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Influence of Agronomic Variables on the Composition of Mate Tea Leaves (*Ilex paraguariensis*) Extracts Obtained from CO_2 Extraction at 30 °C and 175 bar

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The aim of this work is to assess the influence of light intensity (plants with direct sun exposure and in a controlled light intensity) and age of leaves (6−24 months) on the characteristics of the extracts of mate tea leaves obtained from carbon dioxide at high pressures. Samples of mate were collected in an experiment conducted under agronomic control at Industria e Comercio de Erva-Mate Barão LTDA, Brazil. The content of selected organic compounds of the extracts was evaluated by gas chromatography together with mass spectrometry. Quantitative analysis of caffeine, theobromine, phytol, vitamin E, squalene, and stigmasterol was performed, and the results showed that field variables exert a strong influence on the liquid yield and on the chemical distribution of the extracts.

KEYWORDS: Mate tea leaves (*Ilex paraguariensis*); agronomic variables; drying; supercritical extraction; GC/MSD

INTRODUCTION

Mate (*Ilex paraguariensis*) is an important natural product in the economic and cultural context of South Brazil with many relevant properties such as antiinflammatory, therapeutic, antirheumatic, stimulating, and diuretic properties attributed to it (*1*−*4*). To take a glance at the mate market, we consider that only in this region of Brazil can one find more than 40 mate processing industries and about 180 000 medium and small properties crowded together in a narrow area dedicated almost exclusively to cultivating this raw material (*5*, *6*). Considering the fact that all of those industries direct their efforts to produce the same base product, comminuted mate leaves for teas; that processing of mate leaves within industrial environment is nowadays conducted in a very rudimentary way; and that this raw material was only recently available from other countries (*7*), it is not surprising that the strong competition established has required company investments toward producing higher value products.

Recently, the development of new separation techniques has gained increasing importance in the chemical and food industries due primarily to the imposed environmental and public health regulations and the necessity of minimizing energy requirements (*8*, *9*). It is well-known that carbon dioxide is an appropriate solvent for supercritical extraction purposes in food industry since it is nontoxic, nonflammable, nonexplosive, readily available, and has a low critical temperature and pressure that avoid degradation of thermolabile compounds (*9*−*11*). The advantages of using near critical carbon dioxide extraction prevail when small raw material amounts and high quality products are processed.

Despite the importance of mate tea leaves in the social and economic context of South Brazil, the literature is very scarce on works focusing on the extraction of organic compounds of this raw material obtained from supercritical fluid extraction (*3*, *4*, *12*). Saldanã et al. (*12*) presented a study on the extraction of methylxanthines from mate tea leaves using CO_2 as solvent and ethanol as cosolvent in the temperature range of 40−80 °C and pressures up to 400 bar. Esmelindro et al. (*4*) presented an investigation regarding the effect of temperature and solvent density on the extraction yield and on the distribution of chemical components of the extracts. Some works are available in the literature focusing on the content of methylxanthines of distinct populations of *I. paraguariensis* (*13*, *14*). Cansian (*14*) analyzed the chemical profile of 20 *I. paraguariensis* populations that naturally occur in Brazil, indicating low variation in the content of volatile organic compounds among those native populations. The results obtained by these authors suggested that intrapopulation agronomic conditions, like light intensity, age of leaves, and microclimate, may be an important factor concerning the volatile organic compounds distribution in *I. paraguariensis*. It should be noted that both works cited above deal with native populations; that is, they investigate plants without agronomic control.

