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Experimental designs characterizing seasonal variations and solvent effects on the quantities of coumarin and related metabolites from *Mikania laevigata*

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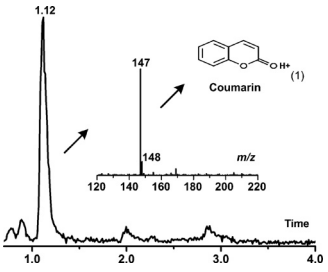
HIGHLIGHTS

- The existence of melilotic acid in *M. laevigata* is reported for the first time.
- Coumarin is found to be most abundant in summer plant extracts.
- *O*-coumaric acid, is predominant in winter and spring samples.
- Statistical mixture models indicate that synergic binary interactions are important.

GRAPHICAL ABSTRACT



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ABSTRACT

Statistical design mixtures of acetone, chloroform, dichloromethane and ethanol were used to study the effects of different solvents and their mixtures on the quantities of coumarin and related metabolites extracted from *Mikania laevigata* samples harvested in each of the four seasons. RP-HPLC-DAD and both positive and negative modes of UPLC-MS analyses were used to determine relative quantities of coumarin, *o*-coumaric acid and melilotic acids in each season for all the mixture design extracts. The existence and measurement of the relative abundances of melilotic acid in *Mikania laevigata* have not been reported previously. Highest coumarin concentrations were encountered in the summer whereas its *o*-coumaric acid precursor and melilotic acid were most abundant in the spring. *O*-coumaric and melilotic acids concentrations were strongly correlated during the year. Also solvent effects were seen to be significant. Ethanol and 1:1 binary mixtures of ethanol and acetone extracted the largest quantities of coumarin whereas ethanolic binary and ternary mixtures with chloroform and dichloromethane provided the best yields of *o*-coumaric and melilotic acids. Statistical mixture models indicated that synergic binary interactions, especially those involving ethanol with acetone or chloroform, are important in the *Mikania* extraction process.

1. Introduction

Mikania laevigata Schultz Bip. ex Baker, popularly called guaco, is widely used as a medicinal plant in South America to treat a variety of ailments [1,2]. Phytochemical and pharmacological studies have shown its leaves have broncodilatory [3,4], antimicrobianic [5], antiulcerogenic [2] e anti-inflammatory [6–8] properties. Recently

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it has been found that its ethanolic extract has significant anecdotal action against the toxic effects of the *Philodryas olfersii* poison [9]. Coumarin, 1,2-benzopyrone, is the main pharmacological substance in this plant and is used as a biomarker for pharmacological formulations [10,11]. Furthermore secondary metabolite derivatives of cinnamic acid and kaurane diterpenes [12–14], also found in this plant have demonstrated synergic pharmacological effects. As can be expected the presence and concentrations of these metabolites are directly related to the therapeutical efficiency of guaco extracts so their monitoring is an important tool for characterization and quality control [15].

The number and concentrations of secondary metabolites in plants depend on many factors such as geographical origin, agronomical and environmental aspects, climate and seasonality [16,17]. Furthermore the determination of metabolite concentrations depends on the extraction technique and solvent extractor. In spite of the pharmacological and commercial importance of the *Mikania laevigata* plant no report has been found in the literature regarding seasonal or solvent extraction effects on its chemical constituents. In this study these two important sources, seasonality and nature of the solvent extractor, of its measured metabolite variability, are investigated.

In recent years our groups have shown that statistical mixture designs are useful tools for the systematic study of solvent effects on natural products [18–20]. They are not only ideal for studying the effects of pure solvents on extraction results but also to determine synergic and antagonistic effects among solvents that are relevant to the extraction procedure. In order to obtain a complete extraction and the largest concentrations of desired products the ideal solvent should have maximum selectivity, compatibility with the properties of the extracted materials and the largest extraction capacities in terms of their saturated substance coefficients in the solvent [21,22].

With the objective of studying solvent and seasonal effects on the quantities of coumarin and related metabolites extracted from guaco, a simplex–centroid mixture design involving four solvents, acetone, chloroform, dichloromethane and ethanol, was performed on guaco samples harvested in each of the four seasons. First a qualitative phytochemical study was undertaken to determine the compositions of the different fractions of extracted material. The organic fraction containing significant quantities of cumarin and related metabolites was then singled out for mixture design analysis of their yields estimated using reversed phase-high performance liquid chromatography with a diode array detector (RP-HPLC-DAD) and ultra-performance liquid chromatography coupled to a mass spectrometer with an electronebulization interface (UPLC–ESI-MS).

2. Experimental

2.1. Plant material

Mikania laevigata Schultz Bip. ex Baker leaves were analyzed. A voucher of this plant was deposited in the herbarium at Londrina State University in Londrina, PR, Brazil and registered under the 44355 FUEL number.

