Food Analytical Methods (2018) 11:1977–1985
https://doi.org/10.1007/s12161-018-1159-4

Fingerprinting *Cynara scolymus* L. (Artichoke) by Means of a Green Statistically Developed HPLC-PAD Method

Otávio Aguiar Souza[1] · Renato Lajarim Carneiro[2] · Thiago Henrick Martins Vieira[1] · Cristiano Soleo Funari[3] · Daniel Rinaldo[1]

Received: 27 September 2017 / Accepted: 14 January 2018 / Published online: 29 January 2018

Abstract
The process to develop a chromatographic method for fingerprinting complex matrices should be performed through a multiparameter approach that could lead to the desired separation and save environmental resources such as organic solvents and energy. In other words, this process should be pursued by employing an optimized experimental design and having a response function which takes into consideration separation parameters together with environmental parameters. Green Analytical Chemistry principles should be pursued during all steps of the research. This work presents a heuristic approach to develop a high-performance liquid chromatography method for fingerprinting an extract from leaves of *Cynara scolymus* L., a food plant consumed worldwide. A fractional factorial design was used to identify relevant chromatographic variables followed by a comprehensive design for optimization purposes (Doehlert design). A response function called green chromatographic fingerprinting response was employed to obtain a compromise between fingerprint quality and low environmental impact of the method. This optimized approach led to the development of a robust and green method for fingerprinting *C. scolymus* by HPLC-PAD. This method proved to be greener than the reference method reported in literature and compatible even with no state of art HPLC instruments because the system backpressure did not exceed 15 MPa and the column temperature was 35 °C.

Keywords Metabolic fingerprinting · Green analytical chemistry · Experimental design · Doehlert · Optimization · Green liquid chromatography

Introduction

Artichoke (*Cynara scolymus* L.) is an herbaceous plant mainly cultivated in Europe, where its fleshy leaves and receptacle (known as "heads") are an important ingredient of the Mediterranean diet, and it has been considered as an Intangible Cultural Heritage of Humanity. It also has been cultivated for over a century in South America, where its production is increasing (López-Molina et al. 2005; Lutz et al. 2011; Unesco 2010; Romani et al. 2006). The artichoke heads (fleshy leaves and receptacle) are consumed fried, boiled, and/or steamed, and used in many recipes due to its pleasant taste. It is a source of proteins, minerals, dietary fibers, and fructo-oligosaccharides, etc. (Romani et al. 2006; Zhu et al. 2004; Pandino et al. 2011; Wu et al. 2013).

Additionally, artichoke leaf extract has been used in folk medicine for centuries, mainly due to its choleretic, diuretic, and hypocholesterolemic activities (Rodriguez et al. 2002; Fritsche et al. 2002; Bundy et al. 2008). These traditional uses have been supported by scientific investigations over the years. Antimicrobial, antioxidant, antiglycemic, and digestive properties etc. have been reported for this plant (Zhu et al. 2004; Zapolska-Downar et al. 2002; Fantini et al. 2011; Marakis et al. 2002; Holtmann et al. 2003). Its pharmacological activities have been associated with compounds such as caffeoylquinic acids (e.g., 1,3-Di-*O*-caffeoylquinic acid, or cynarin), flavonoids (e.g., luteolin-7-rutinoside), and sesquiterpene lactones (e.g., cynaropicrin) (Zhu et al. 2004; Bundy et al. 2008). A variety of pharmaceutical preparations and food

✉ Daniel Rinaldo
danielrinaldo@fc.unesp.br

[1] School of Sciences, São Paulo State University (UNESP), 14-01 Engenheiro Luiz Edmundo Carrijo Coube Ave., Vargem Limpa, Bauru, São Paulo 17033360, Brazil

[2] Department of Chemistry, Federal University of São Carlos (UFSCar), Washington Luís highway (SP-310 km 235), São Carlos, São Paulo 13565905, Brazil

[3] School of Agricultural Sciences, São Paulo State University (UNESP), 1879 Doutor José Barbosa de Barros St., Jardim Paraíso, Botucatu, São Paulo 18610307, Brazil

supplements containing artichoke leaves and flowers are offered on the market (Schütz et al. 2006).

The development of safe and efficient medicines from plants is a difficult task because plant materials can have multiple compounds that can even work synergistically (Liang et al. 2004; Liang et al. 2010; Qian et al. 2007; Xie et al. 2007). A multicomponent approach should be preferred over a single component one for standardizing and quality controlling a medicinal plant and its derived products. A proper chromatographic fingerprint is suggested by agencies such as the US Food and Drug Administration (FDA), the European Medicines Evaluation Agency (EMEA), and even World Health Organization for this purpose (Li et al. 2010; The European Agency for the Evaluation of Medicinal Products 2006; Tistaert et al. 2011; US Food and Drug Administration 2004; World Health Organization 1991).

Thus, development of a chromatographic method for quality control of a medicinal plant should be driven by a heuristic approach, and design of experiments (DoE) can fit this goal. As opposed to a univariate (or trial-and-error) proposal, DoE is a multiparameter approach that performs simultaneous, impartial, and systematic evaluation of all factors of interest, where the number of experiments is minimized while maximum information is obtained from experimental data, saving time and resources. DoE allows researchers to identify interactions among the factors and to build useful response surfaces to predict the optimal experimental conditions (Myers et al. 2016; Montgomery 2017). The selection of the factors to be evaluated, the response function to be monitored, and even the experimental design itself should consider green analytical chemistry (GAC) concepts and principles (Gałuszka et al. 2013). In other words, a resource-saving approach should be pursued from the method development process.

This work aimed to offer an efficient, green, and affordable HPLC-PAD method for fingerprinting extracts of leaves of *C. scolymus*. For that, (1) a multiparameter and multianalyte approach was adopted, (2) the design of experiments was rigorously selected to save energy and solvent during all the method development process, (3) ethanol was selected as the organic modifier, (4) standard HPLC conditions were employed during all method development to be compatible even with non-state-of-the-art instruments, and (5) the environmental performance of the method was compared with a reference method previously reported.

