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Article

Supercritical Fluid Extraction and Chromatographic Analysis (HRGC-FID and HRGC-MS) of *Lupinus spp*. Alkaloids

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Os extratos de alcalóides de *Lupinus spp*., obtidos por métodos convencionais (maceração/sonicação – extração em fase sólida; maceração/sonicação –extração líquido-líquido) e por SFE (extração com fluido supercrítico) usando CO_2 e CO_2 modificado (CO_2/MeOH, CO_2/EtOH, CO_2/^{i}PrOH e CO_2/H_2O) foram analisados por CGAR-DIC (cromatografia gasosa de alta resolução com detector de ionização de chama) e CGAR-EM (cromatografia gasosa de alta resolução acoplada à espectrometria de massas). As análises quantitativas por CGAR-DIC foram feitas pelo método do padrão interno, para a quantificação de lupanina, multiflorina e um alcalóide derivado da esparteína. CGAR-EM permitiu a identificação dos constituintes químicos (alcalóides e outras substâncias) destes extratos.

The alkaloid extracts from *Lupinus spp*., obtained by conventional methods (maceration/sonication - solid phase extraction; maceration/sonication - liquid-liquid extraction) and SFE (supercritical fluid extraction) using CO_2 and modified CO_2 (CO_2/MeOH, CO_2/EtOH, CO_2/^{i}PrOH and CO_2/H_2O) were analysed by HRGC-FID (high resolution gas chromatography - flame ionization detector) and HRGC-MS (high resolution gas chromatography - mass spectrometry). The HRGC-FID quantitative analyses were performed with an internal standard method for quantification of lupanine, multiflorine and a spartein-like alkaloid. HRGC-MS allowed identification of the chemical constituents (alkaloids and other compounds) from these extracts.

Keywords: supercritical fluid extraction (SFE), alkaloids, *Lupinus spp*., high resolution gas chromatography (HRGC)

Introduction

Lupinus spp. has been investigated in several countries as a potential alimentary source due to its relatively high protein and oil content (35 - 40% and 8 - 12%, respectively), high productivity and low cost. Although lupine protein levels are similar or larger than that of the soya bean, the main problem is the quinolizidine alkaloids, which are known to provide a bitter taste and toxicity to the seeds. An usual procedure for the elimination of these alkaloids is washing the seeds with flowing water; despite simple, this method requires large volumes of water for the commercial scale *Lupinus* processing[1-3].

SFE (supercritical fluid extraction) is a valuable method both for industrial scale food processing and also for analytical scale studies, as an alternative extraction method to reduce the use of liquid solvents (mainly organic solvents). However, analytical scale SFE of polar compounds is still underexplored, mainly due to the low diffusibility and low polarity of supercritical CO_2. Some moderately polar natural products such as alkaloids and flavonoids have been extracted by SFE[4, 5].

In this work, the extraction of *Lupinus spp*. alkaloids by SFE is compared with conventional methods (maceration/sonication - solid phase extraction, and maceration/sonication - liquid-liquid extraction). The extracts were analysed by HRGC-FID (high resolution gas chromatography - flame ionisation detector), for the quantification of lupanine, multiflorine and a spartein-like alkaloid. HRGC-MS (high resolution gas chromatography coupled with mass spectrometry) analysis allowed the identification of the chemical constituents (alkaloids and other compounds) from the extracts obtained by different methods from *Lupinus* samples obtained from markets in São Paulo, SP.

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Experimental

Materials

Analytical reagent grade ethanol, ethanol, isopropanol, dichloromethane and acetone were purchased from Merck, Darmstadt, Germany. The carbon dioxide and nitrogen gas were supplied by White Martins, Rio de Janeiro, Brazil.

Samples

Seeds of *Lupinus mutabilis*, *L. albus* and *L. sp.* (Leguminosae- Papilonaceae) were collected in Chile by one of the authors (D. von B.). Commercial lupine samples were bought from local markets in São Paulo, SP, Brazil. The samples were dried (*ca.* 40°C), powdered and ground (70- 100 mesh).

Sample extraction: conventional method (maceration/sonication)

Extraction was performed as schematically represented in Figure 1. Each sample was extracted in triplicate, and the extracts were analysed by HRGC-FID and HRGC-MS. The yield of each extraction was determined after drying (room temperature, under nitrogen flux, followed by drying in a vacuum oven at room temperature) until constant weight.

