* (L.) Gaertn.) seeds are lines and were provided by PMA28 (France).

2.2. Chemicals

All solvents and reagents for extraction and LC analysis were of analytical grade or the highest available purity and were purchased from Fisher Scientific (Illkirch, France). Deionized water was purified by a Milli-Q water-purification system from Millipore (Molsheim, France). All solutions prepared for HPLC were filtered through 0.45 µm nylon syringe membranes prior to use. Standards (silymarin, silybin B) and methoxyflavone (internal standard) were purchased from Sigma (France).

2.3. LC-MS

All flavonolignans and taxifoline were quantified using a LC-MS analysis performed on a Water 2695 Alliance coupled with a single quadrupole mass spectrometer ZQ. LC-ESI-MS. Data acquisition and processing were performed with MassLynx 4.0 software. The separation was performed as described in Drouet et al 2018 [31].

2.4. Extraction

2.4.1. Apparatus and general procedure

1000 mg milled achene or whole fruit was extracted in 40 mL of ethanol solvent. The ultrasonic bath used was a USC1200TH (Prolabo; inner dimension: 300mm × 240mm × 200 mm) with a maximal heating power of 400W (i.e. acoustic power of 1W/cm2), equipped with a digital timer, a frequency and a temperature controller. The extraction was conducted during an extraction time ranging from 20 to 60 min at an operating temperature ranging from 25°C to 60°C. Prior to HPLC injection, the extract supernatant was filtered through 0.45 µm nylon syringe membranes. The optimized USAE method was compared with maceration in the same condition without ultrasound.

2.4.2. Experimental Design

A factorial experiment design and the resulting response surface plots were used to identify the optimal extraction conditions for all flavonolignans using XLSTAT2015 software (Addinsoft, Paris, France). Variables were coded at three levels (−1, 0 and +1; Table 1). The three independent variables were EtOH concentration (X1 values were 50, 75 and 100%), ultrasound power (X2 values were 15, 30 and 45 kHz) and extraction time (X3 values were 20, 40 and 60 minutes) (Table 1). Here, twenty-seven batches were obtained by using the DOE (design of experiment) function of XLSTAT 2019 which take values of selective variables at different levels (Table 2). The experiments were carried out in triplicate. Equations of the models were calculated using XLSTAT 2019 DOE analysis tool. Surface plots showing the response as a function of the simultaneous variation of the independent variables were obtained with 3D option of XLSTAT 2019.

2.4.3. Method validation

The method precision, repeatability and stability were evaluated as described by Corbin et al. [15]. The precision, repeatability and stability were expressed in content (mg/g) and relative standard deviation (RSD, %).

2.5. Antioxidant activity

2.5.1. CUPRAC Assay

Cupric ion reducing antioxidant capacity (CUPRAC) was used [32]. Briefly, 10µl of an extract was mixed with 190 µl of the CUPRAC solution (composed of 10mM Cu(II); 7.5mM neocuproine and 1M acetate buffer pH7; ratio 1:1:1 (v/v/v)). Following incubation during 15 minutes at room temperature (25 ± 2 °C), the absorbance value at 450nm of the reaction mixture was measured (BioTek ELX800 Absorbance Microplate Reader).

2.5.2. Inhibition of Advanced Glycation End Products (AGEs)

The inhibitory capacity of AGE formation was determined as described by Kaewseejan and Siriamornpun [33] using a 20 mg/mL BSA (Sigma Aldrich) solution prepared in 0.1 M phosphate buffer (pH 7.4), a 0.5 M glucose (Sigma Aldrich) solution prepared in phosphate buffer and a 0.1 M phosphate buffer at pH 7.4 containing 0.02% (w/v) sodium azide. Incubation was performed at 37 °C for five days in the dark. The amount of fluorescent resulting from the formation of AGEs was determined using 330 nm excitation wavelength and 410nm emission wavelength conditions (VersaFluor fluorometer; Bio-Rad, Marnes-la-Coquette, France). The percentage of anti-AGEs formation was expressed as a % of inhibition relative to the control (addition of the same volume of extraction solvent).

2.6. Anti-aging activity

2.6.1. Collagenase Assay

Collagenase from *Clostridium histolyticum* (Sigma Aldrich) was used. The collagenase activity was determined using N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA; Sigma Aldrich) as a substrate following the protocol of Wittenauer et al. [34]. Absorbance decrease was followed at 335 nm during 20 min thank to a microplate reader (BioTek ELX800; BioTek Instruments, Colmar, France). The collagenase activity in presence of each extraction conditions was determined in triplicated and the anti-collagenase activity was expressed, for each extract, as an inhibition percentage relative to corresponding control (adding same volume of extraction solvent).

2.6.2. Elastase Assay

Elastase assay was performed by using the porcine pancreatic elastase (Sigma Aldrich). The elastase activity was determined using N-Succ-Ala-Ala-Ala-*p*-nitroanilide (AAAVPN; Sigma Aldrich) as a substrate as described by Wittenauer et al. [34]. The release of *p*-nitroaniline at 410 nm

using a microplate reader (BioTek ELX800; BioTek Instruments). Triplicated measurements were performed and the anti-elastase activity was expressed, for each extract, as an inhibition percentage relative to the corresponding control (adding same volume of extraction solvent).

2.7. Statistical treatment of data

The means and the standard deviation were used to present the data composed of three to five independent replicates. Student's *t*-test was performed for comparative statistical analyses. Here, significant thresholds at $p < 0.05$, 0.01 and 0.001 were used for all statistical tests and represented by *, ** and *** respectively. Model analysis (ANOVA) and 3D plots resulting from the combination of variables were conducted using XLSTAT 2019 and R. The correlation values and corresponding *p*-values were obtained with Past by using the Pearson parametric correlation test.

3. Results

3.1. Preliminary single factor experiments and selection of limiting parameters

Several extraction parameters have been described to possibly affecting the extraction efficiency of polyphenols from various plant matrixes [35]. Here, using a single-factor experiment approach, the influence of 5 independent parameters (ethanol concentration, extraction duration, ultrasound frequency, extraction temperature and liquid to solid ratio) on the SILM extraction yield from the mature fruit of *S. marianum* were evaluated. The objective of these preliminary experiments being to identify the extraction limiting factors.

