Vol. 20, No. 3(2021) Bio2404
Revista Mexicana de Ingeniería Química

Enzymatic extraction of limonene, limonin and other relevant compounds from *Citrus sinensis* (orange) and *Citrus aurantiifolia* (lime) by-products

Extracción enzimática de limoneno, limonina y otros compuestos relevantes a partir de subproductos de *Citrus sinensis* (naranja) y *Citrus aurantiifolia* (limón)

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Received: March 12, 2021; Accepted: June 1, 2021

Abstract

The byproducts from orange (*Citrus sinensis*) and lime (*Citrus aurantiifolia*) constitute nearly 50 wt. % of the fresh fruit and, unfortunately, these are discarded. These residues could be utilized to obtain extractable compounds of high added value, such as limonene and limonin.

The extraction of limonene from peels and limonin from seeds, from both orange and lime, was evaluated with a commercial enzyme (Macerex). It was found that the yield of limonene was 17-fold higher (4.0 and 4.7 mg/g-peel from orange and lime respectively) using the enzymatic treatment as compared to blank. More bioactive compounds and in a higher concentration were obtained from peels using the enzymatic treatment as compared to blank.

Limonin yield was twofold when the enzymes were applied to orange and lime seeds, as compared to blank (2.5 mg/g-orange seeds and 3.0 mg/g-lime seeds). The amounts of extracted limonene and limonin had a correlation with the amount of sugars released during degradation of the vegetal tissue.

Enzymatic extraction of bioactive compounds (limonene and limonin) from citrus by-products exhibits high yields, similar to traditional extraction treatment such as hydrodistillation, but under milder conditions.

Keywords: limonene, limonin, *Citrus sinensis*, *Citrus aurantiifolia*, enzymatic extraction.

Resumen

Los subproductos de naranja (*Citrus sinensis*) y limón (*Citrus aurantiifolia*) constituyen cerca del 50% en peso de la fruta fresca, lamentablemente, estos se desechan. Estos residuos podrían emplearse para obtener compuestos de alto valor agregado, como limoneno y limonina.

Se evaluó la extracción de limoneno de cáscaras y limonina de las semillas, de naranja y limón con una enzima comercial (Macerex). Se logró obtener 17 veces más limoneno por tratamiento enzimático (4.0 y 4.7 mg/g-cáscara, de naranja y limón respectivamente), en comparación con el blanco. Con el tratamiento enzimático se obtuvo una mayor cantidad de compuestos bioactivos y en mayor concentración de las cáscaras respecto al blanco. La recuperación de limonina fue dos veces mayor cuando se aplicaron las enzimas respecto al blanco, 2.5 mg/g-semilla naranja y 3 mg/g-semilla de limón. La cantidad de limoneno y limonina extraídos, mostraron una correlación con la liberación de monosacáridos, derivados de la degradación del tejido vegetal.

La extracción enzimática de compuestos bioactivos (limoneno y limonina) a partir de residuos de cítricos, muestra altos rendimientos, semejantes a los obtenidos por los tratamientos tradicionales como la hidrodestilación, y las condiciones de extracción son más suaves.

Palabras clave: limoneno, limonina, *Citrus sinensis, Citrus aurantiifolia,* extracción enzimática.

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Tel/Fax: +52 33 13785900 ext 27505
https://doi.org/10.24275/rmiq/Bio2404
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

Along with mangoes, grapes, apples and bananas, citrus are on the top five crops with the highest worldwide production (Sánchez-Mesa et al., 2020), as 120 million tons are harvested in about 100 countries, orange being the most prevalent (FAO, 2017, Ramos-Ibarra *et al*., 2017). Interestingly, nearly half of the orange and lime weight is the peel and bagasse, which can be utilized as low-cost materials for effectively reduce production costs. As a matter of fact, citrus residues are utilized as food for livestock, for the production of biogas, ethanol, fatty acids, microbial biomass, hydrolytic enzymes, extraction of citric acid, flavonoids and essential oils (Mahato *et al*., 2019, Ambriz-Pérez *et al*., 2021). In Mexico, about 3 million tons of citrus waste are generated per year, which are an excellent source of essential oils (Solleiro, 2019).