Leaf collection was carried out in 2011, always in the morning, in the garden of the Chemistry Department of Londrina State University in the months of January, April, July and October during the summer, autumn, winter and spring of the southern hemisphere. The collection was made carefully, rejecting leaves damaged by fungus, insects or mechanical means. Drying was carried out in the shade for twelve days at room temperature with leaves protected from humidity and possible attacks by fungus, insects and rodents. The leaves were then ground in a blender with the aim of obtaining small fragments for subsequent extraction.

2.2. Reagents

All the reagents used for extract preparation were of analytical grade and bought from VETEC. Methanol and acetonitrile, both of HPLC grade, were bought from VETEC Tedia and J. T. Baker, respectively. A Millipore Milli-Q Gradiente system was used for preparation of the mobile phase.

2.3. Extract preparation and fractioning

The crude extracts were prepared using mixtures of acetone, chloroform, dichloromethane and ethanol whose proportions were varied according to the simplex–centroid mixture design presented in Table 1 and illustrated in Fig. 1. Extraction mediums consisting of the four pure solvents, their six binary 1:1 mixtures, four ternary 1:1:1 mixtures and their quartenary 1:1:1:1 mixture were investigated. For each season 19 crude extracts were prepared in random order including five replicates at the (1:1:1:1) design center point so that experimental error could be determined.

Each crude extract was prepared adding 60 mL of extractor solvent to 12 g of crushed and dried leaves. The mixtures remained in an ultrasound bath for 30 min with the bath water being changed every ten minutes to maintain constant temperature. Then the extracts were filtered through common filter paper with the leaves being consecutively remacerated eight more times.

The crude extracts were concentrated in a rotary evaporator at 60 °C (±3 °C) and maintained in the shade under forced ventilation for 15 days to remove all solvent. After reaching constant weight, the extracts were fractionated by liquid–liquid extraction according to the scheme given by Soares et al. [23]. The crude extracts were redissolved in about 10 mL of their respective extraction mixtures, Table 1. The resulting mixture was homogenized with 24 mL of methanol and 6 mL of water for 10 min in an ultrasound bath and then filter through filter paper. The filtrate was transferred to a separation funnel and acidified with a few drops of concentrated sulfuric acid. Later about 15 mL of chloroform (in triplicate) was added resulting in the appearance of two phases, a dense organic phase and a lighter water/alcohol phase. The organic phase was transferred to a round-bottom flask and submitted to concentration in a rotary evaporator at a temperature of 50 °C (±3 °C), resulting in the separation of the organic phase of intermediate polarity that is rich in coumarin and phenolic compounds. The gravimetric results of these fractions were obtained after reaching constant weight.

2.4. Chromatographic analysis

These organic fractions were submitted to RP-HPLC-DAD and UPLC–ESI-MS analyses. 10 mg of each fraction was redissolved in 200 μL of methanol. Then 20 μL of this solution was diluted with 980 μL (1:50 v/v) of mobile phase consisting of (17.5:17.5:65.0 v/v/v) methanol:acetonitrile:water. The diluted samples were filtered through 0.22 μm Millipore Millex membrane filter. The HPLC analyses were performed with a Thermo Model LC Pump Plus high performance liquid chromatograph coupled with a Finnigan Surveyor PDA Plus diode array detector. The chromatographic conditions employed were: Gemini C_{18} ODS PN0380 Phenomenex (250 mm × 4.6 mm) column, with 5 μm particle size, 23 °C, 20 μL injection volume, 1 mL min^{-1} isocratic elution and monitoring for 25 min at the 274 nm wavelength of the cumarin maximum absorption.

To confirm and identify the secondary metabolites previously detected by RP-HPLC-DAD, a Waters ACQUITY UPLC™ system (Waters, Milford, MA, USA) equipped with ACQUITY UPLC BEH C_{18} column (50 mm × 2.1 mm; 1.7 μm) and coupled to a Quattro Micro triple-quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray ionization (ESI) interface was used.

Table 1
Mixture design solvent proportions, maximum absorbances, averages, standard deviations and replicate standard deviations obtained from the spectral profiles of coumarin in the four seasons.

Fraction	Solvents				Coumarin absorbance/274 nm (10^5)			
	Ethanol	Acetone	Dichlor.[a]	Chlorof.[b]	Summer	Fall	Winter	Spring
e	60	0	0	0	2.45	1.40	1.65	1.32
a	0	60	0	0	0.77	1.18	1.04	0.96
d	0	0	60	0	0.36	1.01	0.48	0.52
c	0	0	0	60	0.55	0.87	0.93	0.34
ae	30	30	0	0	2.08	1.72	1.96	0.87
de	30	0	30	0	0.39	1.04	0.62	0.72
ce	30	0	0	30	1.96	1.53	2.02	0.75
ad	0	30	30	0	0.87	0.46	0.71	0.69
ac	0	30	0	30	0.72	0.81	0.77	0.83
cd	0	0	30	30	1.46	1.16	0.36	0.99
ade	20	20	20	0	1.65	1.23	0.48	0.85
cde	20	0	20	20	1.27	0.17	0.56	0.47
ace	20	20	0	20	1.21	1.07	1.01	0.95
acd	0	20	20	20	0.57	0.82	0.40	0.37
r_1	15	15	15	15	1.16	1.39	1.04	1.16
r_2	15	15	15	15	1.75	1.26	1.55	0.95
r_3	15	15	15	15	1.59	0.99	2.04	1.00
r_4	15	15	15	15	0.79	0.75	1.11	0.82
r_5	15	15	15	15	1.12	0.94	0.81	0.91
Average					1.20	1.04	1.03	0.82
Standard deviation					0.60	0.36	0.56	0.25
Replicate standard deviation					0.39	0.25	0.48	0.12

e: ethanol; a: acetone; d: dichloromethane; c: chloroform.
[a] Dichloromethane.
[b] Chloroform.