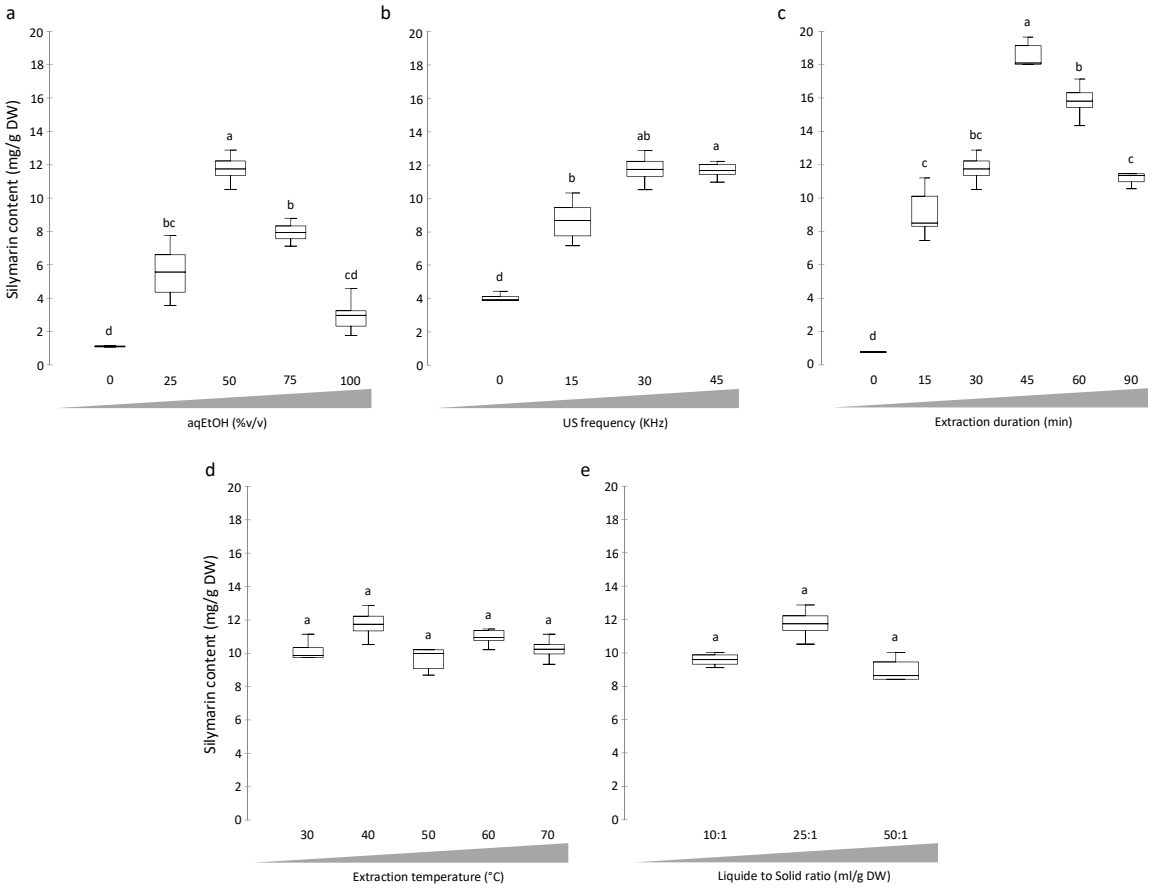

Figure 2. Silymarin (SILM) contents extracted from mature fruits of *S. marianum* as of function of (a) aqueous ethanol concentration (% (v/v)), (b) ultrasound frequency (kHz), (c) extraction duration (min), (d) extraction temperature (°C) and (e) liquid to solid ratio (ml/g DW). The complete description of each extraction conditions is presented in the text. Values are means ± SD of 6 independent replicates. Different letters (a-d) represent significant differences between the various extraction conditions ($p < 0.05$).

The choice of the solvent is certainly the most important parameter to fix for the development of an extraction method. In the literature, it appeared that several organic solvents can be considered for the extraction of plant polyphenols. For this purpose, methanol, ethanol or acetone are the most commonly considered extraction solvents [36]. Here, considering our objective to propose these extracts for future cosmetic applications, and in respect with the development of a green chemistry extraction method, ethanol was chosen. Indeed, ethanol is non-toxic to humans and an environmentally friendly solvent [35]. Moreover, its extraction capacity and efficiency can be modulated easily by the addition of water, thus making it an ideal solvent for the extraction of a wide range of polyphenols with low to high polarity. Interestingly, these two universal solvents are inexpensive, another reason for their common uses for food and/or cosmetic applications [35,36]. The SILM extraction capacity of various ethanol: water mixtures were evaluated (Figure 2a). In these preliminary experiments, 5 concentrations of aqueous ethanol solutions (0%, 25%, 50%, 75%, and 100% (v/v) ethanol in water) were assayed. The other extraction parameters were arbitrary fixed to: 25:1 mL/g DW liquid to solid (L/S) ratio, 30 minutes for the extraction duration, 30 kHz for the ultrasound frequency and 45 °C for the extraction temperature. Ethanol concentration appeared to significantly impact the SILM extraction yield from milk thistle fruits. An optimal extraction yield was obtained for an ethanol concentration of 50% (v/v). Extreme values for the ethanol concentrations (i.e., 0 and 100 % (v/v)) resulted in a 4- to 10-times decreases in SILM, respectively, whereas ethanol concentrations of 25 and 75 % (v/v) resulted in intermediary SILM contents.

The ultrasound frequency is also known to potentially impact the extraction efficiency. This parameter act though the modulation of both the cavitation effect and the diffusion coefficient of the targeted compounds into the extraction solvent. This could result in an increased solubilization of the target compound in the considered extraction solvent, and to an increase of the extraction efficiency [36]. Moreover, the increase of ultrasound frequency could result in a decreased extraction duration, and therefore of energy consumption [37]. However, application of high ultrasound frequencies could also change or destroy the molecular structure of the targeted compound. This could lead to a decrease in the extraction yield, but also a reduction (sometime a complete a loss) of its biological activity [38]. As a consequence, the ultrasound frequency appears as a crucial parameter to consider during the development of an UAE method. In our hands, we evaluated the impact of 4 different ultrasound frequencies on the SILM extraction yield (Figure 2b). The other extraction parameters were arbitrary fixed to: 50% (v/v) aqueous ethanol concentration, 25:1 mL/g DW liquid to solid (L/S) ratio, 30 minutes for the extraction duration and 45 °C for the extraction temperature. A significant impact of ultrasound frequency was noted, with a highest yield observed for a 30 kHz frequency. Lower US frequency (15 kHz) or even non application of US frequency resulted in a low extraction efficiency, whereas highest US frequency (45 kHz) have led to a decreased SILM extraction yield certainly because of a deterioration of these compounds.