Limonene represents up to 90 wt. % of the essential oil in oranges (*C. sinensis*) and limes (*C. aurantiifolia*) peels; it is widely used as flavoring, cleansing agent and as insecticide; it also has antimicrobial and antibacterial properties. It can be used as biodegradable solvent, adhesives, in pharmaceutical processes, chemical synthesis, and so on (Zoccali *et al*., 2019; Ravichandan *et al*., 2018; Kerton, 2009).

Limonin is the main limonoid in citrus seeds. It has been reported that this compound possesses considerable antioxidant activity and antiinflamatory response; also, it is hepatoprotective, anticancer, antiviral, antiparasitic and also has immunomodulatory properties (Rouseff and Nagy, 1982; Chinapongtitiwat *et al*., 2013).

Common methods for extraction of valuable compounds from citrus include, among others, reflux distillation, shaking, stirring, microwave and ultrasonic extraction. Enzymatic treatments have a great potential, because they use low operating temperatures and pressures; such treatments rely on hydrolysis of the components of the plant cell wall (namely cellulose, hemicellulose and pectin), which act as a structural barrier that limits the release of components embedded in plant tissues (Mahato *et al*., 2019; Azmir *et al*., 2013).

In this contribution, a commercial enzyme preparation was applied to peels and seeds from orange and lime, for extraction of limonene, limonin and other bioactive compounds. To the best of our knowledge, this is the first report on the enzymatic extraction of limonin from citruses seeds and of limonene from lime peels.

2 Materials and methods

2.1 Materials

Freshly squeezed oranges (*C. sinensis*) and limes (*C. aurantiifolia*) were collected in Guadalajara (Mexico) from local farmer markets.

Limonene, limonin, α-pinene, β-pinene, myrcene, 3-carene, γ-terpinene, linalool, geraniol and geranyl were purchased from Sigma-Aldrich (Milwaukee, USA). Macerex PM, a commercial enzyme preparation, was purchased from Enmex S.A. de C.V. (Mexico City, Mexico).

2.2 Extraction and quantification of limonene

The extraction of limonene from orange and lime peels was carried out using the commercial enzyme preparation (Macerex PM), which contains pectinase and cellulase obtained from *Aspergillus niger* and *Trichoderma reesei*. The commercial enzymes were diluted to 5% (v/v), as suggested by the supplier. The diluted enzyme preparation had 1 U/mL cellulose, 2.27 U/mL xylanase and 2.95 U/mL pectinase. The conditions for extraction of limonene were determined using a 2^3 experimental design with central points, expanded to a central composite design with the following factors: Temperature (40 - 60 ºC), maceration time (2 - 6 h) and enzyme dose (60 - 80 % v/w: mL enzyme/g peel). All the experiments were carried out at pH 5. The peels were cut into pieces of 1.5 ± 0.5 cm^2. The diluted enzyme preparation was added accordingly to the percentage established in the factorial design and shaken at 150 rpm for the established time. A blank was prepared at the same conditions as the experimental design but adding deionized water instead of the enzyme preparation. Once the reaction time was completed, the solid residue was separated from the liquid and the supernatant was utilized to determine limonene by gas chromatography. In order to compare the results of the enzymatic extractions, limonene was also extracted by hydro-distillation (Ferhat *et al*., 2006), and determined by gas chromatography (GC).

To determine limonene by GC, 5 mL of hexane were added to 30 mL of supernatant, vortexed for 2

min, and centrifuged at 10,000 rpm for 5 min. Then, 1.5 mL were taken from the organic phase, passed through a 0.45 μm membrane and injected into a gas chromatograph (Agilent 7890B) with a FID, injector split/splitless, autosampling 7650 ALS, with a SLB-5ms column 10 m×0.10 mm×0.10 μm film thickness (Supelco) at 50 - 190 °C (ramp 14 °C/min) using hydrogen at 0.5 mL/min as gas carrier. Limonene, α-pinene, β-pinene, myrcene, 3-carene, γ-terpinene, linalool, geraniol and geranyl were used as standards for identification.