The chromatographic conditions employed were: 25 °C, 5 μL injection volume and 3 μL min^{-1} isocratic elution with methanol:water (7:3 v/v) as the mobile phase for 4 min. The MS analyses were carried out in both the positive and negative ionization modes owing to the acidic and basic natures of the secondary metabolites in the *Mikania* plant. The data were obtained in the *SCAN* mode within the 120–220 *m*/*z* interval. Unfortunately, there was no opportunity to perform analysis in the MS/MS mode. The ionization source conditions were: 3 kV capillary voltage, 150 °C source temperature, 80 L h^{-1} cone gas flow, 800 mL h^{-1} dissolution gas flow and 350 °C dissolution temperature. The nitrogen nebulization gas was 99% pure. The data were processed using the MassLynx v4.1 software.

3. Statistical methods

Response surface analysis has been used to model and optimize processes involving vegetal material [24–27]. Here linear, quadratic and special cubic models are investigated [28,29]. Linear models are given by the first summation in the equation,

$$\hat{y} = \sum_i b_i x_i + \sum_i \sum_{<j} b_{ij} x_i x_j + \sum_i \sum_{<j} \sum_{<k} b_{ijk} x_i x_j x_k$$

where, $\hat{y}$ is the predicted absorbance or intensity value, x_i the solvent proportion and b_i, the linear blending model coefficients. The second order term is included in the quadratic model. Significant synergic and antagonistic binary effects, given by the b_{ij} coefficients, occur when the spectral signal of a binary mixture is significantly different than the average of its corresponding pure solvent signals. A significantly larger mixture signal indicates synergism while a smaller one occurs when there is an antagonistic interaction. The b_{ijk} coefficients represent possible ternary effects and are significant when ternary mixtures give significantly different response values compared to those of their corresponding pure solvent and binary mixtures. The simplex–centroid design is recommended since it is capable of determining all of the above models including the special cubic one whereas the simplex lattice model is restricted to obtaining linear and quadratic models.

The statistical models and ANOVA regression results were performed using Statistica 6.0 (Statistica for Windows 6.0, Statsoft, Tulsa, OK, USA, 1999).

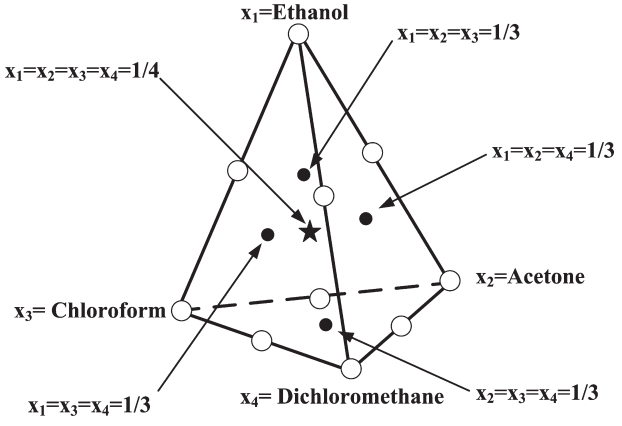

Fig. 1. Simplex–centroid mixture design for the ethanol, acetone, chloroform and dichloromethane solvents.

4. Results and discussion

4.1. Identification of secondary metabolites

Fig. 2 shows the RP-HPLC-DAD chromatograms of the organic fractions of the *Mikania* crude extracts for all four seasons. The presence of two major peaks can be observed for each season.

The most intense peak with a 13.2 min retention time was detected in all the analyzed fractions and presented a spectral profile that is consistent with the structure of coumarin with maximum absorptions at 202, 276 and 312 nm. In order to confirm the presence of coumarin the organic fractions were analyzed in the positive ionization mode by UPLC-MS. In all analyses a peak with a retention time of 1.12 min was detected and analysis of its mass spectra showed a predominant peak of greatest relative abundance