Experimental

Chemicals and Reagents

EtOH (HPLC grade; Tedia®, USA), ultrapurified water (Millipore®, USA), and acetic acid (AcOH, AR grade; Synth®, Brazil) were used as mobile-phase components and extraction media in this work.

Plant Material

Authentic leaves of *C. scolymus* were kindly provided by Centroflora Group (Lot 200112.122), located at the city of Botucatu, SP, Brazil.

Extraction and Concentration

A portion of approximately 10 g of dry *C. scolymus* leaves was extracted by maceration with three aliquots of 100 mL of the binary mixture of EtOH/H_2O (7:3, *v*/*v*) at 40 °C, with constant stirring and replacement of the extractive solution every 48 h, with a total extraction time of 144 h. The fluid solutions were collected and concentrated at 40 °C by an R-300 rotary evaporator (Buchi®, Switzerland) to obtain the dried hydroalcoholic extract of *C. scolymus* leaves.

Pre-Treatment Using Solid Phase Extraction (SPE)

Samples were treated before injection in the HPLC system by solid phase extraction (Sep-Pak C_{18} cartridge, Strata-E, 500 mg/3 mL; Phenomenex®, USA). The stationary phase was activated with 6 mL of EtOH and equilibrated with 6 mL of EtOH/H_2O (7:3, *v*/*v*). The equilibrated stationary phase was then loaded with 50 mg of the hydroethanolic dry extract of *C. scolymus* leaves (HECL) and eluted with 2 mL of the equilibrating solution in order to eliminate chlorophylls and other low-polarity compounds. This procedure was adopted because the objective was to obtain a selective fingerprint of the high and middle polarity compounds with phytotherapeutic properties. The sample at a concentration of 25 mg mL^{-1} was filtered through a PTFE filter (0.45 μm, 25 mm; Phenomenex®, USA).

High-Performance Liquid Chromatography Analyses

HPLC analyses were performed using an HPLC-PAD apparatus (Jasco®, Japan), equipped with quaternary pump (model PU-2089 plus), photodiode array detector (model MD-2010 plus), automatic injector (model AS-2055 plus), and column oven (model CO-2060 plus). Separations were achieved in a C_{18} column (XBridge, 150 × 4.60 mm i.d. × 5 μm; Waters®, Ireland) coupled to a C_{18} guard column (4 × 3 mm, 5 μm; Phenomenex®, USA). The optimized method employed 0.5% acetic acid aqueous solution (A) and EtOH (B) at 1 mL min^{-1} and 35 °C at the following gradient: 5–50% B (0–30 min); 50–100% B (30–31 min); 100% B (41 min). Equilibration of the stationary phase was achieved with 5% B for 30 min. A 2-μL preheater (Thermo Fischer Scientific®, USA) was coupled to the pre-column and kept in the column

oven. Injection volume and column equilibration time were fixed at 20 μL and 30 min, respectively. Chromatographic data were accessed, processed, and handled employing ChromNAV (Jasco®, Japan) and Openchrom® (Lablicate GmbH, Germany) software. Statistical analyses, validation of the empirical models, normal probability, and response surface plots were performed using GNU Octave 4.2.1, Matlab 2010a (Mathworks®, USA), Microcal Origin 6 (OriginLab®, USA), and LibreOffice 5 (The Document Foundation, USA) software.

Multivariate Analyses—Fractional Factorial Design and Doehlert Design

Initially, five chromatographic factors were screened by means of a fractional factorial design ($2v^{5-1}$) (Table 1). Later, the statistically significant factors were inserted in a Doehlert design (Table 2) with the aim of finding a mathematical model that could indicate an optimal point. Two responses were monitored: number of peaks (n) and green chromatographic fingerprinting response (GCFR, Eq. 1).

$$GCFR = n^2(FP/MP)(n/t) \quad (1)$$

where n is the total number of peaks of the chromatogram, t is the total chromatographic run time, FP is the number of peaks in the half-part of the chromatogram with fewer number of peaks, and MP is the number of peaks in the other half-part of the chromatogram with more peaks. The higher the GCFR score, the better by means of global chromatographic optimization. Further information about GCFR is provided elsewhere (Funari et al. 2014; Ji et al. 2005). Although any manifestation with signal/noise (S/N) higher than 3 is considered a peak in HPLC, only those with S/N higher than 100 (Openchrom® software) were taken into consideration for both responses monitored in this work because this ratio allowed UV spectra to be recorded, an important parameter in this work.

Table 1 Factors and levels investigated in Fractional Factorial Design on Screening Variables

Factors	Levels		
	−1	0	+1
X1. Initial % of EtOH	5	12.5	20
X2. Final % of EtOH	25	37.5	50
X3. Temperature of analysis (°C)	35	57.5	80
X4. % acetic acid in H_2O	0	0.25	0.5
X5. Flow rate (mL min^{-1})	0.6	0.8	1.0

Measuring the Environmental Impact of the Optimized Method

A metric called Environmental Assessment Tool (HPLC-EAT, Eq. 2) was employed to estimate the environmental impact of the method compared with other methods described in the literature for similar purposes (Gaber et al. 2011).

$$HPLC\text{−EAT} = S1m1 + H1m1 + E1m1 + S2m2 + H2m2 + E2m2 + \ldots + Snmn + Hnmn + Enmn \quad (2)$$

where S, H, and E refers to a score attributed to each solvent $(1-n)$ based on the impact on safety, health, and environment, respectively. Each score is multiplied by the mass (m) of the solvent. Detailed information is provided elsewhere (Gaber et al. 2011). The HPLC-EAT final score for a method is easily calculated with free software provided by the authors (Gaber et al. 2011). For this metric, a higher score indicates a high environmental impact; therefore, the lower the score, the better.

Validation of the Optimized Method

The validation of the optimized method followed International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Harmonised Tripartite Guideline 2005) until repeatability analysis. The monitored parameters were selected based on those proposed by Funari et al. (2014) for complex matrices. Thus, the quality of the method was checked by taking the relative standard deviation (RSD) among (1) the total peak areas of the chromatograms and (2) the ratios between the retention time of any peak with an area > 4% of the total peak area and the retention time observed for a reference peak (indicated with an asterisk in Fig. 3).