In this context, the aim of this work is to assess the influence of some agronomic variables (light intensity and age of leaves) on the characteristics of the extracts obtained from high pressure CO_2 extraction of mate tea leaves. It should be noted that this

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work is not focused on the comparison of different extraction methods but on evaluating the effects of the mentioned agronomic variables on the content of some compounds presented in the extracts. The extraction method selected was high pressure carbon dioxide extraction due to the purity (solvent-free) of the extract obtained and also the low temperatures employed in this process. Of course, the extraction temperature and pressure (solvent density) exert a strong influence on the extraction yield and on the chemical profile of the extracts (see ref *8*). In this sense, as this work is focused on the evaluation of agronomic variables and drying method on the liquid yield and chemical profile of mate tea leaves extracts, the extraction experiments were performed in a semibatch laboratory-scale unit keeping the temperature constant at 30 °C and the pressure constant at 175 bar. In addition, the influence of drying method (microwave and vacuum oven) is also presented in this work. The quantitative analyses of caffeine, theobromine, phytol, squalene, vitamin E, and stigmasterol were conducted using gas chromatography/mass spectrometry (GC/MS). The extraction yield, extraction kinetics, and chemical composition of the extracts are reported in this work.

MATERIALS AND METHODS

Sample Collection and Materials. Mate tea leaves samples were collected in an experiment conducted under agronomic control at Industria e Comercio de Erva-Mate Barão LTDA. At the beginning of the experiment, all plants were about 7 years old, and all leaves and ramifications were totally cut off. At this time, the age of leaves was set to zero months. All plants were handled under identical fertilization conditions. Half of the experiment was covered with a covering device that absorbed 75% of the natural light incidence. Seven plants were selected to assemble the treatment sample, where fractions from the top, middle, and bottom of each tree were sampled and homogenized to form the sample of each treatment. The samples were dried in two distinct methods: in a vacuum oven (24 h at 35 °C) and in a domestic microwave oven (5 min). The final moisture of all samples was around 2%. After they were dried, the samples were stored under a nitrogen atmosphere until the extraction to prevent oxidation. The CO_2 employed in the extractions (White-Martins, 99.5% in liquid phase) was used without further treatment.

Apparatus and Extraction Procedure. The experiments were performed in a laboratory-scale unit, discussed in detail by Rodrigues et al. (*8*), which consisted basically of a CO_2 reservoir, two thermostatic baths, a syringe pump (ISCO 500D), a 0.1 dm^3 jacketed extraction vessel, an absolute pressure transducer (Smar, LD301) equipped with a portable programmer (Smar, HT 201) with a precision of ±0.125 bar, a collector vessel with a glass tube, and a cold trap. Typically, amounts of around 25 g of comminuted mate tea leaves were loaded into the extraction vessel. The CO_2 was pumped at a constant flow rate of 2 g min^{-1} into the bed, which was supported by two 300 mesh wire disks on both ends, and was kept in contact with the herbaceous matrix for at least 1 h to allow the system to stabilize. Afterward, the essential oil was collected by opening the micrometering valve and the CO_2 mass flow was accounted for the pump recordings. After that, the mass of the extracted oil was weighed, the glass tube was reconnected to the equipment, and this procedure was performed until no significant mass was extracted or, as in some cases, the extraction period exceeded a preestablished limit. The experiments were accomplished in approximately 400 min, isothermally at constant pressure. All experiments were performed at fixed temperature and pressure conditions: 30 °C and 175 bar, respectively. Duplicate runs were performed for all conditions, leading to an average standard error in the extraction yields of 0.03.

Extract Characterization. The extracts were analyzed through a GC/MS (Shimadzu QP5050A), using a capillary column DB5 (30 m, 0.25 mm, 25 μm); a flow rate of 1 mL min^{-1}; an electronic impact mode of 70 eV; and a split mode (split ratio 1:10). Forty milligrams of the extract was dissolved in 1 mL of dichloromethane, and 1 μL of this solution was injected into the GC/MS system. The column temperature was programmed 70 °C/3min, 3 °C/min to 230 °C, and 4 °C/min to 310 °C/15 min. Helium was the carrier gas, and the injection and detector temperatures were 290 and 310 °C, respectively. The identification and quantification of some compounds were accomplished through the analytical standards injection and biphenyl as internal standard by comparing the mass spectra and GC retention time. The analytical standards caffeine, theobromine, phytol, vitamin E, squalene, stigmasterol, and biphenyl were from Aldrich (Palo Alto, CA). For each standard, solutions were prepared (from 250 to 4000 mg L^{-1}) using dichloromethane (Merck, HPLC level) and stored under refrigeration. In all samples, an internal standard (biphenyl 100 mg L^{-1}) was added. Every analysis was replicated at least three times.