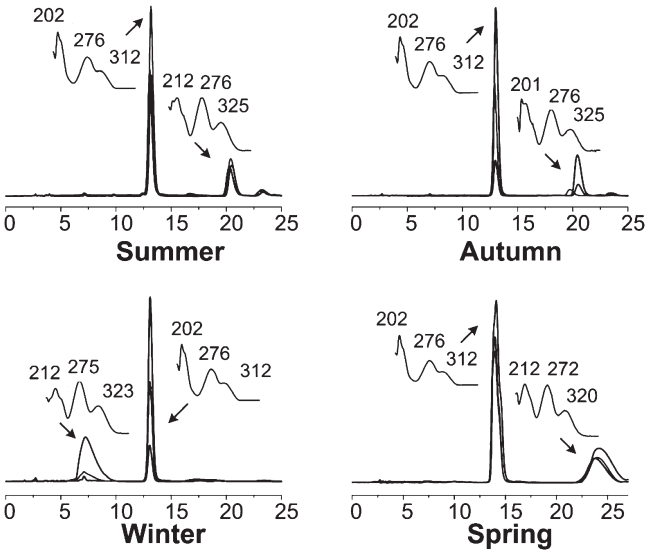

Fig. 2. Chromatograms of the organic fractions of the *Mikania laevigata* plant and their relevant UV profiles obtained at the replicated design center point, (1:1:1:1) ethanol–acetone–dichloromethane–chloroform extraction mixture, for the four seasons.

for a principal ionic species with m/z of 147 ($[M + H]^+$), characteristic of protonated coumarin. This can be seen in Fig. 3 that shows a total ions chromatogram with the mass spectra of the peak corresponding to the retention time of 1.12 min for the (1:1:1:1) ethanol, acetone, dichloromethane and chloroform mixture.

The second less intense peak in Fig. 2 does not have good resolution and has variable retention times depending on the season of sample collection, about 6 min for winter, 24 min for spring and 21 min for summer and autumn. However, in spite of the large variations in the elution times their spectral profiles are very similar between 240 and 370 nm with maximum absorptions around 276 and 323 nm. These spectral bands are consistent with the structures of some phenolic derivatives of cinnamic acid, such as *o*-coumaric acid that is a precursor of coumarin [30]. In order to confirm the presence of this acid the organic fractions were also analyzed in the negative ionization mode by UPLC-MS. In all of the total ion chromatograms of the analyzed fractions two peaks with 1.24 and 1.56 min retention time were observed, Fig. 4.

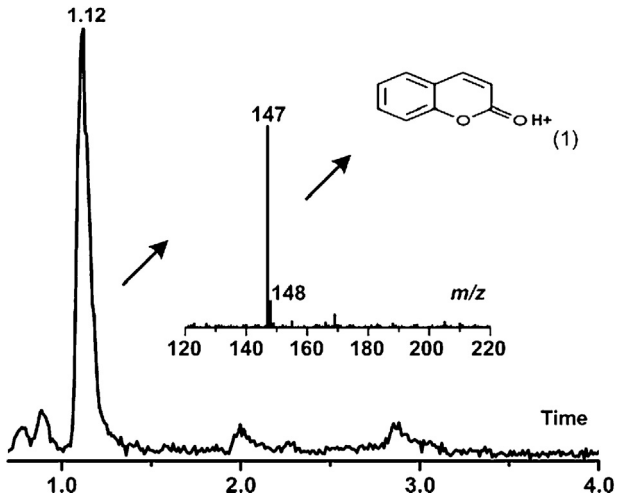

Fig. 3. Total ions chromatogram and ESI mass spectra of the ion corresponding to the 1.12 min retention time peak in the organic phase of the crude *Mikania* spring extract for the quartenary mixture of the simplex–centroid design.

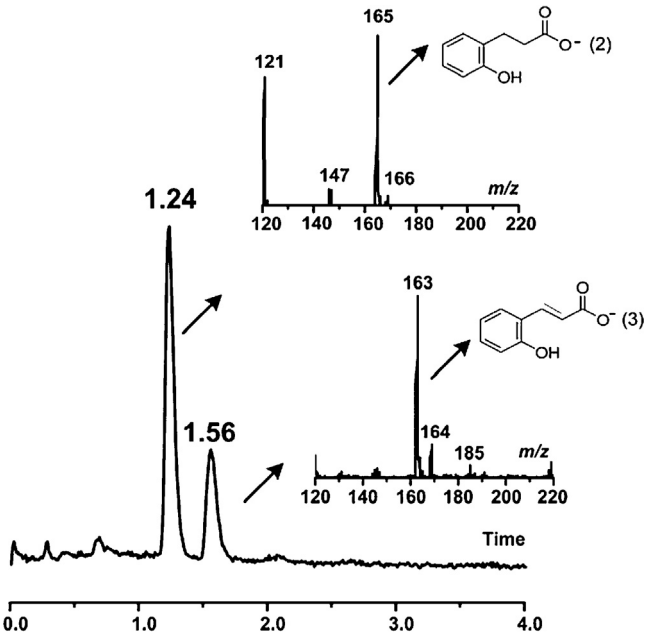

Fig. 4. Total ion chromatograms and ESI mass spectra of the ion corresponding to the peaks with retention times of 1.24 and 1.56 min for the organic fraction of the crude *Mikania* spring extract for the quaternary mixture of the simplex–centroid design.