Results

Screening Variables—Fractional Factorial Design $2v^{5-1}$

According to normal probability plots (Fig. 1), variables X1, X3, and X4 proved to be the most important variables for number of peaks response, whereas the variables X1, X3, and X5 proved to be the most important ones for GCFR response. Although X5 did not present high significance in the normal probability plot for GCFR (Fig. 1b), a statistical evaluation of results (ANOVA) shows that this variable could be influential in the model; therefore, it was kept for the Doehlert

Table 2 Four-factor Doehlert design factors and levels investigated with the following results

Run	Factors[a,c]				Terms of Eq. 1					Results[d]	
	X5	X1	X3	X4	n^2	FP	MP	FP/MP	n/t	n	GCFR
1	1 (1.0)	0 (12.5)	0 (57.5)	0 (0.25)	81	0	9	0	0.30	9	0.00
2	0.5 (0.9)	0.87 (20.0)	0 (57.5)	0 (0.25)	64	0	8	0	0.27	8	0.00
3	0.5 (0.9)	0.29 (15.0)	0.82 (80.0)	0 (0.25)	100	0	10	0	0.33	10	0.00
4	0.5 (0.9)	0.29 (15.0)	0.2 (63.1)	0.79 (0.50)	100	0	10	0	0.33	10	0.00
5	− 1 (0.6)	0 (12.5)	0 (57.5)	0 (0.25)	169	2	11	0.18	0.43	13	13.32
6	− 0.5 (0.7)	− 0.87 (5.0)	0 (57.5)	0 (0.25)	324	3	15	0.2	0.6	18	38.88
7	− 0.5 (0.7)	− 0.29 (10.0)	− 0.82 (35.0)	0 (0.25)	324	4	14	0.29	0.6	18	55.54
8	− 0.5 (0.7)	− 0.29 (10.0)	− 0.2 (51.0)	− 0.79 (0)	225	2	13	0.15	0.5	15	17.31
9	0.5 (0.9)	− 0.87 (5.0)	0 (57.5)	0 (0.25)	289	2	15	0.13	0.57	17	21.84
10	0.5 (0.9)	− 0.29 (10.0)	− 0.82 (35.0)	0 (0.25)	361	4	15	0.27	0.63	19	60.97
11	0.5 (0.9)	− 0.29 (10.0)	−0.2 (51.9)	− 0.79 (0)	196	2	12	0.17	0.47	14	15.24
12	− 0.5 (0.7)	0.87 (20.0)	0 (57.5)	0 (0.25)	64	0	8	0	0.27	8	0.00
13	0 (0.8)	0.58 (17.5)	− 0.82 (35.0)	0 (0.25)	256	2	14	0.14	0.53	16	19.50
14	0 (0.8)	0.58 (17.5)	− 0.2(51.9)	− 0.79 (0)	81	0	9	0	0.30	9	0.00
15	− 0.5 (0.7)	0.29 (15.0)	0.82 (80.0)	0 (0.25)	121	0	11	0	0.37	11	0.00
16	0 (0.8)	− 0.58 (7.5)	0.82 (80.0)	0 (0.25)	121	1	10	0.10	0.37	11	4.44
17	0 (0.8)	0 (12.5)	0.61 (74.4)	− 0.79 (0)	81	0	9	0	0.30	9	0.00
18	− 0.5 (0.7)	0.29 (15.0)	0.2 (63.1)	0.79 (0.50)	121	0	11	0	0.37	11	0.00
19	0 (0.8)	− 0.58 (7.5)	0.2 (63.1)	0.79 (0.50)	169	2	11	0.18	0.43	13	13.32
20	0 (0.8)	0 (12.5)	− 0.61 (40.6)	0.79 (0.50)	196	2	12	0.17	0.47	14	15.24
CP1[b]	0 (0.8)	0 (12.5)	0 (57.5)	0 (0.25)	121	1	10	0.10	0.37	11	4.44
CP2[b]	0 (0.8)	0 (12.5)	0 (57.5)	0 (0.25)	100	1	9	0.11	0.33	10	3.70
CP3[b]	0 (0.8)	0 (12.5)	0 (57.5)	0 (0.25)	121	1	10	0.10	0.37	11	4.44
CP4[b]	0 (0.8)	0 (12.5)	0 (57.5)	0 (0.25)	144	1	11	0.09	0.40	12	5.24

[a] X5, flow rate (mL min^{-1}); X1, initial percentage of EtOH; X3, temperature (°C); X4, percentage of acetic acid on water

[b] Central point and correspondent replicate number

[c] Codified values are given without brackets and their following experimental values are indicated in brackets

[d] Collected at 350 nm

design. Variable X2 does not appear as significant, so it was fixed at 50% of EtOH for the next step. Using these four selected variables, linear models were calculated and the analysis of variance (ANOVA) at 95% of confidence revealed the R^2 value (coefficient of determination) of 0.92 and 93.8% of explainable variance for number of peaks response and R^2 of 0.90 and 92.1% of explainable variance for GCFR response.

Although the linear models did not find lack of fit, this type of design is employed as an initial step of investigation, as it does not allow evaluation of the curvature of the

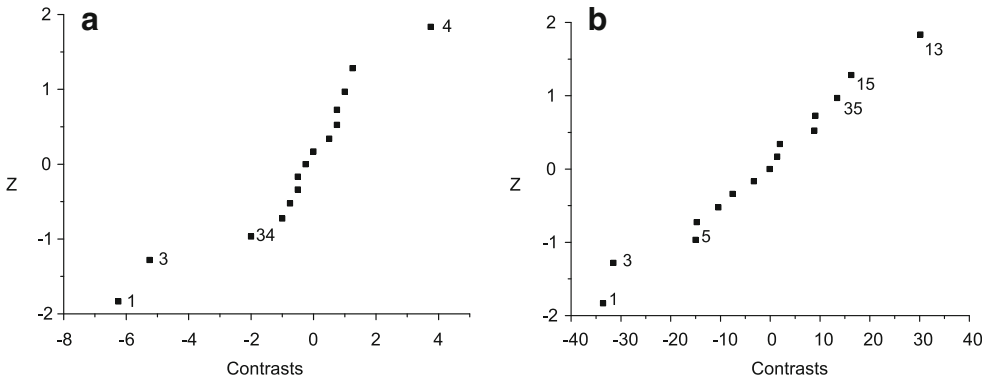

Fig. 1 Normal probability plots for contrasts regarding **a** number of peaks and **b** GCFR responses in fractional factorial design

response surface. This kind of model is employed mainly to identify the relevant variables.