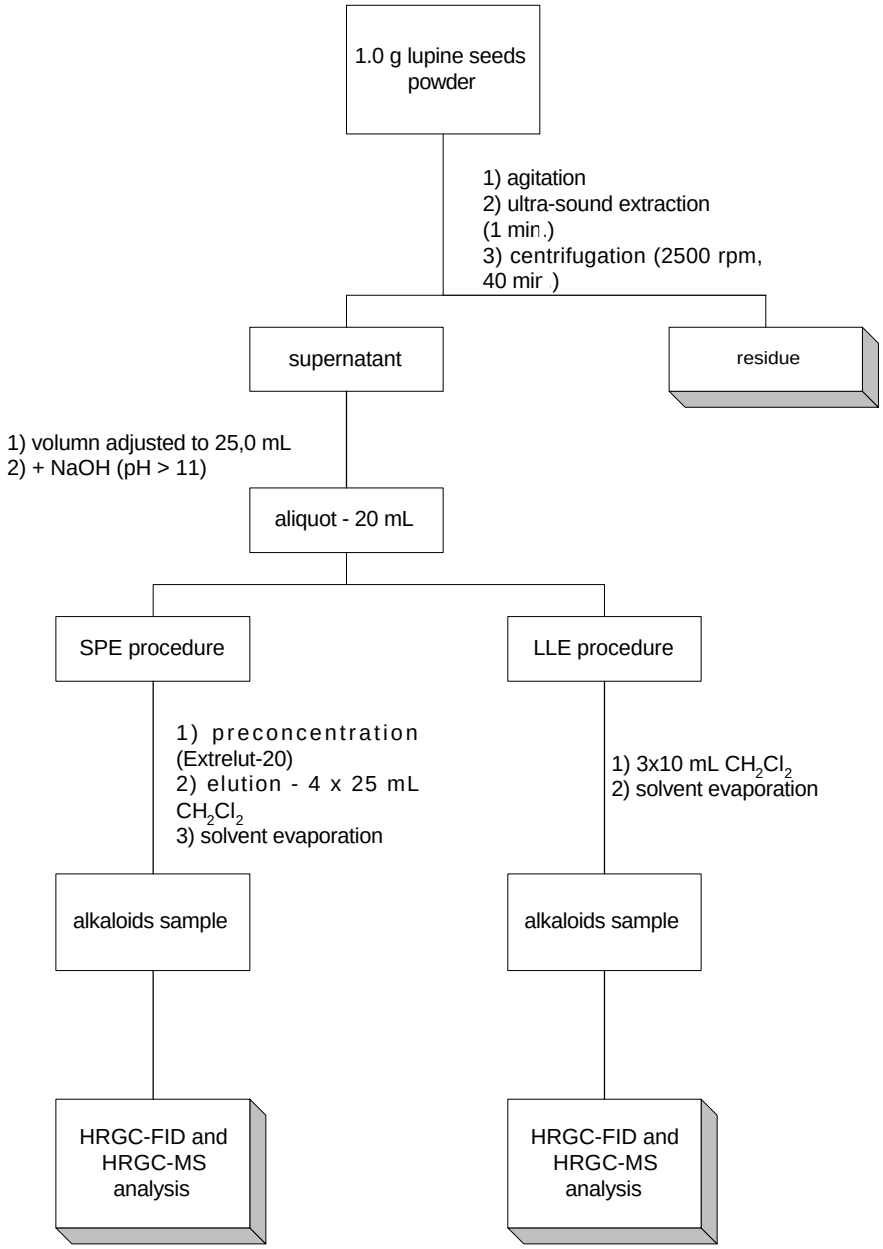


Figure 1. Schematic drawing of the conventional extraction procedure and clean-up for *Lupinus* samples.

Sample extraction: SFE

Supercritical fluid extractions were performed on an analytical scale "home-made" system, previously described[6] . Powdered *Lupinus* samples (0.5 g each) were extracted for approximately 20 minutes in a dynamic mode, using as extraction fluids pure CO_2 or CO_2 modified with different solvents, as indicated in Table 1.

Table 1. Conditions of the fluid mixtures used in SFE experiments.