For the more intense peak at 1.24 min. two mass/charge signals were observed (2), one with m/z of 165 ($[M - H]^-$) and the other less intense one at m/z of 121 ($[M - H]^-$), that is probably the result of a loss of CO_2 characteristic of the phenolic acids. These data are consistent with the presence of melilotic acid [31], 3-(2-hydroxyphenyl) propionic acid. The chromatographic peak at 1.56 min showed the existence of one predominant ion (3) with a m/z signal of 163 ($[M - H]^-$) indicative of deprotonated *o*-coumaric acid. These substances have very similar UV spectral profiles consistent with those observed in Fig. 2 [32–34].

4.2. Seasonal and solvent effects

The seasonal and solvent extraction effects on coumarin, the principal active substance of the *Mikania* plant, *o*-coumaric acid, the coumarin biosynthesis precursor, and melilotic acid were determined from the simplex–centroid design results. For each RP-HPLC-DAD analyzed extract the intensity of maximum absorption at 274 nm was measured. Table 1 contains these intensity values, their averages and standard deviations for each season. The last line in the table presents the standard deviations of the quintuplicate experiments, r_1–r_5 which furnishes estimates of the experimental error of the extraction process.

Comparison of the averages indicates that the plant in summer with an average intensity of 1.20 au contains more coumarin than in the fall and winter that have similar average intensities, 1.04 and 1.03 au. Spring appears to have the smallest amount of coumarin with an average of only 0.82 au. However a 0.34 au error estimate in the absorbance determinations in Table 1 is obtained from the pooled standard deviation of the individual replicate standard deviations. As such a 0.08 au error value is estimated for the average values in Table 1. Since this value is comparable to the differences in the average values, paired *t*-tests were performed to determine if the seasonal effects are indeed significant.

The low average for the spring is seen to be statistically different from all the other averages above the 90% confidence level. In fact the difference between the summer and spring averages is statistically significant well above the 99% confidence level. The summer average is statistically larger than the fall and winter

averages above the 80% confidence level. These results confirm the ordering in the average values indicating that a significant seasonal effect on the coumarin amount in the *Mikania* plant does indeed exist. In the summer the *Mikania* plant is in its most productive vegatative stage characterized by elevated biomass [35] especially for the yonger leaves. The greater efficiency of the extraction procedure in the summer may be directly related to a higher proportion of younger leaves during this season. These results are in agreement with those observed by Biavatti et al. [36] and Bertolucci et al. [37].

Fig. 5a and b show bar graphs of the intensities at maximum absorbance of the guaco summer and fall extracts for each simplex–centroid design mixture. Remembering that the estimated error in the intensities of the extraction procedure is ±0.34 au one sees that the only media that appear to be clearly extracting higher amounts of coumarin are pure ethanol and the 1:1 ethanol mixtures with ethyl acetate and chloroform in the summer and perhaps the fall. Ethanol is an amphiprotic solvent with a predominant behavior of forming hydrogen bonds as either a proton donor or acceptor.

Seasonal and solvent extraction effects were also investigated for *o*-coumaric and melilotic acids. Table 2 contains the intensity values of the strongest mass spectral signal/lines of these compounds along with their average, standard deviation and