The effects of the agronomic variables on the quantitative content of the selected compounds were statistically analyzed by an analysis of variance coupled with a Tukey's test at a 5% confidence level. All statistical analysis were performed in the Statistica software (*15*).

Table 1. Extraction Liquid Yield of Mate Tea Leaves with CO_2 at High Pressures[a]

run	light intensity[b]	age of leaves (months)	drying method	extraction yield[c] ± standard error ($g_{extract}$/100 $g_{rawmaterial}$)	no. of replicates (*n*)
1	high	6	microwave	0.60 ± 0.04	2
2	low	6	microwave	0.22 ± 0.01	2
3	high	12	microwave	1.59 ± 0.03	2
4	low	12	microwave	0.90 ± 0.01	2
5	high	12	vacuum	2.00 ± 0.05	2
6	high	18	microwave	1.88 ± 0.06	3
7	low	18	microwave	0.99 ± 0.05	2
8	high	18	vacuum	2.56 ± 0.04	2
9	low	18	vacuum	1.50 ± 0.03	2
10	high	24	microwave	1.15 ± 0.04	2
11	low	24	microwave	0.19 ± 0.01	2
12	high	24	vacuum	1.76 ± 0.03	2
13	low	24	vacuum	1.33 ± 0.02	2

[a] Extraction conditions: temperature of 30 °C and pressure of 175 bar. [b] High light intensity means that plants are not protected from natural light incidence, whereas low light intensity means that plants were covered with an apparatus that retained 75% of the natural incident light. [c] Average value of the extraction yield obtained from *n* replicates.

RESULTS AND DISCUSSIONS

Extraction Yield. Table 1 presents the extraction liquid yield of the mate tea leaves with carbon dioxide at high pressures. The extraction yield (liquid yield) was defined as the mass of the extract obtained (g of extract) by 100 g of the raw material (mate tea leaves) loaded into the extractor. In fact, the gain of extract mass is a matter of time or, in other words, a matter of carbon dioxide flow through the mate tea leaves bed. In this sense, the extraction yield reported in **Table 1** was calculated after 300 min (600 g of carbon dioxide) in all extractions. From this table, it is clear that the agronomic variables investigated exert a pronounced effect on the amount of volatile matter extracted.

The effects of the investigated variables can be better analyzed from **Figures 1**−**3**. **Figure 1** presents the influence of the age of leaves on the kinetics of the extraction of mate tea leaves, i.e., on the mass gain as a function of time. The coordinates of the curves express the extraction yield by carbon dioxide mass flowed through the bed, as these variables are in fact process variables and can easily be converted into extraction mass or time. For instance, the extraction yield is a variable that does not depend on the amount of raw material used in the extraction,