SFE fluid mixture (v:v)	Pressure / atm	Temperature /°C
CO_2	80.0	40.0
CO_2/ 10% MeOH	80.0	60.0
CO_2/ 15% MeOH	80.0	60.0
CO_2/ 10% EtOH	80.0	60.0
CO_2/ 10% i_{Pr}OH	80.0	60.0
CO_2/5 % H_2O	90.0	70.0

Sequential mode extractions were done firstly with pure CO_2, followed by extraction of the same *Lupinus* sample using CO_2 modified with 10% EtOH. Temperature and pressure conditions of each step were as reported in Table 1.

All the extracts were collected in analytical grade CH_2Cl_2 (Merck, Darmstadt, Germany) contained in a test tube, in an ice bath. The solvent was removed at room temperature, under nitrogen flux, followed by drying in a vacuum oven at room temperature until constant weight. Whenever possible, extractions were made in triplicate (see remarks in Tables 2 and 3). Residues were dissolved in analytical grade methanol (Merck, Darmstadt, Germany) prior to HRGC analysis.

Clean up

Clean up was done by percolating the extract (obtained by conventional extraction or SFE), solubilized in 5.0 mL analytical grade methanol, through a glass Pasteur-type pipette containing 0.5 g silica gel 60, 70 - 230 mesh (Merck, Darmstadt, Germany) and 0.5 g active charcoal (Reagen, Rio de Janeiro, Brazil).

Extracts obtained by SFE using aqueous mixtures have been submitted to SPE (solid phase extraction), using Sep-Pak C-18 cartridges (Waters), preconditioned with 8.0 mL methanol followed with 8.0 mL H_2O. The extract was

Table 2. Yield for total extracts from *Lupinus* samples obtained by conventional and SFE extraction methods (expressed in mg of alkaloid /g plant material).

Extraction method	(x ± s.d.)	% s.d.
Brazilian commercial Lupinus sample - *seeds*		
Conventional (solid phase preconcentration)	(0.23 + 0.01)	4.35
Conventional (liquid-liquid extraction)*	(0.29 ± 0.01)	3.45
SFE CO_2*	(2.69 ± 0.90)	33.46
SFE CO_2/10% MeOH (with clean-up)*	(4.29 ± 0.56)	13.05
SFE CO_2/10% MeOH (without clean-up)*	(17.44 ± 1.72)	9.86
SFE CO_2/15% MeOH (with clean-up)*	(0.34 ± 0.03)	8.82
SFE CO_2/15% MeOH (without clean-up)*	(4.39 ± 0.32)	7.29
SFE CO_2/10% EtOH (with clean up)*	(2.62 ± 0.14)	5.33
SFE CO_2/10% EtOH (without clean up)*	(13.85 ± 3.23)	23.32
SFE (sequential mode) CO_2 , CO_2/10% EtOH *	(7.98 ± 0.87)	10.90
SFE CO_2/10% i_{Pr}OH (with clean-up)*	(8.11 ± 0.48)	5.92
SFE CO_2/10% i_{Pr}OH (without clean-up) *	(13.16 ± 0.44)	3.34
SFE CO_2/5% H_2O *	(0.86 ± 0.16)	18.60
Brazilian commercial Lupinus sample - *seeds peel*		
SFE CO_2/ 10% EtOH *	(16.42 ± 3.90)	23.78
SFE CO_2/ 5% H_2O *	(0.29 ± 0.03)	10.34
Chilean Lupinus sp. *seeds sample*		
SFE CO_2/10% EtOH *	(16.07 ± 2.19)	13.63
SFE CO_2/ 5% H_2O *	(1.06 ± 0.02)	1.97
Chilean Lupinus albus *seeds sample*		
SFE CO_2/10% EtOH *	(9.99 ± 1.26)	12.61
SFE CO_2/5% H_2O *	(1.00 ± 0.10)	10.00
Chilean Lupinus mutabilis *seeds sample*		
SFE CO_2/10% EtOH **	(39.78 ± 5.86)	14.73
SFE CO_2/5% H_2O **	(1.88 ± 0.09)	4.95

* n= 3; **n= 2; s.d. = standard deviation; % s.d. = relative standard deviation

Table 3. Content of alkaloids in *Lupinus* samples (expressed in mg of alkaloid /g plant material).