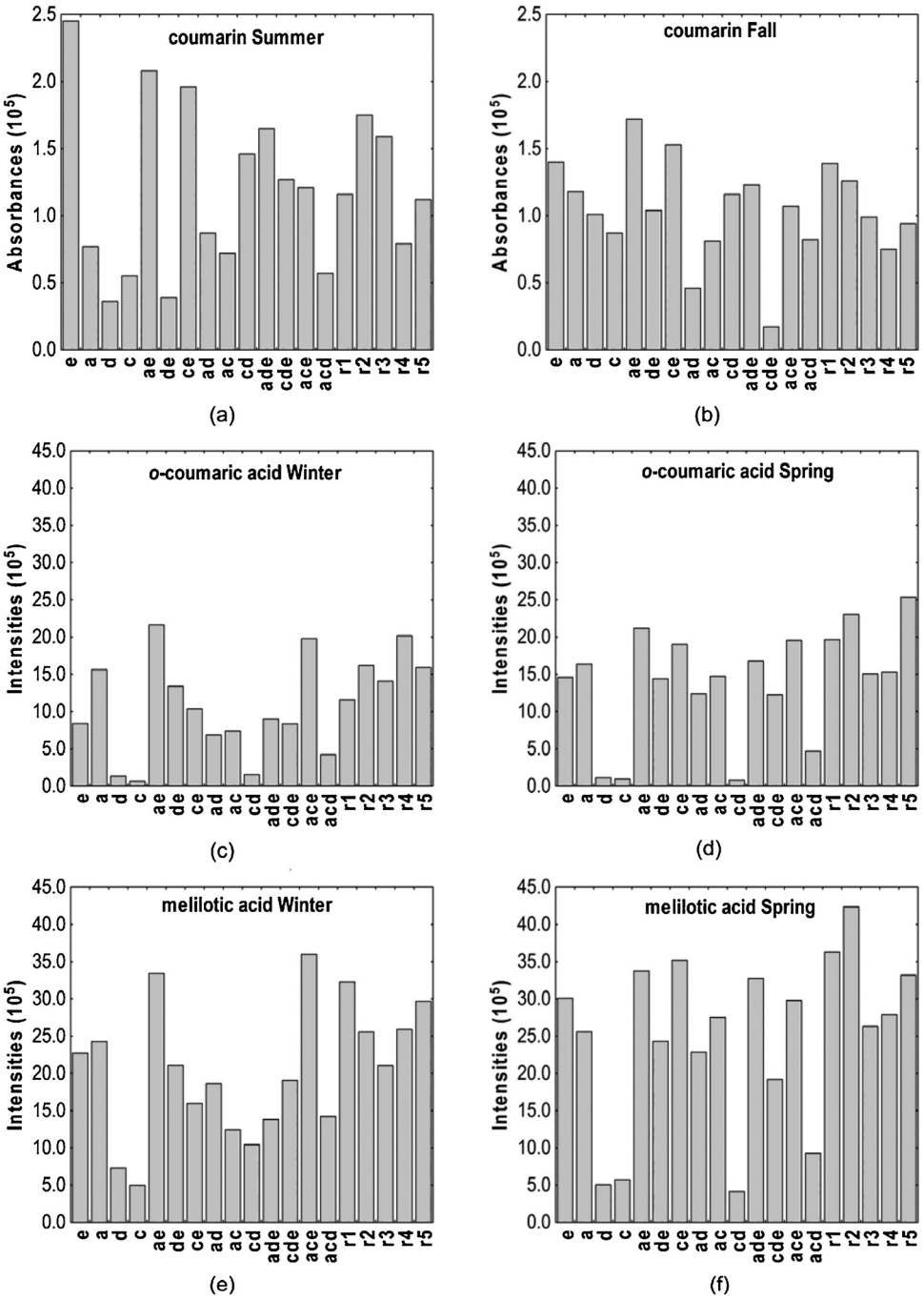

Fig. 5. (a) Maximum absorbances of the coumarin spectral profile for each solvent extractor of the mixture design for the summer and (b) fall, (c) intensities of the strongest UPLC-MS peaks for each solvent extractor for o-coumaric acid in the winter and (d) spring, and (e) for melilotic acid in the winter and (f) spring. The e, a, c and d letters at the bottom of the bar graphs represent the ethanol, acetone, chloroform and dichloromethane solvents.

Table 2
Intensities of the strongest UPLC-MS lines of I-coumaric and melilotic acids obtained for each extract of the mixture design in the four seasons.

Fraction	Intensity *o*-coumaric acid (10^5) *m*/*z* 163 ($[M-H]^-$)				Intensity melilotic acid (10^5) *m*/*z* 165 ($[M-H]^-$)			
	Summer	Fall	Winter	Spring	Summer	Fall	Winter	Spring
e	2.67	4.76	8.41	14.59	5.04	8.11	22.73	30.08
a	3.67	5.43	15.64	16.37	0.85	6.36	24.26	25.59
d	0.29	1.04	1.34	1.15	1.41	6.19	7.302	5.05
c	0.51	0.55	0.66	0.95	2.38	3.80	4.980	5.73
ae	4.69	14.22	21.64	21.2	5.38	10.04	33.41	33.74
de	2.69	6.59	13.38	14.43	4.92	27.40	21.09	24.29
ce	2.87	8.96	10.35	19.04	11.58	13.38	15.98	35.20
ad	3.50	1.79	6.85	12.39	5.27	4.19	18.62	22.87
ac	2.95	3.64	7.39	14.71	6.02	6.53	12.45	27.53
cd	0.82	1.47	1.55	0.800	4.51	9.48	10.42	4.14
ade	4.91	3.32	8.98	16.79	12.38	5.16	13.84	32.76
cde	3.79	6.92	8.38	12.27	10.57	12.42	19.09	19.20
ace	5.94	9.32	19.8	19.56	13.97	7.89	35.97	29.79
acd	1.61	3.93	4.22	4.680	3.29	7.54	14.23	9.27
r_1	3.90	7.71	11.57	19.67	11.34	6.72	32.27	36.28
r_2	5.16	4.02	16.17	23.05	11.66	1.61	25.56	42.36
r_3	5.2	7.16	14.09	15.05	12.68	4.18	21.06	26.32
r_4	5.21	5.43	20.17	15.32	9.26	4.99	25.94	27.89
r_5	5.09	3.25	15.91	25.34	12.78	5.36	29.69	33.19
Average	3.44	5.23	10.87	14.19	7.91	7.97	21.52	23.23
Standard deviation	1.71	3.37	6.44	7.44	4.79	5.54	10.75	12.10
Replicate standard deviation	0.57	1.93	3.16	4.58	3.14	1.89	10.52	6.50

replicate standard deviation values. The average intensity values of the *m*/*z* 165 ($[M-H]^-$) melilotic acid ion are much larger than those of the *m*/*z* 163 ($[M-H]^-$) *o*-coumaric acid one. For both substances the average intensities in the winter and spring are more than twice those of the summer and fall. For *o*-coumaric acid the seasonal effect appears to be large throughout the year since all of the averages are significantly different from one another at or above the 99% confidence level. On the other hand the averages for the melilotic acid ion in the summer and fall are statistically the same whereas those for the winter and spring are statistically different at the 95% confidence level. So the abundance of melilotic acid appears to be about three times larger in the winter and spring compared with the summer and fall. The role of the biosynthetic precursor of coumarin, *o*-coumaric acid, seems especially relevant since it appears to have its highest concentrations in the spring and coumarin abundance was found to be greatest in the summer.