Optimization of Chromatographic Conditions by Doehlert Design

Once the four most relevant variables were determined, a Doehlert design was applied to optimize the chromatographic conditions. The design itself as well as the outputs can be found in Table 2. From this set of experiments, the variables X4 and X5 were revealed to be not significant at 95% of confidence. Thus, the following mathematical models were built as a function of initial percentage of *B* (X1), the temperature of the chromatographic run (X3) for number of peaks (*n*, Eq. 3), and green chromatographic fingerprinting response (GCFR, Eq. 4):

$$n = 11.68_{(\pm 0.75)} - 4.56_{(\pm 1.31)}x_1 - 4.20_{(\pm 1.31)}x_3 + 3.34_{(\pm 2.12)}x_3^2 \quad (3)$$

$$GCFR = -18.92_{(\pm 5.65)}x_1 - 24.12_{(\pm 5.65)}x_3 + 21.57_{\pm (8.06)}x_1^2 + 29.02_{(\pm 7.58)}x_3^2 + 32.27_{(\pm 15.37)}x_{13} \quad (4)$$

R^2 and maximum explainable variance for Eq. 3 were 0.84 and 95.3% and for Eq. 4 were 0.91 and 96.7%. In addition, experimental *F* values for number of peaks (*n*) model were 35.89 (regression/residual) and 2.31 (lack of fit/pure error), with critical values of 3.10 ($F_{3,20,95\%}$) and 2.98 ($F_{10,10,95\%}$), respectively. For GCFR model, experimental *F* values were 48.69 (regression/residual) and 2.31 (lack of fit/pure error), whereas critical values were 2.90 ($F_{4,19,95\%}$) and 2.95 ($F_{8,11,95\%}$), respectively, considering the degrees of freedom and pure error related to replicates in central point and additional replicates obtained after the elimination of non-significant coefficients (Pereira Filho 2015). These *F* tests showed that the regression is statistically significant because the experimental *F* (regression/residual) is around 12 and 17 times the critical value for *n* and GCFR responses, respectively. No lack of fit was observed for both models since $F_{\text{values}} < F_{\text{critical}}$. Response surfaces were built from the Eqs. 3 and 4 (Fig. 2). They predicted a common optimal for number of peaks and GCFR with initial percentage of EtOH (X1) and temperature of analysis (X3) at 5% and 35 °C, respectively. This should lead to 21 peaks and a GCFR score of 95.09. Thus, the whole chromatographic condition tested was 5–50% of EtOH in 30 min, at 1 mL min^{-1} and at 35 °C. The percentage of acetic acid in water was fixed at 0.5% (*v*/*v*). This condition was applied in triplicate, and the observed experimental values were 19.33 ± 0.58 peaks and a GCFR score of 92.53 ± 18.8, which are very close to the predicted values. A representative chromatogram for the optimum point is shown in Fig. 3.

Validation of the Optimized Method

Instrumental Precision

The instrumental precision was evaluated with nine consecutive injections of the same sample at the concentration of 25 mg mL^{-1} and the same vial (ICH Harmonised Tripartite Guideline 2005). The RSD of the total peak area was 3.83%, whereas the maximum value for RSD regarding relative retention time was 0.58%.

Repeatability

Repeatability was also determined using nine consecutive injections, varying sample concentrations in three levels and three replicates for each level. Three samples with three different concentrations of vegetal material (5, 15, and 25 mg mL^{-1}) from HECL were prepared and each one of those, in three different vials, were injected three times (ICH Harmonised Tripartite Guideline 2005). The maximum RSD of the total peak area was 2.77%, whereas the maximum RSD observed for relative retention time was 1.01%.

Discussion

Fractional factorial design was able to indicate four statistically relevant variables in a reduced number of experiments. The Doehlert design led to satisfactory mathematical models for both responses (number of peaks and GCFR). The common predicted optimal point was later experimentally confirmed. The optimal GCFR found increased 52% relative to the best result obtained for the experiments circumvented in the original experimental design (run 10, Table 2). This result confirmed the high quality of the mathematical models.

From the original set of experiments (Table 2), it became clear that the synergistic interaction between X1 and X3 strongly influenced the distribution of the peaks across the chromatogram (second term of GCFR, Eq. 1) as well as the number of peaks per time (third term of GCFR, Eq. 1). From Table 2, the highest values for these terms were obtained when X1 and X3 were investigated in the lowest levels. The best scores for both monitored responses (run 10 followed by the runs 7 and 6, Table 2) were achieved when factors X1 or X3 was investigated at − 0.87 and − 0.82, respectively, or 5% of ethanol and 35 °C of oven temperature.

The fingerprint of HECL (Fig. 3) at 350 nm was found to be dominated by compounds with UV spectra typical of compounds derived from caffeic acid (peaks 1, 2, 4, and 5) and flavones (peaks 3 and 6), which are classes of substances that have already been reported for *C. scolymus* (Negro et al. 2012). This was important as the reported pharmacological activities for this species rely on compounds belonging to