To reduce energy consumption in a context of a green chemistry approach, optimizing extraction duration is essential [37]. As already mentioned for US frequency, increasing extraction duration not necessary results in a gain in terms of extraction yield since a prolonged duration to US can lead to a deterioration of the compounds [38]. We considered 6 extraction duration (0, 15, 30, 45, 60 and 90 min) with the other parameters arbitrary fixed to: 50% (v/v) aqueous ethanol concentration, 25:1 mL/g DW liquid to solid (L/S) ratio, 30 kHz US frequency duration and 45 °C for the extraction temperature. Under these conditions, we observed a gradual increase of SILM extraction from milk thistle fruit, with a maximum extraction efficiency after 45 min, followed by a significant decrease with 60 and 90 min extraction time using these conditions (**Figure** Figure 2c). This observation is in global agreement with other studies that also report on a possible degradation of antioxidant phenolic compounds following ultrasound treatment [35,37,38].

Different extraction temperature (30, 40, 50, 60 and 70 °C) were next evaluated for their influence on SILM extraction yield (**Figure** Figure 2d). In our hands, using the other parameters fixed (ethanol

concentration 50% (v/v), S/M ratio 25:1 mL/g DW, extraction time 45 min and ultrasound frequency 30 kHz), the extraction temperature did not appeared as a limiting parameter significantly impacting the SILM extraction yield. The hot spot theory could explain this observation. Indeed, according to this theory, the cavitation bubbles are considered as a microreactor generating a local environment in the surrounding liquid after their collapse with high temperature (*ca* 4500 °K) and pressure (*ca* 1000 atm) that could justify the low impact of few dozen temperature degrees [36].

Finally, the 3 liquid to solid (L/S) ratios (10:1, 25:1 and 50:1, in ml of 50 % (v/v) aqueous ethanol per gram of DW material) evaluated (using an extraction time of 30 min, a sonication frequency of 30 kHz and a extraction temperature of 45 °C) only resulted in a slight non-significant difference in SILM extraction yield with slightly best result for the ratio 25:1 (**Figure** Figure 2e). This ratio was therefore used hereafter, but this parameter was not considered as limiting parameter, and as such not further optimized.

3.2. Development of a Multifactorial Approach

The preliminary experiments evidenced the significant impacts of ethanol concentration, extraction duration and US frequency (Figure 2) which were therefore selected for further optimization. Here, in order to take into account, the possible interactive influence of these parameters, we employed an experimental factorial design (design of experiment, DOE) with statistical analysis. This strategy is known to be more effective, precise and rapid to integrate a large number of extraction conditions allowing to evidence possible interaction between independent variables as compared with a single factor approach [39]. Taking into account the preliminary experiments, we decided to limit the 3 influencing variables as follow: the concentration of aqueous ethanol (variable X1, ranging from 25 to 75 % (v/v)), the US frequency (variable X2, ranging from 15 to 45 kHz) and the extraction duration (variable X3, ranging from 20 to 60 min). Both their coded levels and experimental values are presented in Table 1. Given the results of the preliminary experiments, an L/S ratio of 25:1 and an extraction temperature of 45 °C were used.

Table 1. Identity, code unit, coded level and experimental values of the 3 independent variables.

Independent variable	Code unit	Coded variable levels		
		-1	0	+1
ethanol concentration (% v/v)[1]	X_1	25	50	75
US frequency (kHz)	X_2	15	30	45
Extraction duration (min)	X_3	20	40	60

[1] % of ethanol concentration in mixture with HPLC grade ultrapure water.

A full factorial design was used to optimize this extraction process in owing to its high reproducibility as a consequence of the real measurement of a large number of experimental conditions compared to other DOE approaches [40]. *In silico* drove the 27 different bath conditions (run ID) were determining with their corresponding independent process variables, and randomized (run order) as presented in Table 2. Each batch condition was assayed in independent triplicates. The SILM extraction yield (Table 2) as well as individual composition in each flavonolignan (Table S1) were determined.

Following HPLC analyses (Figure 3), the SILM content extracted from mature fruit of *S. marianum* ranged from 1.80 (run ID#13) to 17.98 (run ID#26) mg/g DW (Table 2). The individual composition of the SILM of each extract was also determined. The separation was based on the method described by Drouet et al. [14], here further optimized (see Materials and Methods, sections 2.3 and 2.4) to allow a higher resolution for the separation of the different peaks as shown on Figure 3.

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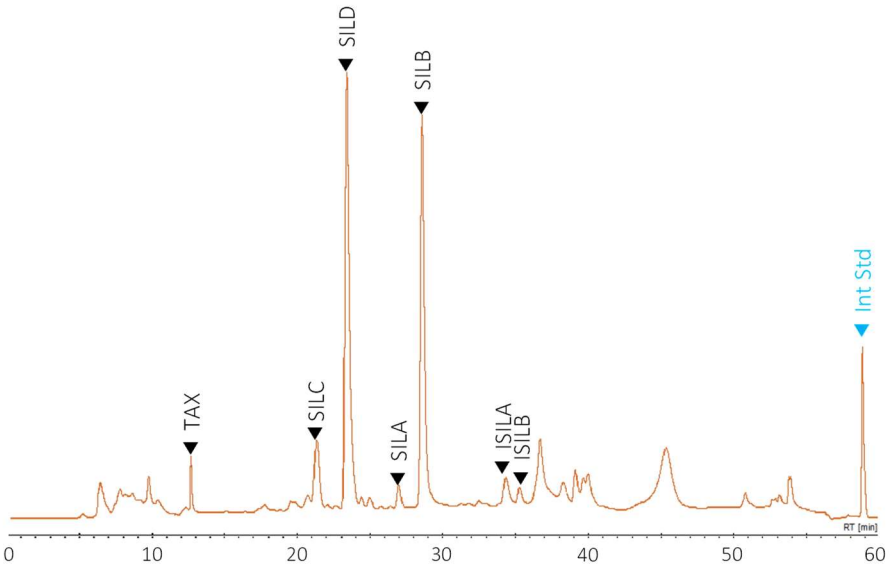

Figure 3. Representative HPLC chromatogram of an extract prepared by USE of a mature fruits (achenes) of a milk thistle (*S. marianum* (L.)) commercial cultivar. The main compounds considered in this study are the flavonoid taxifolin (TAX) and the flavonolignans: silychristin (SILC), silydianin (SILD), silybin A (SILA), silybin B (SILB), isosilybin A (ISILA) and isosilybin B (ISILB). Int Std: internal standard (6-methoxyflavone).