2.3 Extraction and quantification of limonin

Limonin was extracted from the seeds of fresh oranges and limes. The seeds were rinsed with water to clean their surface of sugars and then dried at room temperature; once dried, the seeds were grinded in a grain mill (Coffee Grinder, Hamilton Beach, model 80365).

A 2^3 experimental design was used to analyze the extraction of limonin by varying temperature (40 - 60 ºC), enzyme load (60 - 80%, v/w : mL enzyme/g seeds, % based on the weight of milled seeds) and maceration time (2 - 8 h). The volume of enzyme was taken from the commercial preparation diluted to 5% (v:v). Grinded seeds were mixed along with the enzymes by stirring at 150 rpm for a determined time. Once the time of treatment was reached, the solid residue was separated from the liquid and the supernatant was utilized to determine the amount of limonin obtained by HPLC. 10 mL of chloroform were added to the supernatant obtained from the enzymatic hydrolysis applied to seeds, and then the mixture was vortexed for 2 min. This mixture was left standing for 5 min in order to allow phase separation and recover the chloroform from the bottom. The organic phase was then totally vacuum evaporated at 40 ºC in a rota-evaporator. The residue was then re-suspended in 2 mL of acetonitrile and filtered in a 0.45 μm Millipore membrane for injection in a HPLC system. A blank was prepared at the same conditions as the experimental design but adding deionized water instead of the enzyme preparation.

Also, solvent extraction was carried out as follows: 15 g of ground seeds were placed in an EM flask along 400 mL EtOH (70 vol. %) and subjected to ultrasound at 50 ºC for 3 h. Then, the sample was centrifuged and the supernatant was rota-evaporated at 55 ºC (Liu *et al*., 2012). The determination of limonin was carried out in the same way as in the supernatant of the enzymatic treatments.

Limonin was determined in a HPLC system, which consisted of a Waters® e2695 separation module provided with a Waters® 2998 diode array detector and a Beckman Coulter UltrasphereTM ODS (C18) separation column (5 μm particle size, 250 mm x 4.6 mm). Isocratic elution was carried out with water/acetonitrile (56/46 %) at 1 mL/min. The injection volume was 10 μL and detection was carried out at 210 nm with the diode array detector. Data acquisition was carried out with the software Empower Pro. Limonin identification and quantification was achieved by comparing the retention time with a calibration curve prepared with limonin standard (Sigma-Aldrich) at 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 mg/mL.

2.4 Determination of sugars

Carbohydrates released during enzymatic macerations were quantified by HPLC as follows: Samples were centrifuged at 10,000 rpm for 10 min, the supernatant was passed through Sep-Pack C18 cartridges (Waters®) and a 0.45 μm membrane (Millipore). The HPLC system consisted of a Waters® e2695 separation module and a refractive index detector Waters® 2414, a column (Bio-Rad Aminex HPX-87P, 300 x 7.8 mm). HPLC grade water was utilized as eluent at 0.6 mL/min flow rate at 60ºC. For quantification, calibration curves were prepared for glucose, cellobiose, xylose and arabinose from 0.3 to 5 g/L. Data acquisition was carried out with the software Empower Pro.

2.5 Chemical composition of orange and lime peels

Hemicellulose, pectin and lignin in orange and lime peels were determined according to a method described elsewhere (Aravantinos-Zafiris *et al*., 1994).

2.6 Statistical analysis

Statistical analysis was carried out in each experiment in order to assess statistical significance at P<0.05 (ANOVA) using Statgraphics XV.