Extraction Method	Lupanine	Multiflorine	spartein derivative (**3**)
	Brazilian commercial Lupinus *sample - seeds*		
Conventional (SPE)*	$(1.77 \times 10^{-1} \pm 9.51 \times 10^{-3})$	$7.63 \times 10^{-5} \pm 2.94 \times 10^{-6}$	#
Conventional (LLE)*	$9.47x\ 10^{-2} + 1.83 \times 10^{-3}$	#	#
SFE CO_2*	$(9.84 \times 10^{-4} \pm 2.90 \times 10^{-5})$	#	$(9.45 \times 10^{-7} \pm 1.96 \times 10^{-5})$
SFE CO_2/10% MeOH (with clean-up)*	$(1.05 \times 10^{-3} \pm 3.99 \times 10^{-6})$	#	#
SFE CO_2/10% MeOH (without clean-up)*	$(1.31 \times 10^{-2} \pm 1.40 \times 10^{-4})$	#	$(6.65 \times 10^{-3} \pm 8.14 \times 10^{-1})$
SFE CO_2/15% MeOH (with clean-up)*	$(5.03 \times 10^{-3} \pm 4.70 \times 10^{-4})$	#	#
SFE CO_2/15%MeOH (without clean-up)*	$(2.68 \times 10^{-2} \pm 6.70 \times 10^{-4})$	$4.60 \times 10^{-6} \pm 5.47 \times 10^{-7}$	$(5.03 \times 10^{-3} \pm 6.60 \times 10^{-4})$
SFE CO_2/10% EtOH (with clean up)*	$(2.99 \times 10^{-3} \pm 3.10 \times 10^{-4})$	#	$(1.20 \times 10^{-5} \pm 2.74 \times 10^{-3})$
SFE CO_2/10% EtOH (without clean up)*	$(1.20 \times 10^{-2} \pm 1.94 \times 10^{-3})$	$1.44 \times 10^{-5} \pm 1.08 \times 10^{-6}$	$(1.34 \times 10^{-3} \pm 1.89 \times 10^{-3})$
SFE (sequential mode) CO_2; CO_2/10% EtOH *	$(1.01 \times 10^{-2} \pm 2.60 \times 10^{-4})$	$6.41 \times 10^{-6} \pm 2.48 \times 10^{-7}$	$(5.76 \times 10^{-4} \pm 3.63 \times 10^{-4})$
SFE CO_2/10% i_{Pr}OH (with clean-up)*	$(2.91 \times 10^{-3} \pm 1.90 \times 10^{-4})$	#	#
SFE CO_2/10% i_{Pr}OH (without clean up)*	$(9.46 \times 10^{-3} \pm 5.51 \times 10^{-4})$	$4.50 \times 10^{-7} \pm 1.03 \times 10^{-8}$	$(2.91 \times 10^{-5} \pm 2.68 \times 10^{-4})$
SFE CO_2/5% H_2O *	$(1.30 \times 10^{-1} \pm 6.10 \times 10^{-3})$	$5.23 \times 10^{-5} \pm 1.23 \times 10^{-6}$	#
	Chilean Lupinus sp. *seeds sample*		
SFE CO_2/ 10% EtOH*	$(1.00 \times 10^{-1} \pm 7.05 \times 10^{-2})$	$1.92 \times 10^{-5} \pm 4.88 \times 10^{-6}$	$(1.38 \times 10^{-1} \pm 3.65 \times 10^{-2})$
SFE CO_2/ 5% H_2O *	$(3.48 \times 10^{-1} \pm 4.40 \times 10^{-2})$	$6.60 \times 10^{-5} \pm 1.13 \times 10^{-5}$	#
	Chilean Lupinus albus *seeds sample*		
SFE CO_2/ 10% EtOH *	$(1.43 \times 10^{-1} \pm 4.21 \times 10^{-2})$	$5.31 \times 10^{-5} \pm 2.57 \times 10^{-6}$	$(1.44 \times 10^{-3} \pm 5.95 \times 10^{-2})$
SFE CO_2/ 5% H_2O *	$(4.92 \times 10^{-1} \pm 6.51 \times 10^{-2})$	$9.75 \times 10^{-5} \pm 2.31 \times 10^{-6}$	#
	Chilean Lupinus mutabilis *seeds sample*		
SFE CO_2/ 10% EtOH **	$(7.93 \times 10^{-2} \pm 1.51 \times 10^{-3})$	$3.32 \times 10^{-4} \pm 1.62 \times 10^{-5}$	$(9.80 \times 10^{-2} \pm 1.77 \times 10^{-3})$
SFE CO_2/ 5% H_2O **	$(2.82 \times 10^{-1} \pm 3.89 \times 10^{-2})$	$5.75 \times 10^{-4} \pm 2.61 \times 10^{-5}$	#