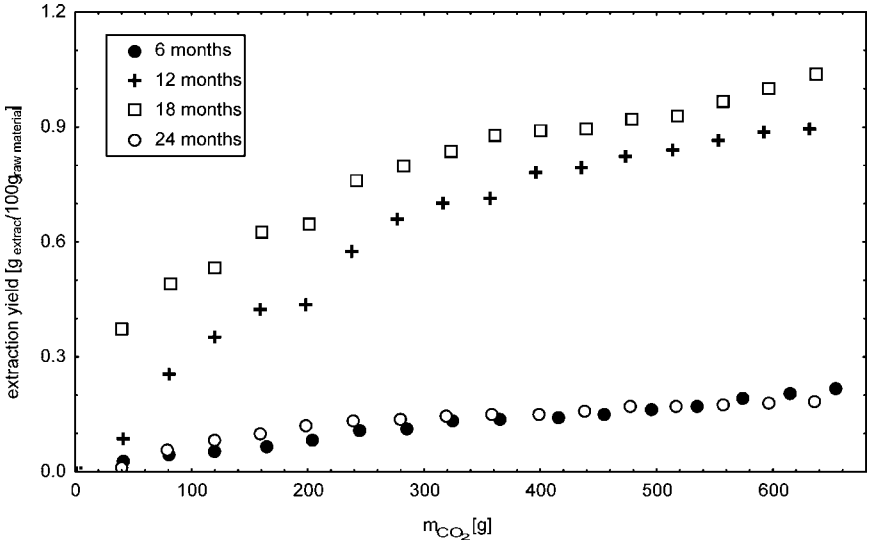

Figure 1. Effects of age of leaves on the kinetics of extraction of mate tea leaves with carbon dioxide at 30 °C and 175 bar. Plants were protected from natural light incidence, and a microwave oven was applied as a dryer.

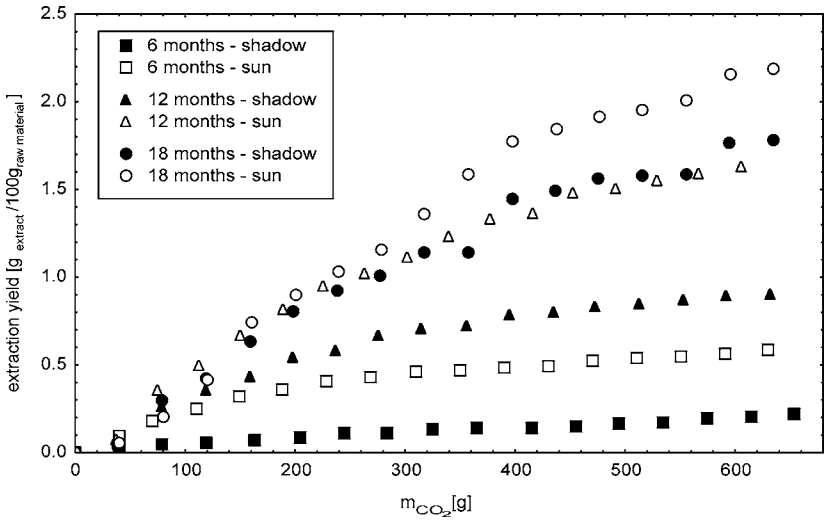

Figure 2. Effects of light intensity on the kinetics of mate tea leaves extraction with carbon dioxide at 30 °C and 175 bar. Samples were dried in a microwave oven.

as is the case of the extraction mass obtained at each point. Also, as the carbon dioxide mass flow rate is kept constant around 2 g min^{-1}, the extraction time is easily converted into mass of carbon dioxide used at each collected point. This figure is related to plants protected from natural light incidence (75% of light retention). A sharp increase in the amount of volatile matter extracted from 6 to 12 months can be observed, followed by an additional increase from 12 to 18 months. For 24 month old leaves, the extraction yield is reduced to similar levels to those of 6 months. These results can be explained in terms of the lifetime of the leaves. Mosele (*6*) pointed out that mate leaves could be down after 18−20 months, and in this sense, there were no leaves of 24 months, but in fact, new young growing leaves. One should note that in commercial mate tea leaves, leaves compose around 75% of the final product, while plant ramifications are responsible for the other part. As the major contribution in the extracts of the plants comes from the leaves and not from the ramifications, the extraction yields are very similar between samples of 6 and 24 months.

Figure 2 presents the effects of light intensity on the kinetics of the extraction of mate tea leaves with carbon dioxide at high pressures, where the samples were dried in a microwave oven. It can be noted that plants directly exposed to sun produce a higher content of volatile matter that can be extracted independently of the age of the leaves. This fact can be attributed to the faster metabolism of the plant at higher light intensity conditions. An inspection of **Table 1** reveals that this observation occurs for all samples investigated.