The bar graphs in Fig. 5c–f show the intensity values of Table 2 for *o*-coumaric and melilotic acids for the winter and spring. The profiles of all four graphs are very similar. Indeed the correlation coefficients between the *o*-coumaric and melilotic acids intensities in this table are 0.88 and 0.96 for winter and spring, respectively. Highest amounts of these acids are extracted by mixtures containing both ethanol and acetone. In fact these mixtures appear to be extracting more of these substances than either pure ethanol or acetone. Notably, very small amounts of these acids are extracted by pure chloroform or dichloromethane or their 1:1 binary mixture. However mixtures of each of these solvents with acetone or ethanol results in large increases in the amounts of extracted acid. These behaviors suggest the existence of synergic effects between solvents and the importance of solvent molecule–solvent molecule interactions in the extraction process.

To confirm the existence of significant synergic effects between solvent molecules mixture models were fitted to the intensity data of coumarin, *o*-coumaric and melilotic acids. The general equation, presented earlier, contains a linear term for each solvent. If no significant solvent–solvent type interaction exists, i.e., a linear model is adequate to represent the data, the coefficients of these terms indicate the relative extraction capacity of each individual solvent. If a quadratic model is more adequate the 2nd order coefficients provide information about the most significant binary solvent–solvent interactions. The last terms in the equation are cubic ones and their coefficients represent possibly important ternary solvent interactions. The coefficients of the mixture models are presented in Table 3. All the mixture models there do not suffer from statistical lack of fit at the 95% confidence level as determined by an analysis of variance (ANOVA) of the regression results. Since the models for coumarin in the fall and spring do present lack of fit their coefficients are not reliable and therefore not presented in the table.

The linear ethanol and acetone coefficients, b_e and b_a, are significant at the 95% confidence level in almost all the models. The two coumarin models in Table 3 for summer and winter are linear with no evidence of significant interaction coefficients among the solvents. The linear blending ethanol coefficients are about twice as large as the acetone one indicating that ethanol is the solvent of choice for extracting coumarin from the *Mikania* plant. In Fig. 5a and b ethanol exracts more coumarin in the summer and fall than does any other pure solvent. Although the amounts extracted by the acetone–ethanol and chloroform–ethanol 1:1 binary mixtures are comparable to the quantities extracted by ethanol no significant binary interactions are predicted by the model.

For the *o*-coumaric acid models in all the seasons the b_a coefficient is larger than b_e indicating that acetone has superior linear blending properties for this extraction. The *o*-coumaric profiles in Fig. 5c and d show that pure acetone extracts more *o*-coumaric acid than pure ethanol in spring and winter. The models for summer, fall and winter have significant interactions between acetone and ethanol as can be seen in Table 3. Inspection of the winter bar graph in Fig. 5c shows that the quantity of *o*-coumaric acid extracted by the 1:1 binary ethanol–acetone mixture is much larger than the average of the quantities extracted by pure ethanol and acetone. On the other hand, the spring model of *o*-coumaric acid does not contain a significant synergic interaction between these two solvents. The *o*-coumaric intensity of 21.2 is mostly accounted for by the linear effects given by the average of the pure acetone and ethanol intensities, 15.5.

The *o*-coumaric acid summer, fall and spring models predict significant synergic interactions for chloroform and ethanol. This can be understood by inspecting Fig. 5d. The 1:1 binary

Table 3
Significant mixture model coefficients for validated models of coumarin, *o*-coumaric acid and melilotic acid.

	Coumarin		*O*-coumaric acid				Melilotic acid			
	Summer	Winter	Summer	Fall	Winter	Spring	Summer	Fall	Winter	Spring
b_e	2.4	1.9	2.2	5.4	–	13.8	5.0	8.1	22.8	28.9
b_a	1.0	1.1	3.4	6.0	15.0	16.0	–	6.4	24.3	24.9
b_d	0.9	1.0	–	–	–	–	–	6.2	–	–
b_c	–	–	–	–	–	–	–	–	–	–
b_{ae}	–	–	11.2	25.3	44.3	–	–	–	–	–
b_{de}	–	–	–	–	–	–	–	81.5	–	–
b_{ce}	–	–	11.0	21.5	–	51.6	32.0	38.2	–	70.6
b_{ad}	–	–	–	–	–	–	17.0	–	–	–
b_{ac}	–	–	–	–	–	–	18.1	–	–	–
b_{cd}	–	–	–	–	–	–	–	–	–	–
b_{ade}	–	–	–	–	–	–	155.6	−332.0	–	–
b_{cde}	–	–	–	–	–	–	–	–	–	–
b_{ace}	–	–	–	–	–	–	112.5	227.2	463.1	–
b_{acd}	–	–	–	–	–	–	–	–	–	–

