Optimization process using response surface analysis of extract yield and coumarin concentration of Guaco (*Mikania laevigata* and *Mikania glomerata*) obtained by supercritical carbon dioxide extraction.

Alessandra Lopes de Oliveira[*1], Letícia Nasser Lauandos Pozza[1], Eliana Setsuko Kamimura[1], Eduardo Vicente[2], Fernando Antonio Cabral[3]

[1] Department of Food Engineering, Faculty of Animal Science and Food Engineering, University of São Paulo (FZEA-USP), Av. Duque de Caxias Norte, 225, Pirassununga, SP, 13635-900, Brazil. E-mail:alelopes@usp.br, Fax: +5519 3565 4284.

[2] Center of research and advance in food chemistry and nutrition applied, Institute of Food Technology (CCQA-ITAL), Av. Brasil, 2880, Campinas, SP, CEP: 13070-178, Brazil.

[3] Department of Food Engineering, University of Campinas (DEA-UNICAMP), Cidade Universitária Zeferino Vaz, Campinas, SP, CEP:13083-862, Brazil.

Originally from South America, the Guaco (*M. glomerata* and *M. laevigata*) is a climbing plant with aromatic leaves. The main components are caurenoic acids, cinammic acid grandiflora, stigmasterol, and coumarin resin. Therapeutic uses have been attributed to its anti-coughing, anti-asthmatic, bronchodilator activities in addition to treat dermatitis, mycoses, cancer, ulcers, and dental caries.

Coumarin is a white crystal with a similar flavor to vanilla. It is used in perfumes, detergents, toothpaste, cigarettes, and as a food additive. It has been used in medicine as antimicrobial, anti-inflammatory, and bronchodilators agents.

SFE is a technique used to obtain extracts from plants in various segments of food, cosmetics and pharmaceutical industry, and research. CO_2 has been used in supercritical extraction of natural sources, mainly by the low temperature process which preserves the thermo sensitive components and avoids waste.

Aiming to check the pressure (*P*) and temperature (*T*) to obtain extracts from Guaco with high concentration of coumarin, a study was prepared using a complete central composite 2^2 factorial experimental design and response surfaces analysis.

Response surfaces analysis, regardless of the Guaco species used, showed that SFE provides extracts with high yields, specifically for conditions in which higher *P* and *T* were employed (up to 175 bar and up to 45^{o}C). Under those conditions, in which high yields were obtained, the extracts were richer in coumarin. This behavior showed that coumarin is highly soluble in supercritical CO_2, and when it is better solubilized it increases the mass of total extract. The maximum concentration of coumarin in the *M. laevigata* (13.18 mg/g) was considerably higher than the maximum found by *M. glomerata* (0.049 mg/g). The minimum concentration of coumarin in the extract (8.67 mg/g for *M. laevigata* and 0.012mg/g for the *M. glomerata*) was considerably higher than that obtained by extraction with ethanol (1-2 μg/mL).

INTRODUCTION

Carbon dioxide is used in supercritical extraction of natural products since the critical conditions of *P* and *T* are easily accessible. It can ensure good stability of the extraction process, and, in addition, at low critical *T* (31^{o}C) it avoids the degradation of thermosensitive compounds. SFE is a powerful technique that has been used in the separation process of a large number of natural products in different segments of industry and scientific research. It exhibits physicochemical properties intermediate between liquid and gas. Its high density

increases its power as a solvent while its low viscosity and high diffusivity promote a considerable penetration power in the matrix of the solute [1, 2].
Guaco (M. *laevigata and M. glomerata*), originally from South America, develops as a climbing plant with green-bright rigid leaves, almost triangular. These leaves exude a strong aroma when scrubbed and this plant can be harvested at any time of year, especially before blooming when it increases its levels of bioactive ingredients [3].
The most important components of Guaco are the diterpenics: caurenoic acids, cinamoil grandifloric acid, stigmasterol, and resin coumarin [4]. Essential oils, tannins, astringent substances, and pigments [3] are important components as well. Some of its main therapeutic uses are due to its expectorant and antiasthmatic bronchodilator activity. It can also be used applied topically (onto the skin) to treat dermatitis and mycoses [4]. Studies have also showed the Guaco effects against cancer, ulcer, and infections by microorganism as well as its power to prevent of dental cavities and plaque [5]. The species *M. laevigata* has more antiulcerogenic effects due to the greater concentration of coumarin, which has been tested as antiulcerogenic and bronchodilators activities. However, the *M. glomerata* has higher antimicrobial activity. For anticancer activity, there were no significant differences between the two species since the diterpenic acids, whose this action is attributed, are present in equal quantity in the two species [5].
Coumarin (1,2-Benzopyrone, 2H-1-Benzopyran-2-one, cis-o-Coumarinic acid lactone, Benzo-α-pyrone, Coumarinic anhydride, and Cumaru) is a white crystal at room temperature and vanilla-like aroma. It can be found in various plants from different species and families. It is also present in honey, in which it is one of the constituents responsible for its characteristic aroma [6]. Coumarin is used as fixative agent or to emphasize the fragrance in perfumes, detergents, toothpaste, cigarettes, and alcoholic beverages [7]. Additionally, it is used as a food additive as sweeteners, to enrich essential oils, and in the nickel plating process leading to a lower porosity and greater brightness of the final product [8]. On account of its biochemical properties, coumarin has been used in medicine as antimicrobial [9], anti-inflammatory [10], and bronchodilators [11]. Pharmacological tests have also showed that coumarin can cause inhibition of the effects of intestinal and uterine muscle in vivo [8].
In order to verify the influence of *P* and *T* to obtain Guaco extracts and the concentration of coumarin in these extracts, a study of the optimization process of supercritical extraction was carried out using a complete central composite 2^2 factorial experimental design and response surface analysis.