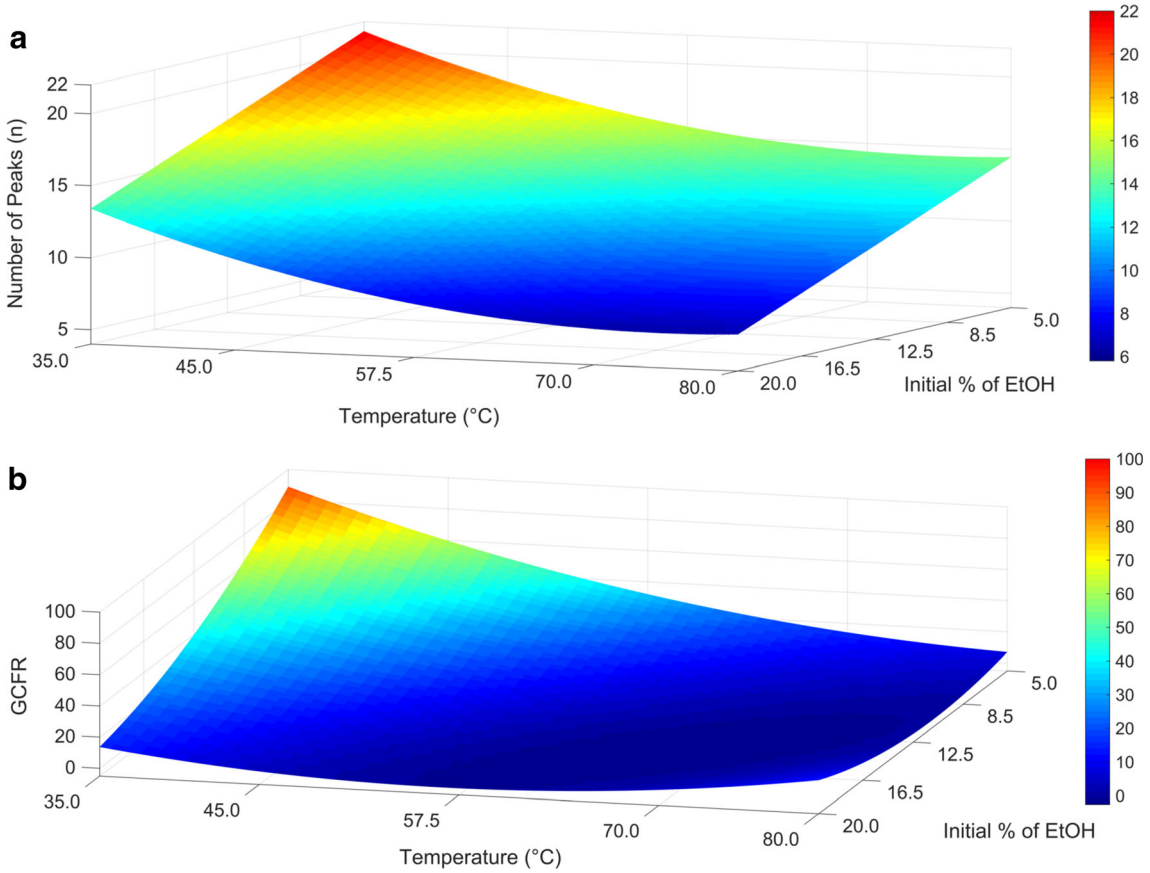

Fig. 2 Response surface plots for **a** number of peaks and **b** GCFR responses at Doehlert design

these classes. With regard to the separation and resolution of the peaks, it is important to emphasize that the developed fingerprint has qualitative purposes. In this way, a degree of overlapping and/or non-Gaussian normal distribution for peaks are accepted if the researcher is able to successfully access the chemical complexity of the sample and handle the related data regarding all detected peaks (Alaerts et al. 2010; Ji et al. 2005; Liang et al. 2010; Klein and Rivera 2000; Gong et al. 2003). In fact, it was possible to obtain a good selectivity, considering that we were able to analyze the UV spectrum of each peak in all samples analyzed in the validation, including in the evaluation of the repeatability parameter, where the sample concentration varied from 5 to 25 mg mL^{-1} and the chromatographic profile did not present changes. In this context, the minor separation factor and resolutions for the developed fingerprint in relation to the reference method did not damage selectivity.

Regarding environmental aspects, Doehlert design is a type of DoE that is considered one of the best alternatives to obtain mathematical model. The four-factor Doehlert design uses a reduced number of experiments compared to central composite design and Box–Behnken. The disadvantage is that this lower number of experiments decreases the number of degrees of freedom for residuals. However, in our case, this was not a problem since we had at least 19 degrees of freedom for residuals. Thus, the selection of a fractional factorial design for screening variables and a Doehlert design for optimization purposes are in accordance with three principles of GAC, which state that the generation of a large volume of analytical waste should be avoided (principle 7), that a multianalyte (both response functions employed fit this point) or multiparameter methods are preferred (principle 8), and that the use of energy should be minimized (principle 9).

The selection of ethanol and acetic acid as organic modifiers instead of the widely used acetonitrile and methanol plus formic or trifluoroacetic acid was in accordance with other three principles of green chemistry which state that reagents obtained from renewable sources should be preferred (principle 10), toxic reagents should be eliminated or replaced (principle 11), and that the safety of the operator should be increased (principle 12).

The multivariate quadratic models (Eqs. 3 and 4) indicated that the lowest investigated levels for initial percentage of the organic solvent (X1) and temperature of analysis (X3) should lead to the best chromatographic fingerprint according to both monitored responses. These also fit principles 7 and 9 of GAC mentioned before. The variable X1 presented a high significance for the optimization of the chromatographic separation for both monitored responses because it is directly related to the force of elution of the mobile phase (Snyder et al. 1997). In