3 Results and discussion

3.1 Limonene extraction from orange and lime peels

The extraction of limonene from orange (OP) and lime peels and (LP), using the enzyme preparation Macerex, was evaluated using a central composite experimental design (Table 1). The largest amount of limonene of orange peels obtained was 4.02 mg/g OP at 40 ºC, 6 h maceration and 80% enzyme load. The three factors studied (temperature, time, and amount of enzyme) were statistically significant (P<0.05). The interactions temperature-time, and amount of enzyme-temperature were significant as well. However, the interaction temperature-time was statistically more significant. Regarding lime peels, the highest yield on limonene found was 4.73 mg limonene/g LP (Table 1) treated at 60 ºC, 6 h and 60% enzyme load. It was found that for the extraction of limonene from lime peels, the maceration time, the enzyme load, the interactions time-enzyme load, temperature-enzyme load, and the quadratic effect of the main three factors are significant at P<0.05. Interestingly, the interaction time-enzyme load has a higher statistical significance, as the P-value was lower (P-value 0.001).

The surface response suggests that the yield of limonene from OP increased by lowering the temperature, increasing enzyme load and extending the time (Figure 1), which can be attributed to the fact that the enzymes showed a higher activity at moderate temperatures, while at higher temperatures they became inactivated as seen elsewhere (Li et al. 2006). For Macerex® it is recommend 50 ºC with an effective range between 2 and 60 ºC.

It has been reported that increasing the amount of enzyme results in a higher proportion of essential oil and phenolic compounds (Dominguez *et al*., 1994; Li *et al*. 2006), which agrees with our study for limonene extraction. However, it has been shown also that a short reaction time of the enzyme (0.33 - 2 h) is sufficient to significantly increase the yield of limonene (Dominguez *et al*., 1994).

The surface response for lime peels (Figure 2) shows that limonene yield can be increased by decreasing enzyme load, and increasing time and temperature. The highest yield of limonene (3.1 mg/g peel) was achieved by increasing the maceration time and lowering the enzyme load; however, about 2.3 mg limonene/g peel yield can be obtained at low maceration time and high enzyme load.

Table 1. Optimization of the enzymatic extraction of limonene from OP and LP by a central composite experimental design methodology.

Temperature (ºC)	**Time (h)**	**Enzyme (%)**	**D-Limonene (mg/g peel)**	
			Orange	Lime
40	2	60	0.59 ± 0.41	2.66 ± 0.15
60	2	60	1.04 ± 0.10	3.35 ± 0.84
40	6	60	2.10 ± 0.10	4.03 ± 0.72
60	6	60	1.74 ± 0.32	**4.73 ± 0.74**
40	2	80	2.45 ± 0.62	2.91 ± 0.20
60	2	80	2.28 ± 0.28	3.10 ± 0.34
40	6	80	**4.02 ± 0.22**	3.32 ± 0.58
60	6	80	1.64 ± 0.20	2.19 ± 0.73
33.3	4	70	0.56 ± 0.20	0.27 ± 0.06
66.8	4	70	0.57 ± 0.16	0.26 ± 0.02
50	0.64	70	0.24 ± 0.06	0.08 ± 0.04
50	7.4	70	0.42 ± 0.08	0.16 ± 0.01
50	4	53.2	0.41 ± 0.02	0.34 ± 0.08
50	4	86.8	0.19 ± 0.06	0.14 ± 0.06
50	4	70	1.42 ± 0.25	2.75 ± 0.85
50	4	70	2.50 ± 0.61	1.66 ± 0.50

[a]Average of four replicates; bold figures indicate the highest extraction of D-limonene from each citrus and enzymatic preparation.