Table 2. Results of full factorial design experiments for the extraction of silymarin (SILM) from mature fruits of *S. marianum* (L.).

Run ID	Run order	X_1	X_2	X_3	SILM (mg/g DW)
Run ID#1	17	-1	-1	-1	2.24 ± 1.80
Run ID#2	24	0	-1	-1	6.99 ± 4.11
Run ID#3	26	+1	-1	-1	4.33 ± 3.09
Run ID#4	21	-1	0	-1	2.55 ± 4.61
Run ID#5	22	0	0	-1	9.66 ± 1.44
Run ID#6	6	+1	0	-1	6.51 ± 3.06
Run ID#7	10	-1	+1	-1	4.37 ± 1.72
Run ID#8	27	0	+1	-1	8.45 ± 4.48
Run ID#9	7	+1	+1	-1	6.88 ± 4.82
Run ID#10	18	-1	-1	0	3.21 ± 0.34
Run ID#11	12	0	-1	0	12.12 ± 1.57
Run ID#12	8	+1	-1	0	6.15 ± 1.08
Run ID#13	25	-1	0	0	1.80 ± 1.50
Run ID#14	1	0	0	0	14.25 ± 0.51
Run ID#15	16	+1	0	0	9.79 ± 2.47
Run ID#16	23	-1	+1	0	2.49 ± 1.59
Run ID#17	11	0	+1	0	12.88 ± 3.35
Run ID#18	14	+1	+1	0	9.13 ± 1.80
Run ID#19	15	-1	-1	+1	2.49 ± 0.33
Run ID#20	3	0	-1	+1	16.00 ± 0.35
Run ID#21	13	+1	-1	+1	7.27 ± 0.36
Run ID#22	9	-1	0	+1	4.20 ± 0.30
Run ID#23	5	0	0	+1	16.71 ± 0.19
Run ID#24	19	+1	0	+1	10.05 ± 0.48

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Run ID#25	4	-1	+1	+1	4.04 ± 0.30
Run ID#26	20	0	+1	+1	17.98 ± 0.66
Run ID#27	2	+1	+1	+1	8.54 ± 0.94

Values are the mean ± RSD of 3 independent replicates except for *, which correspond to the highest SILM content here determined by 6 independent experiments to confirm this value.

In regards to SILM composition from the most to the less abundant (Table S1):

- SILB was detected under each extraction condition and its contents ranged from 1.29 (run ID#1) to 7.52 (run ID#26) mg/g DW;
- the detected SILD contents ranged from 0.40 (run ID# 13) to 4.21 (run ID#20) mg/g DW, whereas SILC was not detected under one extraction condition (run ID#16). This run ID#16 presented the combination of low ethanol concentration (25% (v/v)) and application of high US frequency (45 kHz);
- the detected ISILA contents ranged from 0.45 (run ID#3) to 2.49 (run ID#26) mg/g DW, whereas ISILA was not detected under 9 extraction conditions (run ID#1, #4, #7, #10, #13, #16, #19, #22 and #25). All these run IDs were obtained using with the same (lowest) aqueous ethanol concentration of 25% (v/v) as X1 extraction condition (X1 = -1, Table 1);
- the detected SILC contents ranged from 0.01 (run ID#19) to 1.52 (run ID#26) mg/g DW, whereas SILD was not detected under 4 extraction conditions (run ID#7, #16, #22 and #25). Like run ID#16, the run IDs#16 and #25 presented the same combination of low ethanol concentration (25% (v/v) in water, X1 = -1, Table 1) and high ultrasound frequency of 45 kHz (X2 = +1, Table 1), whereas run ID#22 presented again low ethanol concentration (i.e. 25% (v/v), X1 = -1, Table 1) and intermediate ultrasound frequency of 30 kHz (X2 = 0, Table 1), but during a prolonged period of time (X3 = +1 (i.e., 60 min), Table 1);
- the detected SILA contents ranged from 0.01 (run ID#13) to 1.09 (run ID#26) mg/g DW, whereas SILA was not detected under 3 extraction conditions (run ID#4, #16 and #24). These run IDs used the same low ethanol concentration (i.e. 25% (v/v), X1 = -1, Table 1), whereas run ID#16 and #24 were performed at high US frequency (X2 = +1, Table 1) during a prolonged period of 40 and 60 min, respectively (X3 = 0 or +1, respectively, Table 1)
- TAX was detected under each extraction conditions and its content ranged from 0.16 (run ID#3) to 0.68 (run ID#20);
- and finally, the detected ISILB contents ranged from 0.03 (run ID#9) to 0.55 (run ID#26) mg/g DW, whereas SILA was not detected under 3 extraction conditions (run ID#1-4, #6, #7, #10, #12-16, #19, #22, #24, #25 and #27). We observed that the use of an aqueous ethanol concentration of 25% (v/v) always failed to extract ISILB. These results may also be related to the low accumulation of ISILB in the mature fruit of the considered milk thistle cultivar.

To sum up these results, the hypothesis of low extraction yields of SILM and its constituents as a consequence of their lower solubility in extraction solvent with high polarity and/or of destructive extraction linked to too drastic (high and/or prolonged) US treatment can be made.

To analyse more deeply these results, a model of the SILM extraction yield as a function of the 3 different variables was obtained by multiple regression analysis (Table 3). Using the conditions described in Table 1 and Table 2, the SILM extraction yield (Y_{SILM}) as a function of the 3 different variables (X1: ethanol concentration, X2: ultrasound frequency and X3: extraction duration) in the form of a polynomial equation was: $Y_{SILM} = 13.52 + 2.29X1 + 0.78X2 + 1.96X3 – 7.45X1^2 - 0.86X2^2 – 0.25X3^2 + 0.32X1X2 + 0.55X1X3 – 0.11X2X3$ (Table 3).