MATERIAL AND METHODS

Guaco leaves obtained from *CPQBA* (Multidisciplinary Center for Research in Chemistry, Biology, and Agriculture - UNICAMP, Campinas, SP, Brazil) were washed and dried in an oven with air circulation (35^oC/36h). Low temperature was used to prevent degradation of the thermosensitive components. After drying, the final moisture content was 9.56%. The leaves were grounded and separated by size (0.297 mm).
During the extraction process, dry ground Guaco was mixed with supercritical CO_2 in a cylindrical extractor vessel (300 mL). The equilibrium pressure was monitored by calibrated Bourdon gauges (accuracy ± 0.5%) while the desired temperature was controlled by immersion in a water bath with control accuracy of ± 1^oC (Sulab, Campinas, Brazil). Data from the literature [12, 13] were used as basis for the experimental conditions of pressure (*P*) and temperature (*T*) employed.

Refrigerated CO_2 was pumped into the systems, and *P* was controlled by monitoring the flow using a high pressure pump (Eldex, AA-100-S, Napa, CA) and the micrometering valve (Autoclave Engineers, 10VRMM2812, Erie, USA) in the output of the system. The extract, was continuously separated from CO_2 and collected in a container immersed in an ice bath during 2h. The depressurization of the system, during the collection, was made under ambient conditions with temperatures around 25°C and barometric pressure of 0.94 bar. The average flow volume of CO_2 for these experiments was 1.37 L/min or 0.0023 kg CO_2/min since CO_2 density under those conditions is 0.0383 kgmol/m^3. For the calculation of the yield, the extracts were weighed in analytical scale (Sartorius, BL 210s) and correlated with the mass of the dried leaves in the extractor.

For the analysis of the yield and the concentration of coumarin in the extracts, different conditions of *P*-*T*, defined according to a complete central composite 2^2 factorial experimental design with *P* varying from 83 to 267 bar and *T* from 31 to 59°C as independent variables, were used. 5 levels with 4 star points and three replicates at the central point were considered. The dependent variables obtained as responses were the extraction yield and the concentration of coumarin in the extracts. The results were obtained from response surfaces analysis based on the factorial experimental design used in the regression analysis [14, 15].

The determination of the concentration of coumarin in the extract was performed at the Institute of Food Technology (ITAL, Campinas, SP, Brazil), by means of high performance liquid chromatography (HPLC). The equipment, a Shimadzu liquid chromatograph (SCL-10A VP), was composed of a Rheodyne injection valve with loop sampling of 20μL, isocratic pump (LC-10AT VP), UV-Visible detector (SPD-M10A VP), and software acquisition data (CLASS-VP). The chromatographic column used was a C18 Inertsil 5 ODS-3 Chrompack-Varian (150mm × 4.6mm × 5μm). Prior to the analysis, the extracts were diluted in absolute ethyl alcohol (Mallinckrodt) and filtered in cellulose membrane (0.45 μm, Schleicher & Schull). As mobile phase, it was used acetic acid solution (5%) and methanol (60:40). The calibration curve of coumarin was constructed for pure coumarin (99%) (Synth, C1067.06.AG, Brazil). Using this curve, the dilution factors and the peak areas of the chromatogram obtained for each Guaco leaves extract, the concentration of coumarin (g/100g) in the dried and grounded leaves was determined

RESULTS AND DISCUSSION

Despite of the Guaco species, the response surface analysis showed that the supercritical CO_2 as solvent provides extracts with relatively high yields, especially for conditions in which high *T* and *P* were employed. More particularly for *P* higher than 175 bar in the *T* range studied (Table 1, Figure 1). Under the conditions which produced very high yields, the extracts were also rich in coumarin (Table 1).