*n=3; **n=2; # not detected

percolated through the cartridge and eluted with methanol, acetone, ethyl acetate and chloroform, successively (8.0 mL each). These fractions were collected and combined for total yield determination and chromatographic analysis.

Some samples were not submitted to the clean up step and were directly analyzed by HRGC; they are indicated in Table 3.

Chromatographic analysis

HRGC-MS analyses were performed using a HP 5970 mass selective detector (Hewlett - Packard, USA), (EI, 70 eV), coupled to a HP 5890 GC. The column used was a 95% methyl, 5% phenylpolysiloxane, LM-5 (50 m x 0.25 mm x 0.65 mm) supplied by L & M (São Carlos, Brazil). Samples were injected using the split mode (1:30), with injector temperature and HRGC-MS interface temperature both at 300°C. The column temperature was programmed to rise from 170 °C (3.5 min), at 6 °C min^{-1}, to 300 °C (held during 20 min). Helium was used as carrier gas, at the average linear velocity of 35 cm sec^{-1}; MS data were processed using a CPU HP 7946 / HP 9000-300. Tentative identifications were made by comparison of the obtained spectra with literature data[7,8].

The HRGC-FID analyses were performed on a HP 5890 GC, using the same column and the same temperature program as used for the HRGC-MS analysis. Detector (FID) temperature was 320 °C, split (1:30), injector temperature was 280 °C, data were obtained on HP 3396 A integrator. Hydrogen was used as the carrier gas, at an average linear velocity of 40 cm sec^{-1}. All quantitative analyses were made by the internal standard method, using caffeine as an analytical standard. The *Lupinus* extracts were diluted to 1.0 mL in methanol, and 0.3 mL of a caffeine standard solution (1 mg mL^{-1}) was added to the sample, which was analyzed by HRGC-FID. For each alkaloid quantified a corresponding calibration curve was prepared (injections in triplicate for each concentration), and linearity for internal standard quantification was checked within the range of 0.05 – 0.50 mg caffeine mL^{-1}.

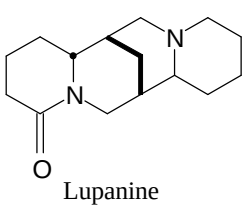
Results and Discussion

Extraction methods

The yields for each extraction procedure were determined using both Brazilian commercial and Chilean samples (Table 2). The conventional extraction gave a similar yield, both by Extrelut preconcentration and liquid-liquid extraction (Figure 1). Most of the SFE procedures gave better yields than the conventional method. Some of the SFE experiments showed a large standard deviation, due to some specific problems of some fluid mixtures. For example, water extracted polar compounds, which plugged the system due to the production of foam. Another problem was the trapping of CO_2 extracts: losses of extracts due to plugging (ice formation in the extremity of restrictor) are inherent to extract collection in solvents (the process herein adopted). The time required for SFE was 20 minutes for each extraction while for conventional methods the total time was several hours for the complete procedure.

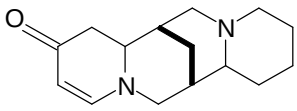
Chemical composition of the extracts

HRGC-MS analysis of the extracts obtained from a commercial *Lupinus* sample allowed tentative identification of three alkaloids, by comparison with literature data[7]: lupanine (**1**), multiflorine (**2**) and a spartein derivative (**3**). Many other compounds were identified as alkaloids, but a more detailed tentative identification was not possible. The main feature of these unidentified alkaloids was a peak at *m/z* = 58, which is found in several lupane-type alkaloids[7]. Some extracts also contained other compounds, mainly fatty acids and long chain hydrocarbons, which were identified by their MS profiles[9]. The CO_2/H_2O mixture required slightly stronger conditions, since the critical constants of H_2O are significantly higher than those of organic solvents[10]. SFE using CO_2 modified with 5% H_2O was the most selective condition for alkaloid extraction; however, the critical conditions of this mixture (Pc = 89.1 atm, Tc = 69.1 °C; calculated according to the literature[11]), require a relatively high temperature for the usual working conditions with natural products. SFE using CO_2 modified with 10% methanol showed the best yield with a