Table 3. Values, standard deviations and statistical analysis of the regression coefficients for the SILM extraction yield (Y_{SILM}) from mature fruits of *S. marianum* (L.) as a function of the 3 different variables (X1: ethanol concentration, X2: ultrasound frequency and X3: extraction duration)

Source	Value	SD	*t*	*P* > |*t*|

Constant	13.52	1.0	13.75	< 0.0001***
X_1	2.29	0.5	5.04	0.0001***
X_2	0.78	0.5	1.70	0.11ns
X_3	1.96	0.5	4.31	0.0004***
X_1^2	-7.45	0.8	-9.44	< 0.0001***
X_2^2	-0.86	0.8	-1.09	0.29ns
X_3^2	-0.25	0.8	-0.31	0.76ns
X_1X_2	0.32	0.6	0.58	0.57ns
X_1X_3	0.55	0.6	0.98	0.34ns
X_2X_3	-0.11	0.6	-0.20	0.84ns

SD standard error; *** significant p < 0.001; ** significant p < 0.01; * significant p < 0.05; ns not significant.

The statistical analysis (Table 3) evidenced the significant impact on the SILM extraction efficiency from mature fruit of *S. marianum* (L.) of the linear coefficients X1 (ethanol concentration) and X2 (extraction time) and the quadratic coefficients $X1^2$. On the contrary, the other linear X3 (ultrasound frequency), quadratic $X2^2$ and $X3^2$ as well as interaction coefficients were not significant ($p > 0.05$). Therefore, ethanol concentration (X1) as well extraction duration (X2) appeared to be the most influent parameters for this extraction process over US frequency (X3) for SIM extraction. The same trend was observed for the individual constituents of the SILM, with the exception of ISILB for which the quadratic coefficients $X1^2$ was the sole significant coefficient (Table S2).

In addition to all these significant coefficients, SILB extraction was also significantly impacted by the US frequency (linear coefficients X2). SILB was therefore the unique compound for which extraction was significantly influenced by this US frequency variable. Nevertheless, we have to keep in mind, here during the DOE in all the extraction conditions US were applied at 3 different frequencies appearing to be in the best range in preliminary experiments. During these preliminary experiments it also became clear that the absence of US treatment drastically reduced extraction efficiency. So, here we can conclude that US frequency X3 variable did not significantly influenced the SILM extraction yield in the selected range of values for this variable, whereas the absence of US had clearly resulted in a less efficient extraction process during the preliminary experiments.

Results of the analysis of variance (ANOVA) and the fit for the models obtained for SILM and its constituents are listed in Table 4 and Table S3, respectively. The high F-value (14.73) and the low *p*-value ($p < 0.0001$) indicated that the model was highly significant and can predict the SILM content as a function of the variable values with a great precision (Table 4). The same trend was recorded for each individual constituent of the SILM (Table S3), with a lower but still significant precision for ISILB. This was also confirmed by the low and non-significant lack of fit values. The model precision in the prediction of the experimental values is evidenced by the predicted *vs*. experimental plot presented in Figure S1, with determination coefficient R^2 of 0.891 (adjusted value of 0.833) for SILM, and ranging from 0.810 for TAX to 0.946 both for SILC and SILD, with the exception of ISILB presenting an R^2 value of 0.589 (Table 4 and Table S3). Finally, this was also confirmed by the coefficient value (CV) indicating the adequacy between the model and experimental values.

Table 4. ANOVA of the SILM extraction model.

Source	Sum of square	df	Mean of square	*F*-value	*p*-value
Model	517.0	9	57.4	15.4	<0.0001
Lack of fit	63.4	17	3.7	0.060	ns
Residual	58.5	17	3.4	-	-
Pure Error	4.9	0	-	-	-
Cor. Error	580.4	26	-	-	-
R^2	0.891				
adj R^2	0.833				

CV %	0.238

df: degree of freedom; Cor. Error: corrected error; R^2: determination coefficient; R^2 adj: adjusted R^2; CV variation coefficient value; ns: non-significant.

In order to better understand the complexity of the model, 3D plots were drawn for SILM (Figure 4) and the individual constituents (Figures S2-8).

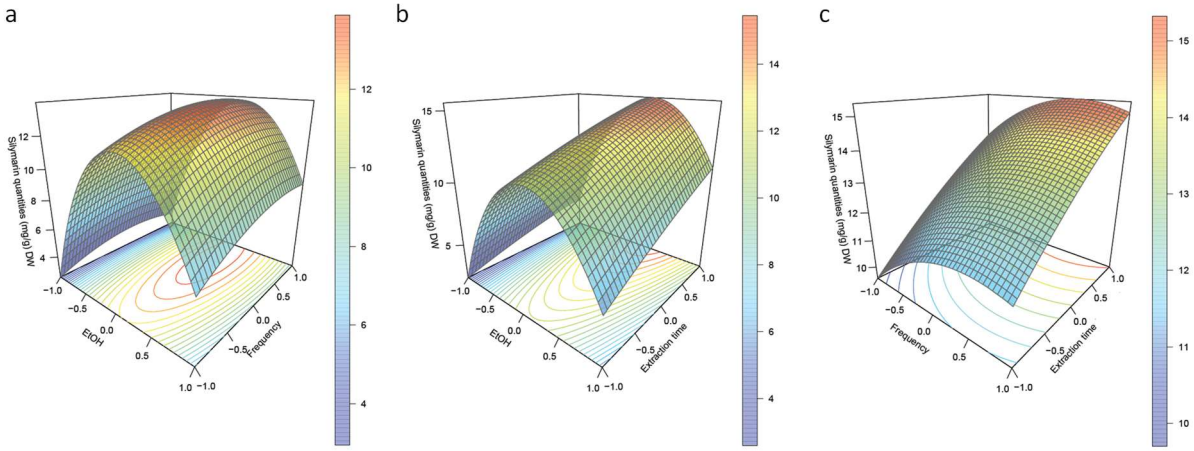

Figure 4. 3D plots from the model predicted SILM extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration.