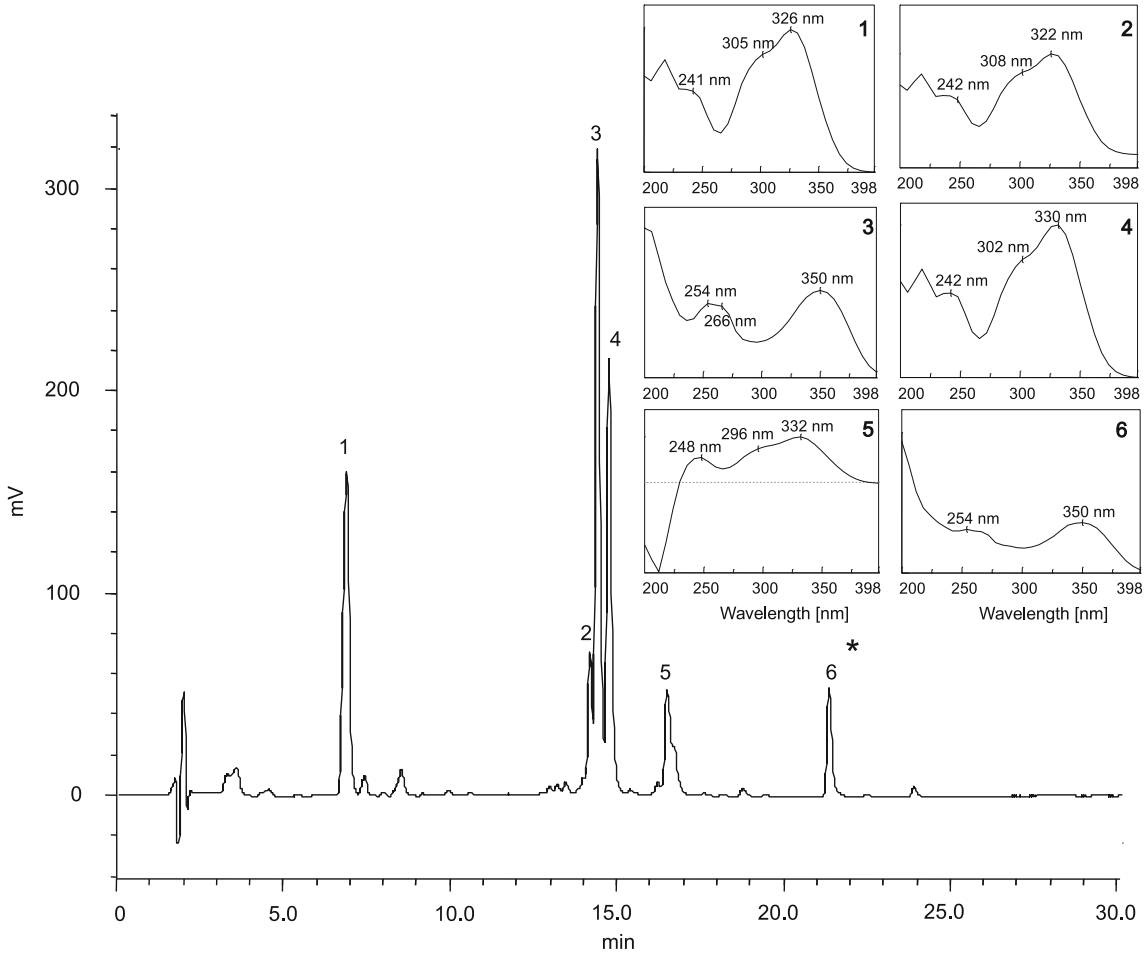

Fig. 3 HPLC-PAD fingerprint of *C. scolymus* (HECL) at $\lambda = 350$ nm. Column: XBridge, 150 × 4.6 mm. Mobile phases: H_2O + 0.5% acetic acid (A) and EtOH (B) at the following gradient: 5–50% B in 30 min. Flow rate: 1 mL min^{-1}. Temperature of analysis: 35 °C; injection volume: 20 μL. UV spectra of the major peaks are provided over the chromatogram

this context, the higher investigated experimental value for X1, the higher is the eluotropic force solvent system as mobile phase and the lower the number of peaks and GCFR score. Regarding the variable X3, where higher experimental values are investigated in the runs, there occurs a decrease in viscosity, changes in dielectric constant and pH of the mobile phase, as well as the arrangement of the stationary phase ligands and diffusion of the analytes (Heinisch and Rocca 2009; Dolan 2002; Snyder et al. 1997). As a consequence, a higher dissolution of the analytes in the solvents and lower interaction with the stationary phase (decrease in the amount of analyte equilibria between the mobile and stationary phases) occur, promoting co-elution of the peaks. This phenomenon also decreases the responses *n* and GCFR.

On the other hand, the levels of variables X4 and X5 were fixed at 0.5% of acetic acid (A) and 1 mL min^{-1}, which were not the best levels from a green chemistry standpoint. From this perspective, 0% acetic acid and 0.6 mL min^{-1} would be preferred, but the decision for 0.5% acetic acid as A and 1 mL min^{-1} was made taking into consideration the improvement of the peak shapes (avoiding ionization of acidic analytes) and resolutions to values considered satisfactory to UV spectra recording (e.g. run 11 in fractional factorial design and the tested possible optimum points). It is well known that employing an acid in the mobile phase can avoid ionization of acidic analytes, as expected for HECL, and, thus, improve peak shapes and resolution (Snyder et al. 1997).

A simple and freely available tool called HPLC-EAT (Eq. 2) was employed to quantify the environmental impact of the method proposed here for the fingerprint of *C. scolymus* (Gaber et al. 2011) compared to the reference method reported in literature by Negro et al. (2012) for a similar purpose. This method was selected as a reference method among other ones available in the literature because in our opinion it presents the best compound separation, resolution, peak shape, and online UV spectra, employing acetonitrile (MeCN) as the mobile-phase organic modifier, which is the most used organic solvent in HPLC (thus, MeCN can be considered a reference solvent in reverse phase liquid chromatography). In previous calculation, the pretreatment of the sample was taken into

consideration. The method proposed here and the reference method presented scores of 19.6 and 81.0, respectively, which evidenced the greenness of the method proposed here compared to the reference method. That can be mainly explained by the favorable scores of EtOH when compared with MeCN regarding safety, health, and environmental impacts (Gaber et al. 2011). EtOH is considered a greener alternative to HPLC due to its relatively low toxicity and to its biodegradability. MeCN is strongly undesired from the green analytical chemistry standpoint since it is toxic to mammals and aquatic life (Hutchinson et al. 2012; Fritz et al. 2009). A minor impact on HPLC-EAT scores was due to organic solvent consumption in the methods under comparison. The method proposed here consumed 20.5 mL of EtOH, whereas the reference method consumed 23.2 mL of MeCN.

Later, the ratios between GCFR and HPLC-EAT were also calculated for both methods. This ratio considers parameters of separation given by GCFR with environmental parameters given by HPLC-EAT, thus providing a more heuristic view of the process of separation (Funari et al. 2014). According to this hybrid metric, the higher the score, the better. Therefore, it was applied for both methods under comparison, leading to 4.72 ± 0.20 and 0.02 ± 0.00 for the method proposed here and the reference method, respectively.