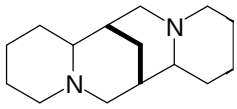
Lupanine

1



Multiflorine

2



Sparteine like alka

3

Table 4. Compounds and respective MS data (EI, 70 eV) found in the extracts of a commercial *Lupinus* sample.

(**a**) extract obtained by SFE using CO_2 modified with 5% H_2O

Peak	Tentative identification	Main fragments, *m/z* (%)
1	lupanine	136 (100), 55 (71), 149 (49), 98 (36), 150 (35), 97 (32)
2	lupane type alkaloid	58 (100), 73 (34), 55 (31), 69 (29), 205 (25), 96 (22)
3	multiflorin	55 (100), 69 (64), 73 (60), 134 (52), 57 (32), 83 (32)
4	ftalate (contaminant)	149 (100)

(**b**) extract obtained by SFE using CO_2 modified with 10% methanol

Peak	Tentative identification	Main fragments, *m/z* (%)
1	carboxilic acid	57 (100), 73 (31), 58 (21), 69 (17), 55 (17), 77 (15)
2	fatty acid ester	55 (100), 69, (74), 74 (70), 87 (37), 59 (33), 67 (32)
3	fatty acid ester	74 (100), 87 (56), 55 (24), 75 (18), 57 (68), 69 (40), 71 (33)
4	carboxilic acid	73 (100), 60 (93), 55 (68), 57 (68), 69 (40), 71 (33)
5	unidentified	
6	hydrocarbon	55 (100), 69 (57), 74 (54), 83 (38), 87 (33), 67 (33)
7	hydrocarbon	55 (100), 69 (60), 83 (42), 67 (35), 57 (35), 56 (32)
8	unidentified	
9	fatty acid	79 (100), 55 (61), 67 (48), 93 (42), 108 (40), 80 (29)
10	lupanine	136 (100), 55 (68), 74 (43), 69 (42), 149 (33), 97 (33)
11	unidentified	
12	hydrocarbon	55 (100), 69 (54), 67 (54), 57 (44), 81 (40), 98 (36)