The linear coefficients of the second-order polynomial equation for X1 ethanol concentration, X2 ultrasound frequency and X3 extraction duration, as well as the interaction coefficients X1X2 (ethanol concentration and ultrasound frequency) and X1X3 (ethanol concentration and extraction duration) were all positives, indicating that the increase of these parameters results in a favourable action on the SILM extraction yield. But, their low values, in association with the negative values recorded for their quadratic coefficients (i.e., $X1^2$, $X2^2$ and $X3^2$) as well as for the interaction coefficient between ethanol concentration and US frequency X2X3 indicate that the extraction of SILM reaches a maximum value before decreasing for higher values of these parameters. We can observe these tendencies on the 3D plots with first a positive action on SILM extraction yield in increasing aqueous ethanol concentration combined with higher US frequency and/or prolonged extraction duration (Figure 4a, b). But, the highest aqueous ethanol concentration, on the one hand, as well as prolonged extraction duration with high US frequency, on the other hand, resulted in a marked decline of SILM extraction yields (Figure 4). Ethanol/water mixtures represent eco-friendly solvents able to extract a wide range of polyphenols, however optimal ethanol/water ratio is highly dependent of the polarity of the considered polyphenol [36]. Applying high ultrasound frequency for a prolonged period of time is known to be potentially destructive and to induce oxidation of natural products, especially when water is used as solvent (or present as mixture with ethanol) that could lead to a decrease in extraction yield as well as to the loss of the biological activities of the compound [27,36,38,41]. According to the adjusted second order polynomial equation, optimal conditions were a USE with 54.5% (v/v) aqueous EtOH as solvent, applying an ultrasound frequency of 36.6 kHz during an extraction time of 60 min, with a fixed extraction temperature of 45°C and liquid to solid ratio of 25:1 ml/g DW. Adjusted to the material, an US frequency of 30 kHz was used. Under these optimized conditions, SYLM extraction yield from the mature fruit of *Silybum marianum* AJQ cultivar reached 20.28 ± 0.41 mg/g DW.

3.3. Validation of the Extraction Method

As shown in Figure 3, the identification and quantification of the SILM different constituents were using the validated separation method described by Drouet et al. [14] by comparison with an authentic commercial standards, and further confirmed by LC-MS. Here, the separation resolution

was further ameliorated following slight modification of the mobile phase (see Materials and Methods, section 2.4), thus allowing a precise quantification of each compound. Coupled with the present optimized extraction method, in order to certify accuracy and precision, the method was then validated according to the recommendations of the association of analytical communities (AOAC) (http://www.aoac.org). The parameter values of this validation procedure are satisfactory in terms of precision, repeatability and stability according to AOAC standards and are presented in Table 5.

Table 5: Precision, repeatability and stability validation parameters of the method.

	Precision (*n=5*)		**Repeatability** (*n=5*)		**Stability** (*n=5*)	
	Content		*Content*		*Content*	
	mg/g DW	*RSD (%)*	*mg/g DW*	*RSD (%)*	*mg/g DW*	*RSD (%)*
SILM	20.28 ± 2.02	2.02	19.12 ± 0.88	4.62	20.16 ± 0.43	2.16
SILC	1.93 ± 0.06	3.00	1.71 ± 0.10	5.72	2.02 ± 0.07	3.40
SILD	2.40 ± 0.13	5.57	2.63 ± 0.08	3.20	2.56 ± 0.10	3.94
SILA	1.06 ± 0.03	3.11	1.02 ± 0.02	2.47	1.17 ± 0.04	3.68
SILB	8.43 ± 0.13	1.54	7.96 ± 0.28	3.58	8.17 ± 0.33	4.02
ISILA	4.17 ± 0.14	3.24	3.89 ± 0.36	9.25	4.14 ± 0.11	2.64
ISILB	2.29 ± 0.13	5.48	1.91 ± 0.15	7.83	2.10 ± 0.07	3.26

RSD: relative standard deviation (expressed in %).

3.4. Evaluation of the biological activities of the extracts relevant to cosmetic

To evaluate the influence of the extraction process, to ensure that the biological activity is retained during this process and to correlate the biological activity with the phytochemicals of the extract, we next determined the antioxidant and anti-aging potential relevant to cosmetic of each of the 27 run ID. Indeed, SILM and its flavonolignans have received a recent interest for their potent antioxidant and anti-aging activities relevant to cosmetic [14–19].

CUPRAC assay have been reported to effectively evidence the antioxidant activity of milk thistle extracts [20]. Here, the antioxidant activity evaluated by the CUPRAC assay ranged from 51.33 (run ID#10 – SILM content of 3.21 mg/g DW) to 183.80 (run ID#26 – SILM content of 17.98 mg/g DW) µM AEAC (Figure 5, Table S4). Oxidative stress has been associated with aging and could lead to the formation of advanced glycation end products (AGEs) [42]. Here, the strong inhibition of AGEs formation confirmed the antioxidant capacity of these extract evidenced by the CUPRAC assay. The inhibition of AGEs formation ranged from 6.64 (run ID#13 – SILM content of 1.80 mg/g DW) to 74.31 (run ID#26 – SILM content of 17.98 mg/g DW) % of inhibition (Figure 5, Table S4). A strong significant correlation was observed between SILM content and CUPRAC antioxidant activity (PCC=0.862) as well as between SILM content and AGEs inhibitory action (PCC=0.997) (Table 6).

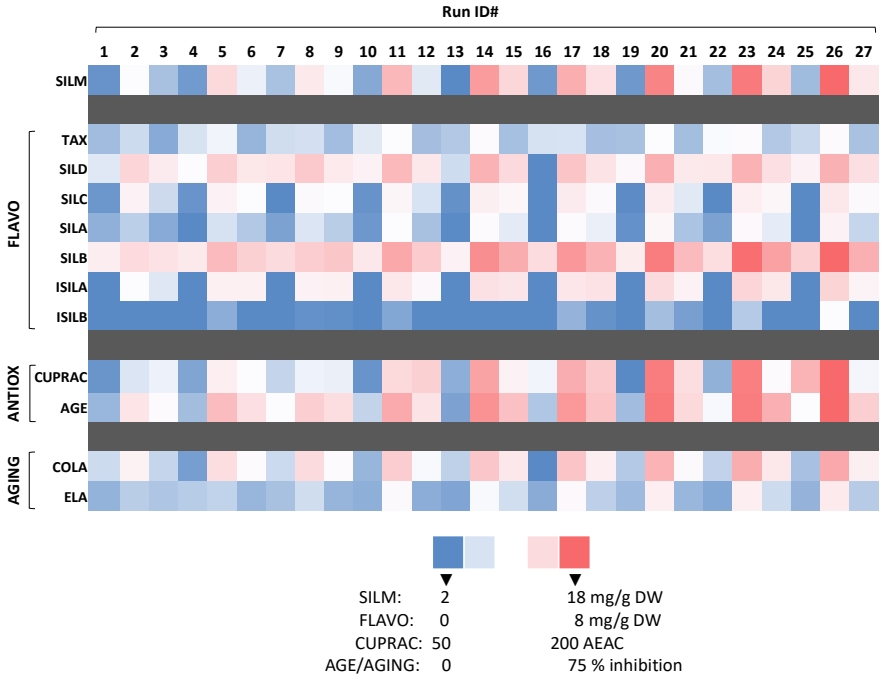

Figure 5. Heatmap showing the phytochemical composition and biological activities relevant to cosmetic of the 27 extracts from *S. marianum* (L.) mature fruit following USE. Two antioxidant assays were conducted: CUPRAC (expressed as ascorbic acid equivalent antioxidant capacity (AEAC, in µM AEAC)) and the inhibition of advanced glycation end product (AGE) formation (expressed in % of inhibition relative to a control obtained by measuring the activity of the corresponding extraction solvent). Two anti-aging assays were conducted by determining the inhibition activity of each extracts toward collagenase (COL) and elastase (ELA) enzymes (expressed in % of inhibition relative to a control obtained by measuring the activity of the corresponding extraction solvent).