Figure 3 presents the influence of the drying method on the kinetics of the extraction of mate tea leaves at high pressures. In this figure, plants are directly exposed to natural light. It can be observed that higher extraction yields were obtained when the samples were dried in a vacuum oven, probably due to the more aggressive drying mechanism imposed on herbaceous samples in the microwave drier, which can evaporate some compounds. Some works in the literature (*16*, *17*) pointed out that unlike conventional heating, where a substance is heated from outside to inside, in a microwave oven, the substances absorb energy in several points and transfer it to the rest of the body. In this way, the heating is nonuniform and occurs from inside to outside of the matrix.

Extract Characterization. Figure 4 presents a typical TIC chromatogram of the extract of mate tea leaves obtained by carbon dioxide at 30 °C and 175 bar. As can be noted in this

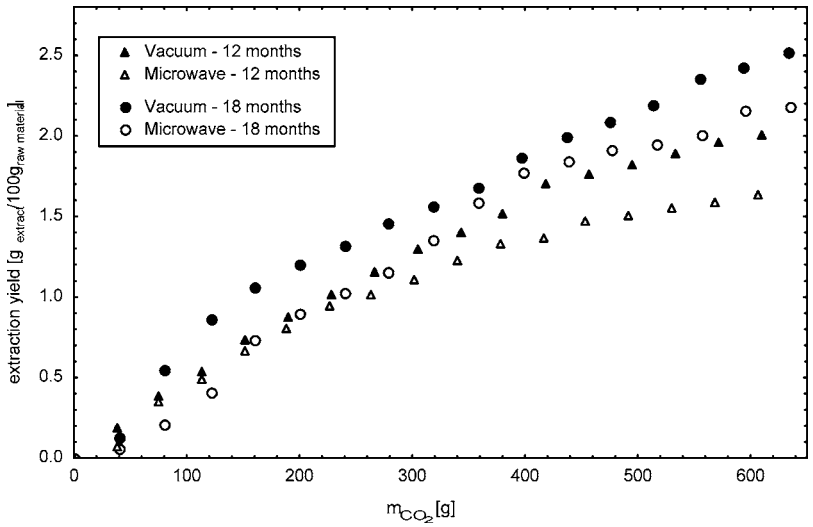

Figure 3. Effects of drying methods on the kinetics of extraction of mate tea leaves with carbon dioxide at 30 °C and 175 bar. Plants received natural light intensity without any sun protection.

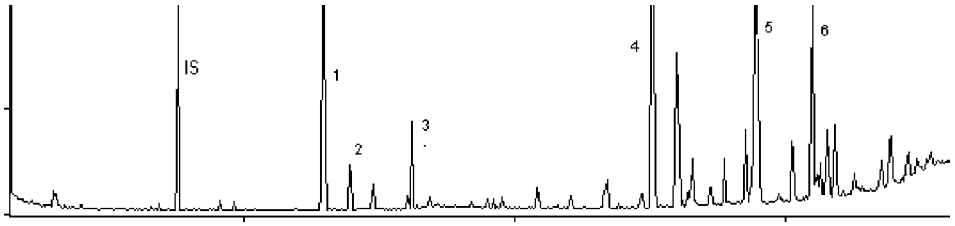

Figure 4. Typical TIC chromatogram of the extract of mate tea leaves obtained by carbon dioxide at 30 °C and 175 bar. 1, Caffeine; 2, theobromine; 3, phytol; 4, vitamin E; 5, squalene; 6, stigmasterol; and IS, biphenyl (internal standard).

Table 2. Quantitative Analysis (Mean Value ± Standard Error) of the Extracts of Mate Tea Leaves Obtained with Carbon Dioxide at 30 °C and 175 bar[a]