Regarding the instrumental precision and repeatability, RSD values compatible with good methods for fingerprinting complex samples were observed (Funari et al. 2014).

Conclusion

This work showed an efficient and less impactful way to obtain a chromatographic fingerprint of a *C. scolymus* leaf extract. This is because the method developed here was obtained from a multiparameter approach and considered separation and environmental parameters at the same time. Throughout the development process, lean experimental designs were selected, as well as a low toxicity and biodegradable organic modifier (ethanol). Although EtOH is more viscous than acetonitrile (the organic solvent employed in the reference method), this showed to be compatible even with non-state-of-the-art instruments since it does not exceed 15 MPa of backpressure and the temperature for analysis is very close to room temperature. It shows that the migration of HPLC methods for greener ones did not require changes in the HPLC instrumentation itself, but it requires a change from a traditional method development approach to a more heuristic one, where the concept of performance considers parameters of separation in conjunction with environmental parameters.

Acknowledgements The authors would like to thank the São Paulo Research Foundation (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support (grants 15-20639/1, 16/08179-8, and 45398 2014-5). The authors also thank Dr. Valdecir Farias Ximenes of the Department of Chemistry of São Paulo State University for the use of the HPLC-PAD instrument.

Funding This study was funded by the São Paulo Research Foundation (FAPESP) (grants 15/20639-1, 16/08179-8) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grant 45398 2014-5).

Compliance with Ethical Standards

Conflict of Interest Otávio Aguiar Souza declares that he has no conflict of interest. Renato Lajarim Carneiro declares that he has no conflict of interest. Thiago Henrick Martins Vieira declares that he has no conflict of interest. Cristiano Soleo Funari declares that he has no conflict of interest. Daniel Rinaldo declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

References

Alaerts G, Dejaegher B, Smeyers-Verbeke J, Heyden YV (2010) Recent developments in chromatographic fingerprints from herbal products: set-up and data analysis. Comb Chem High Throughput Screen 13:900–922. https://doi.org/10.2174/138620710793360284

Bundy R, Walker AF, Middleton RW, Wallis C, Simpson HCR (2008) Artichoke leaf extract (*Cynara scolymus*) reduces plasma cholesterol in otherwise healthy hypercholesterolemic adults: a randomized, double blind placebo controlled trial. Phytomedicine 15:668–675. https://doi.org/10.1016/j.phymed.2008.03.001

Dolan JW (2002) Temperature selectivity in reversed-phase high performance liquid chromatography. J Chromatogr A 965:195–205. https://doi.org/10.1016/S0021-9673(01)01321-8

Fantini N, Colombo G, Giori A, Riva A, Morazzoni P, Bombardelli E, Carai MAM (2011) Evidence of glycemia-lowering effect by a *Cynara scolymus* L. extract in normal and obese rats. Phytoter Res 25:463–466. https://doi.org/10.1002/ptr.3285

Fritsche J, Beindorff CM, Dachtler M, Zhang H, Lammers JG (2002) Isolation, characterization and determination of minor artichoke (Cynara scolymus L.) leaf extract compounds. Eur Food Res Technol 215:149–157. https://doi.org/10.1007/s00217-002-0507-0

Fritz R, Ruth W, Kragl U (2009) Assessment of acetone as an alternative to acetonitrile in peptide analysis by liquid chromatography/mass spectrometry. Rapid Commun Mass Spectrom 23:2139–2145. https://doi.org/10.1002/rcm.4122

Funari CS, Carneiro RL, Andrade AM, Hilder EF, Cavalheiro AJ (2014) Green chromatographic fingerprinting: an environmentally friendly approach for the development of separation methods for fingerprinting complex matrices. J Sep Sci 37:37–44. https://doi.org/10.1002/jssc.201300955

Gaber Y, Törnvall U, Kumar MA, Amin MA, Hatti-Kaul R (2011) HPLC-EAT (environmental assessment tool): a tool for profiling safety, health and environmental impacts of liquid chromatography methods. Green Chem 13:2021–2025. https://doi.org/10.1039/c0gc00667j

Gałuszka A, Migaszewski Z, Namieśnik J (2013) The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices. Trends. Anal Chem 50:78–84. https://doi.org/10.1016/j.trac.2013.04.010

Gong F, Liang YZ, Xie PS, Chau FT (2003) Information theory applied to chromatographic fingerprint of herbal medicine for quality control. J Chromatogr A 1002:25–40. https://doi.org/10.1016/S0021-9673(03)00648-4

Heinisch S, Rocca JL (2009) Sense and nonsense of high-temperature liquid chromatography. J Chromatogr A 1216:642–658. https://doi.org/10.1016/j.chroma.2008.11.079

Holtmann G, Adam B, Haag S, Collet W, Grünewald E, Windeck T (2003) Efficacy of artichoke leaf extract in the treatment of patients with functional dyspepsia: a six-week placebo-controlled, double-blind, multicentre trial. Aliment Pharmacol Ther 18:1099–1105. https://doi.org/10.1046/j.1365-2036.2003.01767.x

Hutchinson JP, Remenyi T, Nesterenko P, Farrell W, Groeber E, Szucs R, Dicinoski G, Haddad PR (2012) Investigation of polar organic solvents compatible with corona charged aerosol detection and their use for the determination of sugars by hydrophilic interaction liquid chromatography. Anal Chim Acta 750:199–206. https://doi.org/10.1016/j.aca.2012.04.002

ICH Harmonised Tripartite Guideline (2005) Validation of analytical procedures: text and methodology Q2(R1). ICH. https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. Accessed 12 July 2017

Ji Y, Xu Q, Hu Y, Heyden YV (2005) Development, optimization and validation of a fingerprint of *Ginkgo biloba* extracts by high performance liquid chromatography. J Chromatogr A 1066:97–104. https://doi.org/10.1016/j.chroma.2005.01.035

Klein EJ, Rivera SL (2000) A review of criteria functions and response surface methodology for the optimization of analytical scale HPLC separations. J Liq Chromatogr Relat Technol 23:2097–2121. https://doi.org/10.1081/JLC-100100475