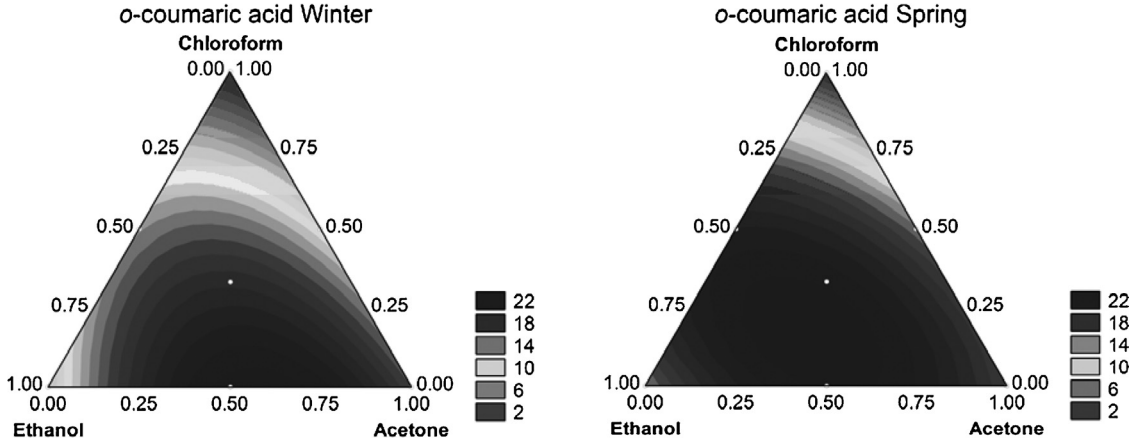

Fig. 6. Response surface model plots for the line intensities of *o*-coumaric acid in the (a) winter and (b) spring.

chloroform-ethanol mixture has an intensity of 19.0 substantially larger than the average of the pure ethanol and chloroform intensities, 7.8. In fact the mass spectral intensity for the acid extracted by pure chloroform is only about 1.0.

In winter the melilotic acid model has $b_a > b_e$ whereas this relation is inverted in the spring. Fig. 5e shows the intensity of this acid extracted with acetone is slightly larger than that for pure ethanol. However the ethanol extraction yield is larger than the acetone one in the spring as shown in Fig. 5f. The spring model has a 95% confidence level significant binary synergic interaction between ethanol and chloroform. As can be seen in this figure the intensity associated with the 1:1 binary chloroform-ethanol mixture, 35.2, is much larger than the average of the intensities for pure ethanol and chloroform, 17.9.

The winter model for melilotic acid does not contain significant binary interactions but does have an important ternary synergic interaction involving acetone, ethanol and dichloromethane. In Fig. 5e the intensity for the ternary 1:1:1 acetone-chloroform-ethanol extraction is the largest whereas the weighted average of the ethanol, acetone and chloroform intensities along with those of their 1:1 binary mixtures is much smaller.

Finally it should be remarked that only one of the significant interaction coefficients, the acetone-dichloromethane-ethanol one in the fall for melilotic acid, is negative which is indicative of an antagonistic interaction involving these three solvents. However it is more than compensated by two synergic binary interactions and one ternary interaction. The fact that all the binary and ternary coefficients in Table 3 are positive, except one, shows that molecular interactions involving more than one solvent can enhance yields of extracted secondary metabolites.

Response surface contour plots are very convenient for illustrating these solvent interactions. Fig. 6 contains contour plots for the *o*-coumaric acid line intensities for the winter and spring. The ethanol-acetone synergic interaction for the winter is very visible with maximum predicted values close to the 1:1 binary ethanol-acetone mixture point. Even though the 1:1 binary ethanol-acetone mixture has a higher line intensity than the 1:1 chloroform-ethanol mixture, the latter solvent pair is the only one with a significant model interaction because chloroform is a very poor extractor.

5. Conclusions

The results indicated that significant seasonal effects exist for coumarin, *o*-cumaric and melilotic acid amounts in the *Mikania* plant. Highest coumarin concentrations were encountered in the summer whereas its *o*-coumaric acid precursor as well as melilotic acid were most abundant in the spring. Also the solvent effects are seen to be significant. In summer, ethanol extracted the highest amount of coumarin in the plant. Highest amounts of *o*-coumaric and melilotic acids were extracted by mixtures containing both ethanol and acetone in the winter and spring. Statistical mixture models indicated that synergic binary interactions, especially those involving ethanol with acetone or chloroform, are important in the *Mikania* extraction process. The chromatographic analysis of mixture design extractions has been shown to provide important

information about secondary metabolite amounts during the four seasons that may be potentially useful in plant applications.

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