run	no. of replicates	caffeine ($mg/g_{extract}$)	theobromine ($mg/g_{extract}$)	phytol ($mg/g_{extract}$)	squalene ($mg/g_{extract}$)	vitamin E ($mg/g_{extract}$)	stigmasterol ($mg/g_{extract}$)
1	4	166.67 ± 11.03	1.16 ± 0.10	29.27 ± 1.03	24.45 ± 2.95	51.15 ± 5.20	129.36 ± 11.59
2	3	535.20 ± 49.55	7.11 ± 1.22	21.70 ± 2.05	9.45 ± 0.53	32.20 ± 0.93	191.74 ± 16.22
3	4	24.84 ± 3.27	0.27 ± 0.04	5.59 ± 0.57	35.97 ± 4.58	52.63 ± 7.85	51.83 ± 5.41
4	5	92.45 ± 6.71	t	14.08 ± 1.40	20.55 ± 2.54	25.64 ± 6.95	49.04 ± 4.15
5	2	9.78 ± 0.75	0.05 ± 0.00	1.36 ± 0.23	35.62 ± 4.47	25.82 ± 4.26	24.18 ± 3.61
6	10	49.85 ± 5.84	0.13 ± 0.01	4.41 ± 0.81	79.03 ± 5.39	49.00 ± 5.05	50.53 ± 4.48
7	7	192.79 ± 24.20	1.42 ± 0.20	8.77 ± 1.16	56.08 ± 12.06	63.29 ± 10.77	111.73 ± 15.83
8	5	11.74 ± 1.34	0.03 ± 0.02	1.05 ± 0.08	85.82 ± 3.73	45.26 ± 2.25	36.96 ± 1.29
9	2	99.50 ± 7.34	1.33 ± 0.17	6.32 ± 0.74	44.10 ± 3.53	38.39 ± 1.56	74.95 ± 2.32
10	5	51.52 ± 5.81	0.27 ± 0.05	11.50 ± 1.58	24.48 ± 3.96	44.43 ± 5.12	52.40 ± 6.83
11	3	162.19 ± 9.38	0.67 ± 0.01	33.49 ± 1.34	24.41 ± 2.80	57.30 ± 4.76	83.07 ± 5.59
12	4	98.26 ± 6.63	0.28 ± 0.07	4.82 ± 0.53	53.43 ± 2.75	31.29 ± 2.34	47.28 ± 5.00
13	2	110.34 ± 1.95	0.33 ± 0.07	2.64 ± 0.11	35.39 ± 0.84	21.66 ± 0.33	32.21 ± 2.11

[a] t, traces (compound amount lower than 0.005 mg of compound per g of extract).

figure, around 30 compounds could be identified in the extracts. In this work, six representative compounds were selected of the extracts that could have potential applications in product formulation: two representing the methylxanthines class (caffeine and theobromine), which are stimulating and energetic compounds; an alcohol (phytol); a steroid (stigmaterol); a major hydrocarbon compound (squalene); and vitamin E, which is a powerful natural antioxidant.

Table 2 presents the quantitative distribution of selected compounds presented in the extracts of mate tea leaves obtained from the extraction with carbon dioxide at high pressures. All analyses were replicated in order to check the experimental reproducibility and to permit the adequate statistical analysis treatment of the results (number of replications of GC/MS analysis of each run is presented in column 2 of the table). The experimental conditions of each run are the same as those labeled in **Table 1**. An analysis of **Table 2** indicates that the chemical distribution of the investigated compounds was strongly affected by the agronomic variables investigated and by the drying method. For instance, when the plants are protected from the natural light incidence (runs 2, 4, 7, 9, 11, and 13), the major compound presented in the extract is always caffeine, while this fact is not verified when the samples come from plants located in shadow areas. **Table 3** presents the analysis of variance of light intensity, age of leaves, and drying method on the quantitative content of selected compounds of the extracts of mate tea leaves obtained from the extraction with carbon dioxide. The values presented in this table are, in fact, mean values of the compound concentrations for each condition. For example, when mate plants are conducted in a shadow area,