Table 6: Pearson coefficient correlation (PCC) linking SILM and its constituents to their antioxidant and anti-aging activities.

	SILM	TAX	SILC	SILD	SILA	SILB	ISILA	ISILB
CUPRAC	0.862***	0.500*	0.768*	0.776*	0.806*	0.887*	0.827**	0.697*
AGE	0.997***	0.604*	0.930**	0.942**	0.976*	0.979***	0.960**	0.766*
COLA	0.976**	0.659**	0.957**	0.927**	0.968**	0.928***	0.908**	0.801*
ELA	0.922**	0.702**	0.893**	0.843*	0.910**	0.894*	0.830*	0.843*

*** significant $p < 0.001$; ** significant $p < 0.01$; * significant $p < 0.05$

All the SILM constituents were also correlated to these antioxidant activities (Table 6). The SILM content and composition of wild ecotypes of *S. marianum* from Pakistan have been correlated with their antioxidant activity measured by CUPRAC assay [14]. Natural antioxidant have attracted a growing interest over the last decade because of their possible use as alternative to the potentially harmful, synthetic antioxidant like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) in different formulations [43–45]. Recently, a SILB palmitate derivative has been synthesized and displayed a pronounced anti-lipoperoxidant activity, inhibiting the formation of conjugated diene production in two different lipophilic media (bulk oil and o/w emulsion) subjected to accelerated storage test [45]. Here, this antioxidant action *in vitro* is further confirmed by the CUPRAC assay correlated to the SILM and SILB contents. In addition, oxidative stress has been associated with aging and age-related diseases [46], in particular leading to the formation of AGE [47]. The ability of natural compounds to inhibit their formation have therefore attracted increasing attention in cosmetic. The high inhibition of AGE formation correlated with SILM content, in particular with SILA and SILB which is of special interest for future applications.

Next step was to evaluate the inhibitory activity of the extracts toward collagenase and elastase. Indeed, the potent inhibitory action of SILM and its flavonolignans toward these enzymes has been recently evidenced [15]. A strong inhibitory effect was observed for collagenase, whereas it was less marked for elastase (Figure 5, Table S4). Indeed, collagenase inhibition ranged from 4.21 (run ID#16 – SILM content of 2.49 mg/g DW) to 49.13 (run ID#26 – SILM content of 17.98 mg/g DW) % of inhibition, while, elastase inhibition ranged from 6.84 (run ID#13 – SILM content of 1.80 mg/g DW) to 22.93 (run ID#26 – SILM content of 17.98 mg/g DW) % of inhibition. However, a strong significant correlation was measured for both enzymatic inhibitory action with SILM content (Table 6). Elastase and collagenase are enzymes acting on the remodelling and/or degradation of the extracellular matrix components in the dermis, thus potentially leading to skin alterations such as skin tonus decrease, formation of deep wrinkles and resilience losses [48–50]. Our results confirmed the potential of silymarin and its constituent as inhibitor of collagenase, and to a less extend elastase. Work aiming at elucidating the inhibition mechanism of each flavonolignans would be of particular interest for future applications.

3.5. Comparison with Conventional Maceration Protocol

To evaluate the efficiency of the present optimized green US extraction procedure, we compare it with a conventional heat reflux extraction method. For this purpose, we used the same aqueous ethanol concentration of 54.5 % (v/v), extraction duration of 60 min, temperature of 45 °C and L/S ration of 25:1. The difference between the two extractions was the application of US frequency at 30 kHz for the optimized US extraction procedure, while for maceration a classical water bath (i.e. no US applied) was used. The results of these extractions are presented in Table 7.

Table 7: Comparison between conventional the present optimized ultrasound-assisted extraction vs conventional heat reflux method.

	USE	Maceration (MAC)	Ratio USE/MAC
SILM	20.28 ± 0.41	3.40 ± 0.14	5.96***
SILC	2.40 ± 0.13	0.94 ± 0.04	2.56***
SILD	1.93 ± 0.06	0.68 ± 0.04	2.84***
SILA	1.06 ± 0.03	0.11 ± 0.02	9.32***
SILB	8.43 ± 0.13	1.31 ± 0.04	6.41***
ISILA	4.17 ± 0.13	0.30 ± 0.01	13.84***
ISILB	2.29 ± 0.12	0.06 ± 0.01	40.37***

Values are the mean ± RSD of three independent replicates expressed in mg/g DW. *** indicate significant differences ($p < 0.001$) between conditions

The results of these two different extraction processes demonstrated that the application our USE protocol resulted in a significant ca 6-times gain in SILM extraction yield compared to maceration. The gains in extraction yields ranged from 2.56 for SILC to 40.37 for ISILB (Table 7). Note that higher extraction yields were obtained when increasing the extraction duration of maceration, but still without reaching values observed with the USE (data not shown). This protocol is therefore of special interest, in the context of green chemistry, in term of reducing energy consumption by using this innovative technology, but also for industrial process. It allows high extraction yields of milk thistle flavonolignans with a reduced extraction costs (reduction in terms of treatment duration and solvent consumption). This efficiency of USE could be a consequence of the hot spot hypothesis: cavitation bubbles acting as a microreactor generating a high temperature and pressure local environment in the surrounding liquid after their collapse resulting in a more efficient rupture of the plant tissue, and therefore a more efficient release and solubilization of the phytochemicals [36].

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3.6. Comparison of the SILM variations in commercial cultivars vs wild ecotypes

Taking advantage of this optimized and validated USE protocol, we finally applied it to compare the content and composition of SILM of 4 commercial cultivars. The results of these extractions are presented in Table 8.

Table 8: Contents of SILM constituents of 4 French commercial milk thistle cultivars.