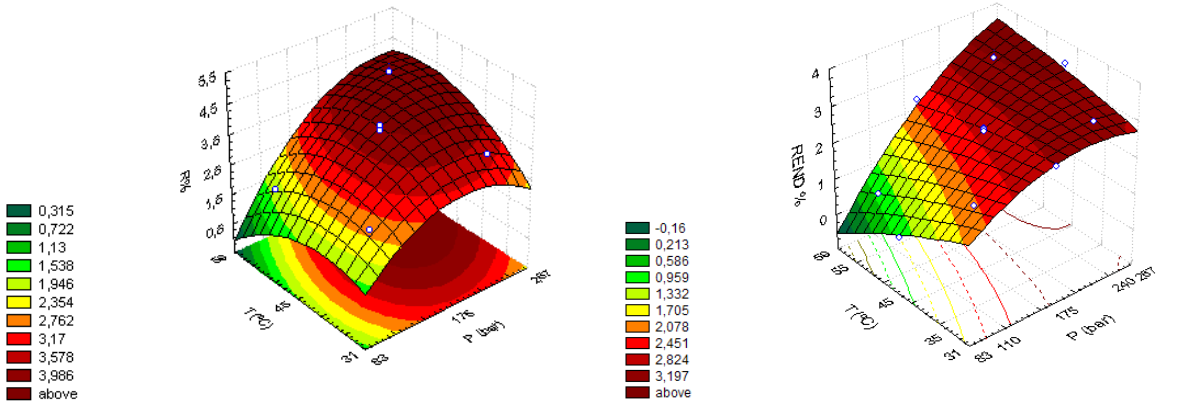
This behavior shows that coumarin, a highly soluble compound in supercritical CO_2, when better solubilized increases the total mass of the extract. However, comparing the two species, the maximum concentration of coumarin in the extract of *M. laevigata* (13.18 mg/g) was considerably higher than the maximum found for *M. glomerata* (0.049 mg/g). In literature, studies that investigate the Guaco activity against diseases reported that the coumarin concentration in *M. laevigata* is higher than in *M. glomerata*. Comparing the results of this study with those of Biavatti et al., 2005 [16], it can be noted that the minimum concentration of coumarin in the extract (8.67 mg/g for *M. laevigata* and 0.012mg/g for *M. glomerata*) was considerably higher than that obtained by the extraction with ethanol (1-2 μg/mL). As mentioned earlier, Coumarin is highly soluble in supercritical CO_2 [13], so it can be said that

this technique is effective in the extraction of this component from Guaco and another natural sources.

Table 1. Complete 2^2 factorial experimental design matrix (including three central points) to study *P* and *T* effects on Guaco extract yield (%) and coumarin concentration (mg/g of extract).

			M. laevigata		*M. glomerata*	
Assay	***P* (bar)**	***T* (ºC)**	**R (%)**	**C (mg/g)**	**R (%)**	**C × 10^{-2} (mg/g)**
1	-1 (110)	-1 (35)	2.60	11.85	2.12	4.90
2	+ 1 (240)	-1 (35)	3.28	8.91	2.94	4.65
3	-1 (110)	+1 (55)	1.75	19.48	0.66	1.16
4	+1 (240)	+1 (55)	4.08	11.03	3.07	4.56
5*	0 (175)	0 (45)	4.03	9.62	2.62	2.73
6*	0 (175)	0 (45)	3.89	10.15	2.59	2.52
7*	0 (175)	0 (45)	4.05	9.46	2.54	1.89
8	- α (83)	0 (45)	1.31	8.67	0.64	7.42
9	+α (267)	0 (45)	4.20	9.40	3.41	1.95
10	0 (175)	- α (31)	2.92	8.81	2.78	9.66
11	0 (175)	+α (59)	3.36	13.18	2.25	1.89

* Central point; α = ± 1,41; R: Yield of the extracts; C: Coumarin concentration (mg/g)



M. laevigata

$$R(\%) = 3{,}99^{*} + 0{,}89P^{*} - 0{,}62P^{2^{*}} - 0{,}43T^{2^{*}} + 0{,}41PT^{*}$$

M. glomerata

$$R(\%) = 2.58^{*} + 0{,}89P^{*} - 0{,}30P^{2^{*}} - 0.26T^{*} + 0{,}40PT^{*}$$

Figure 1. Response surface of temperature and pressure effects on Guaco extract yield for quadratic order 2^2 factorial experimental designs obtained through supercritical CO_2 extraction (*Coefficients with $p \leq 0.05$ were used in the model framework).

CONCLUSION

This work shows optimized operating conditions of *P* and *T* which can be used to obtain extracts rich in coumarin. In the quadratic model (Figure 1) the two variables and the interaction between them significantly intervene ($p \leq 0.05$) in the yield production of extracts. Coumarin is a compound with biological properties, and it is responsible for therapeutic applications of Guaco extracts. Thus, we believe that this study provides a technological advance to obtain highly purified extracts and also rich in this compound since the supercritical CO_2 produces extracts free of organic solvent.

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