Table 3. Statistical Analysis (Analysis of Variance and Tukey's Test) of the Effects of the Agronomic Variables and Drying Method on the Quantitative Content of the Selected Compounds in the Extracts of Mate Tea Leaves Obtained with Carbon Dioxide at 30 °C and 175 bar

	light intensity ($mg_{compound}/g_{extract}$)		age leaves (months) ($mg_{compound}/g_{extract}$)				drying method ($mg_{compound}/g_{extract}$)	
compound	high	low	6	12	18	24	microwave	vacuum
caffeine	58.95 b[a]	198.75 a	350.94 a	42.36 c	88.47 b	105.58 b	159.44 a	65.93 b
theobromine	0.312 b	1.81 a	4.14 a	0.11 d	0.73 b	0.39 c	1.38 a	0.40 b
phytol	8.29 b	14.50 a	25.46 a	7.01 c	5.14 c	13.11 b	16.10 a	3.24 b
squalene	48.40 a	31.66 b	16.95 b	30.71 b	66.26 a	34.43 b	34.30 b	50.87 a
vitamin E	42.80 a	39.75 a	41.68 a	34.69 a	48.99 a	38.67 a	46.95 a	32.48 b
stigmasterol	56.08 b	90.46 a	160.55 a	41.68 d	68.54 b	53.74 c	89.96 a	43.11 b

[a] Equal letters means no significant difference at 5% (Tukey's test).

it is possible to suggest that the content of caffeine in the extract is about three times bigger than when the plants are not protected from the sun. Also in **Table 3**, equal letters between two levels of a factor mean that there is no significant difference at 5% (Tukey's test), as it occurs with vitamin E content regarding age leaves.

Analyzing **Tables 2** and **3**, it can be observed that the light intensity presented a negative effect on the concentration of caffeine, theobromine, phytol, and on the steroid stigmasterol. This fact means that a higher content of methylxantines and steroids can be obtained when the leaves are from plants that grow in shadow areas. An interesting aspect can be addressed to the magnitude of the influence of light intensity on the content of some compounds such as caffeine and theobromine, where their content is increased by a factor of 3. This can be a very important result if one is interested in using this plant for specific applications like teas with low contents of methylxanthines or in energetic drinks, for example. Regarding the content of vitamin E, the effect of the light intensity appears not to be relevant. This is a fascinating aspect, once pure vitamin E is a photosensitive compound. In this sense, the porous structure of the leaves could be acting as a natural protector system to prevent degradation of vitamin E.

Analyzing the influence of the age of leaves on the content of the compounds, it is noted that in general, the content of the compounds analyzed is higher for younger leaves. Furthermore, the metabolic route of each compound is quite different. An important aspect that should be mentioned is the distinction between leaves of 6 and 24 months. As presented in **Table 1**, the extraction yields are very similar between the two samples, but the distribution of chemical substances presents a distinct profile. It should be pointed out that 24 month leaves are, in fact, young leaves that are growing just after the leaves with ages around 18 months or less, but the ramifications are 24 months old. Although the contribution of ramifications in the extraction yield is low, it can be responsible for the differences observed in the chemical composition.

The effects of the drying method applied are also evident in **Tables 2** and **3**. A general tendency of increasing the content of the compounds analyzed when the samples are dried in microwave oven can be observed. This fact might be explained in terms of possible depolymerization and volatilization of high molecular weight compounds (like triterpenes and wax) during the microwave oven drying, which can enrich the extract in products that were not affected by the microwaves. The microwave oven drying technique may also catalyze chemical reactions that can modify the original chemical profile of mate tea leaves extracts. Some works in the literature pointed out peculiar characteristics of the microwave oven on organic synthesis, where the general trends indicate much faster reaction kinetics (*18*−*20*).

CONCLUSIONS

In this work, the effects of sample drying method and some agronomic variables (light intensity and age of leaves) on the extraction yield and chemical composition of extracts of mate tea leaves with carbon dioxide at 30 °C and 175 bar were investigated. The results indicate that all effects exert a remarkable influence on the liquid yield and chemical profile of the extracts and thus an extract with specific characteristics could be obtained by manipulating these variables. For example, mate tea leaves extracts could be used in the formulation of energetic drinks, and in this sense, the leaves should be young with an age ranging around 6 months, in which the content of methylxanthines is at a high level.

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