Cultivars	SILM	TAX	SILC	SILD	SILA	SILB	ISILA	ISILB
APM	35,40 ± 1.31	1.27 ± 0.18	1,79 ± 0.32	9,67 ± 1.45	6,10 ± 0.40	5,30 ± 1.37	1,40 ± 0.10	0,67 ± 0.25
AJN	**43,61 ± 1.61**	1.58 ± 0.13	**3,19 ± 0.64**	**11,77 ± 1.77**	**7,39 ± 1.10**	6,46 ± 1.67	1,23 ± 0.27	0,63 ± 0.10
AJQ	20.28 ± 0.41	0.82 ± 0.05	2.40 ± 0.13	1.93 ± 0.06	1.06 ± 0.03	**8.43 ± 0.13**	**4.17 ± 0.13**	**2.29 ± 0.12**
11E	43,25 ± 1.60	**1.62 ± 0.11**	2,14 ± 0.10	11,35 ± 1.71	6,78 ± 1.56	7,43 ± 1.92	1,65 ± 0.30	0,60 ± 0.16

Values are the mean ± RSD of three independent replicates expressed in mg/g DW.

AJN cultivar is the richest in SILM contents, and accumulated the highest contents in SILC, SILD and SILA, whereas AJQ was the richest in SILB, ISILA and ISILB. The highest accumulation in TAX was measured in 11E. Here, we observed quite restricted variation ranges compared to our previous study with wild ecotypes from Pakistan [14]. It is accepted that the SILM content and composition could vary according to both genetic background and culture conditions [14,51–53]. Strong variations in SILM content and composition was reported for wild ecotypes from Egypt [54], Iran [52], Greece [55] as well as from Poland, Hungary, Bulgaria, and Germany [51]. Culture conditions of the commercial crop are probably more homogenous than natural conditions which could partly explain this led wide range of contents. Here the observed stability in the SILM contents and composition is an important feature for these established cultivars cultivated for commercial purposes. However, the information on the wide range of variations observed in wild ecotypes in relevant for the generation of new cultivars in future breeding strategies for more specific applications.

4. Conclusions

Silybum marianum (L.) (milk thistle) constitutes a unique source of silymarin (SILM), and thus is an attractive starting material for their extraction. SILM is a mixture of flavonolignans accumulated in the mature fruits of *S. marianum*. These compounds have attracted a recent interest for cosmetic applications, and therefore deserve the development of optimized and validated green extraction process. Here, we developed an ultrasound-assisted extraction (UAE) of SILM from mature fruits of *S. marianum* using a design of experiment strategy. The optimal conditions for UAE were: aqueous EtOH 54.5% (v/v) as solvent, an ultrasound frequency of 36.6 kHz during an extraction time of 60 min, at a temperature of 45°C and with a liquid to solid ration of 25:1 ml/g DW. Following optimization, the extraction method was validated according to international standards of the association of analytical communities (AOAC) in order to ensure its precision and accuracy for the quantitation of the individual silymarin components. The efficiency of UAE allowed substantial gains in terms of extraction yields of flavonolignans in comparison to maceration of the same duration. It also allows an efficient extraction in a reduced extraction time. Thus, the present method is of particular interest in a green chemistry context in terms of reducing energy consumption and with the use of a green solvent. High antioxidant capacity of the extracts was evidenced by the *in vitro* CUPRAC assays and inhibition of advanced glycation end products (AGEs). The skin anti-aging action was also confirmed by the strong *in vitro* inhibition capacity of the obtained extract against collagenase and elastase enzymes. The procedure presented here allows a green efficient extraction of native bioactive flavonolignans from the fruits of *S. marianum* with potent antioxidant and anti-aging activities. Altogether these results prove that the US extraction method presented here resulted in high extraction capacity of SILM and its constituents, but also that the native biological activities

of these compounds is retained during extraction. We anticipate that it could allow fast, easy efficient and reproducible extraction of these compounds for future cosmetic applications.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Figure S1** Biplot representation of the linear relation between predicted *vs* measured SILM contents in the 27 sample extracts. Light blue contours represented $p = 0.05$. **Figure S2** 3D plots from the model predicted TAX extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration. **Figure S3** 3D plots from the model predicted SILC extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration. **Figure S4** 3D plots from the model predicted SILD extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration. **Figure S5** 3D plots from the model predicted SILA extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration. **Figure S6** 3D plots from the model predicted SILB extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration. **Figure S7** 3D plots from the model predicted ISILA extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration. **Figure S8** 3D plots from the model predicted ISILB extracted quantities from mature fruits of *S. marianum* as a function of (a) ethanol concentration and ultrasound frequency, (b) ethanol concentration and extraction duration, and (c) ultrasound frequency and extraction duration. **Table S1** Results of full factorial design experiments for the extraction of TAX, SILC, SILD, SILA, SILB, ISILA and ISILB from mature fruits of *S. marianum*. **Table S2** Values, standard deviations and statistical analysis of the regression coefficients for the TAX, SILC, SILD, SILA, SILB, ISILA and ISILB extraction yield from mature fruits of *S. marianum* as a function of the 3 different variables (X1: ethanol concentration, X2: ultrasound frequency and X3: extraction duration). **Table S3** ANOVA results of the TAX, SILC, SILD, SILA, SILB, ISILA and ISILB extraction models. **Table S4** Individual antioxidant and anti-aging activities of the 27 US extract samples.

Author Contributions: conceptualization, C.H. and E.La.; methodology, S.D., E.Le., L.G. and D.T.; formal analysis, C.H.; investigation, S.D., E.Le., L.G., D.T. and A.K.; resources, C.H.; data curation, C.H., D.T., B.H.A and E.La.; writing—original draft preparation, C.H.; writing—review and editing, C.H., E.La., D.T., A.K. and B.H.A; visualization, S.D. and C.H.; supervision, C.H. and E.La.; project administration, C.H.; funding acquisition, C.H.

Funding: This research was part of the MariLine program focusing on the cosmetic application of the milk thistle flavonolignans, supported by Cosmetosciences, a global training and research program dedicated to the cosmetic industry. Located in the heart of the cosmetic valley, this program led by University of Orléans is funded by the Region Centre-Val de Loire.

Acknowledgments: S.D. and L.G. acknowledge research fellowships of Loire Valley Region. D.T. gratefully acknowledges the support of French government via the French Embassy in Thailand in the form of Junior Research Fellowship Program 2018. B.H.A. gratefully acknowledges the support of Cosmetosciences and Le Studium Institute for Advance Study International Research Fellow funded by Region Centre-Val de Loire.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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