Li Y, Wu T, Zhu J, Wan L, Yu Q, Li X, Cheng Z, Guo C (2010) Combinative method using HPLC fingerprint and quantitative analyses for quality consistency evaluation of an herbal medicinal preparation produced by different manufacturers. J Pharm Biomed Anal 52:597–602. https://doi.org/10.1016/j.jpba.2010.01.018

Liang Y, Xie P, Chan K (2004) Quality control of herbal medicines. J Chromatogr B 812:53–70. https://doi.org/10.1016/j.jchromb.2004.08.041

Liang Y, Xie P, Chau F (2010) Chromatographic fingerprinting and related chemometric techniques for quality control of traditional Chinese medicines. J Sep Sci 33:410–421. https://doi.org/10.1002/jssc.200900653

López-Molina D, Navarro-Martínez MD, Rojas-Melgarejo F, Hiner ANP, Chazarra S, Rodríguez-López JN (2005) Molecular properties and prebiotic effect of inulin obtained from artichoke (*Cynara scolymus* L.) Phytochemistry 66:1476–1484. https://doi.org/10.1016/j.phytochem.2005.04.003

Lutz M, Henríquez C, Escobar M (2011) Chemical composition and antioxidant properties of mature and baby artichokes (*Cynara scolymus* L.), raw and cooked. J Food Compos Anal 24:49–54. https://doi.org/10.1016/j.jfca.2010.06.001

Marakis G, Walker AF, Middleton RW, Booth JCL, Wright J, Pike DJ (2002) Artichoke leaf extract reduces mild dyspepsia in an open study. Phytomedicine 9:694–699. https://doi.org/10.1078/094471102321621287

Montgomery DC (2017) Design and analysis of experiments. John Wiley & Sons, Inc., New York

Myers RH, Montgomery DC, Anderson-Cook CM (2016) Response surface methodology: process and product optimization using designed experiments. John Wiley & Sons, Inc., New York

Negro D, Montesano V, Grieco S, Crupi P, Sarli G, Lisi A, Sonnante G (2012) Polyphenol compounds in artichoke plant tissues and varieties. J Food Sci 77:244–252. https://doi.org/10.1111/j.1750-3841.2011.02531.x

Pandino G, Lombardo S, Mauromicale G, Williamson G (2011) Profile of polyphenols and phenolic acids in bracts and receptacles of globe artichoke (*Cynara cardunculus* var. *scolymus*) germplasm. J Food Compos Anal 24:148–153. https://doi.org/10.1016/j.jfca.2010.04.010

Pereira Filho ER (2015) Planejamento fatorial em química: maximizando a obtenção de resultados. EdUfscar, São Carlos

Qian G, Wang Q, Leung KS, Qin Y, Zhao Z, Jiang Z (2007) Quality assessment of Rhizoma et radix Notopterygii by HPTLC and HPLC fingerprinting and HPLC quantitative analysis. J Pharm Biomed Anal 44:812–817. https://doi.org/10.1016/j.jpba.2007.03.029

Rodriguez TS, Giménez DG, Vásquez RP (2002) Choleretic activity and biliary elimination of lipids and bile acids induced by an artichoke leaf extract in rats. Phytomedicine 9:687–693. https://doi.org/10.1078/094471102321621278

Romani A, Pinelli P, Cantini C, Cimato A, Heimler D (2006) Characterization of Violetto di Toscana, a typical Italian variety of artichoke (*Cynara scolymus* L.) Food Chem 95:221–225. https://doi.org/10.1016/j.foodchem.2005.01.013

Schütz K, Persike M, Carle R, Schieber A (2006) Characterization and quantification of anthocyanins in selected artichoke (*Cynara scolymus* L.) cultivars by HPLC-DAD-ESI-MS^n. Anal Bioanal Chem 384:1511–1517. https://doi.org/10.1007/s00216-006-0316-6

Snyder LR, Kirkland JJ, Glajch JL (1997) Practical HPLC method development. John Wiley & Sons, Inc., New York

The European Agency for the Evaluation of Medicinal Products (2006) Note for guidance on quality of herbal medicinal products/traditional herbal medicinal products. EMEA, London

Tistaert C, Dejaegher B, Heyden YV (2011) Chromatographic separation techniques and data handling methods for herbal fingerprints: a review. Anal Chim Acta 690:148–161. https://doi.org/10.1016/j.aca.2011.02.023

United Nations Educational Scientific and Cultural Organization (2010) UNESCO Intangible Heritage Lists. https://ich.unesco.org/en/lists. Accessed 12 Aug 2017

US Food and Drug Administration (2004) Guidance for industry botanical drug product. US Department of Health and Human Services, Rockville

World Health Organization (1991) Guidelines for the Assessment of Herbal Medicines. http://apps.who.int/iris/bitstream/10665/58865/1/WHO_TRM_91.4.pdf. Accessed 12 Aug 2017

Wu J, Qian Y, Mao P, Chen L, Lu Y, Wang H (2013) Separation and identification of phenolic compounds in canned artichoke by LC/DAD/ESI-MS using core-shell C18 column: a comparative study. J Chromatogr B 927:173–180. https://doi.org/10.1016/j.jchromb.2012.11.026

Xie Y, Jiang Z, Zhou H, Cai X, Wong Y, Liu Z, Bian Z, Xu H, Liu L (2007) Combinative method using HPLC quantitative and qualitative analyses for quality consistency assessment of a herbal medicine preparation. J Pharm Biomed Anal 43:204–212. https://doi.org/10.1016/j.jpba.2006.07.008

Zapolska-Downar D, Zapolski-Downar A, Naruszewicz M, Siennicka A, Krasnodębska B, Kolodziej B (2002) Protective properties of artichoke (Cynara scolymus) against oxidative stress induced in cultured endothelial cells and monocytes. L Sci 71:2897–2908. https://doi.org/10.1016/S0024-3205(02)02136-7

Zhu X, Zhang H, Lo R (2004) Phenolic compounds from the leaf extract of artichoke (*Cynara scolymus* L.) and their antimicrobial activities. J Agric Food Chem 52:7272–7278. https://doi.org/10.1021/jf0490192