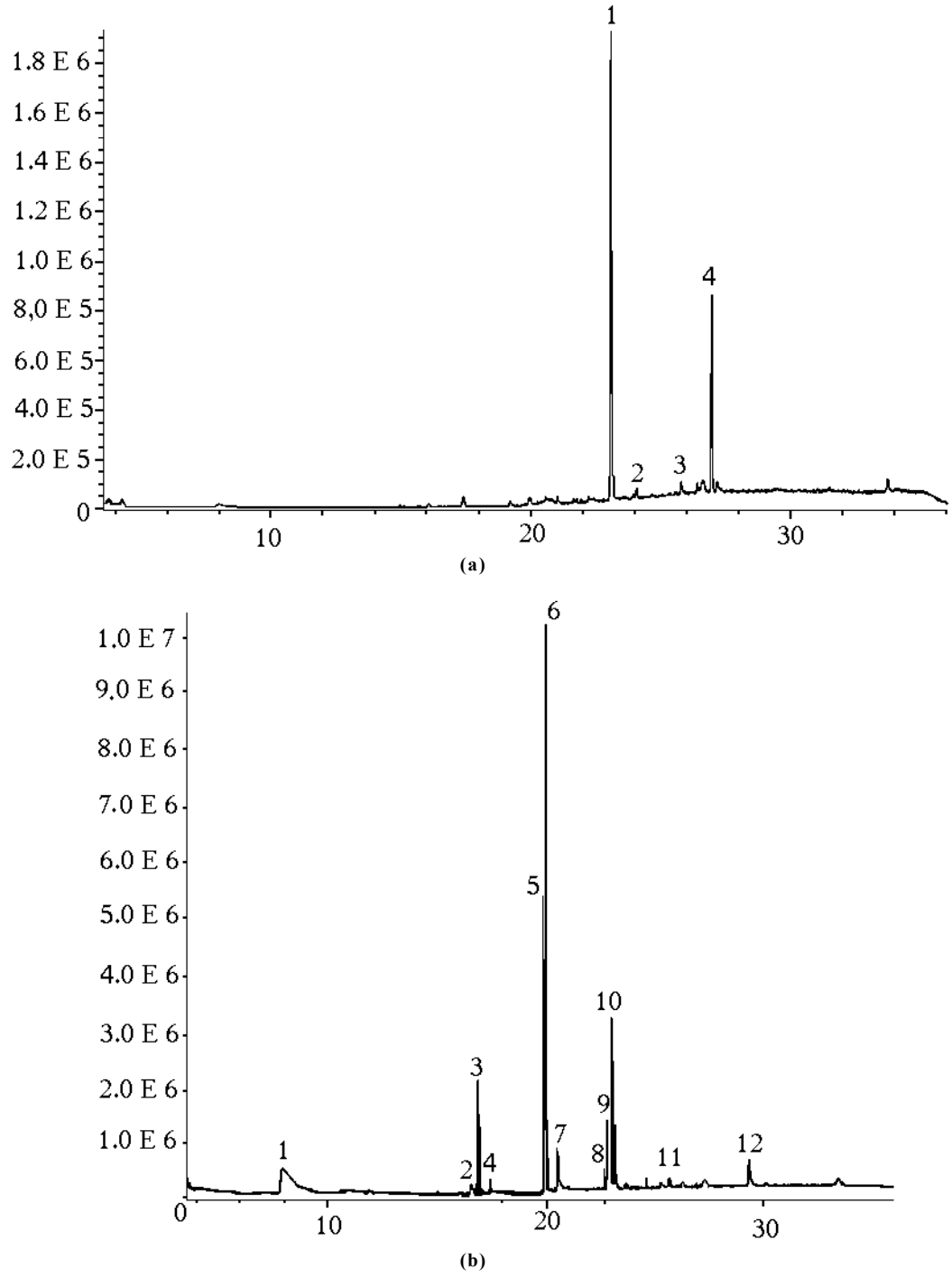


Figure 2. TIC-HRGC-MS (EI, 70eV) of extracts obtained by SFE using CO_2 modified with 5% H_2O (**a**) and SFE using CO_2 modified with 10% methanol (**b**).

lower temperature (Pc = 73.7 atm, Tc = 60.0 ºC). Figure 2 (peaks key on Table 3) shows the TIC-HRGC-MS profile of these two extracts.

Quantitative analysis

Quantitative analyses were made for the main alkaloids. Lupanine was found in all of the *Lupinus* extracts. The regression equations for the analytical curves were y= 0.0359 + 0.6647 x (r=0.999) for lupanine (**1**), y= - 0.0236 + 0.1126 x (r= 0.999) for multiflorine (**2**) and y= 0.06203 + 0.02893 x (r= 0.968) for the spartein derivative (**3**). The average percentage standard error for the peak areas for replicate injections was less than 5%, showing good reproducibility. The content of each alkaloid in all *Lupinus* extracts is shown in Table 3.

The sum of the alkaloid content in the extract obtained by SFE using CO_2 modified with 10% ethanol and not submitted to clean-up before HRGC-FID was the greater of all extraction methods. Unfortunately EtOH usually shows problems in reproducibility as a SFE modifier, since commercial ethanol has a significant (for SFE) variation in water content.

Utilization of isopropanol as a modifier showed no significant improvement in extraction process (yield or selectivity), so this solvent should be considered only as a third option in the choice of modifiers, due to its cost and the difficulty of removing residual solvent from extracts.

Conclusions

The present results indicate that SFE can be used as an alternative to conventional methods for extraction of alkaloids from *Lupinus*. It is faster and had greater total yields for the extracts, and methanol was shown to be the best modifier for *Lupinus* extraction. Lupanin was the most abundant alkaloid in all the extracts (SFE and conventional method) from the samples studied. The utilization of water as a modifier may be a useful tool for the extraction of polar alkaloids, but only for qualitative analysis for the alkaloids herein analyzed.

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