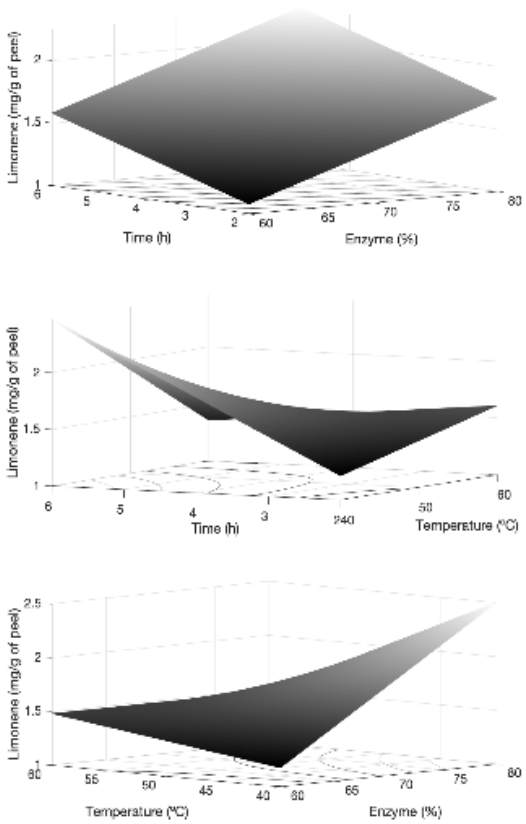


Fig. 1. Surface-response for extraction of limonene from orange peels.

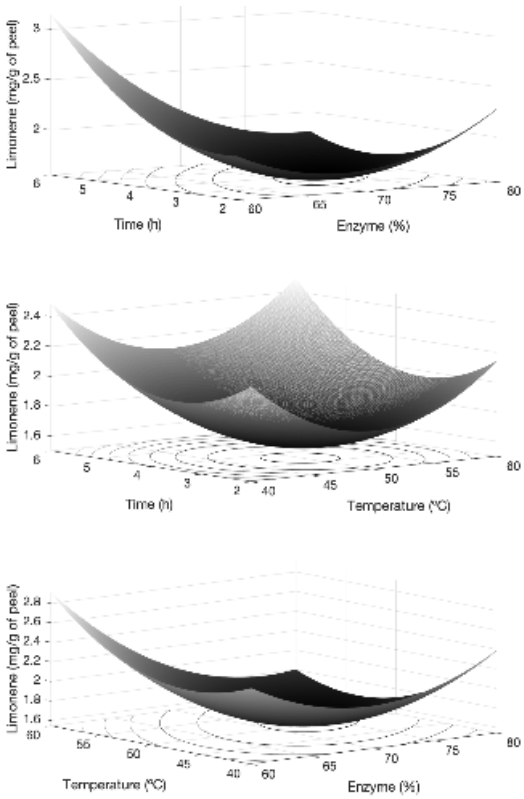


Fig. 2. Surface-response for extraction of limonene from limes peels.

The different behaviors in the extraction of limonene from OP and LP under the tested extraction conditions, might be explained by the fact that the chemical composition of LP is quite different from OP; LP has a higher content of pectin (30.2%), lignin (7.8%) and less hemicellulose (4.5%) as compared to OP (17.4% pectin, 3.8% lignin and 7.9% hemicellulose). Macerex is mainly composed of pectinase and cellulase, which synergistically degrade vegetal biomass thus requiring a lower enzyme load (Beldman *et al*., 1984).

Table 2 shows the maximum yields obtained from the citrus peels extracted by hydrodistillation, blank and the use of enzymatic extracts. It can be seen that the yields for the enzymatic extraction with Macerex and hydrodistillation were similar for both citrus and comparable to the values reported for OP (4.9 mg limonene/g OP, Mamma *et al*., 2014). The yields from the enzymatic extracts were higher as compared to the blank due to the applied enzymatic treatment. Table 2 also shows a comparison of yields of limonene from citrus obtained by different extraction methods. It stands out that the yield obtained in this contribution is twice compared to the enzymatic treatment obtained by Choi *et al*. (2015), while it was nearly as high as other methods that are energy intensive such as hydrodistillation and steam explosion (Mamma *et al*., 2014, Choi et al., 2015, Davidowski and DiMarco, 2009, Boluda and López, 2013). Also, it is worthwhile to consider that the compounds obtained by enzymatic means are more stable towards oxidation as compared to hydrodistillation and steam explosion (Mahato *et al*., 2019).

Interestingly, no reports were found in the available literature on the enzymatic extraction of limonene from LP.

3.2 Chemical composition of extracts

Oil citrus are a significant source of terpenes and phenolic compounds. The effect of enzymatic treatments of citrus peels on the extraction and variety of chemical compounds, and their concentration in the extracts, has been scarcely studied.

Moreover, the reports on this matter are unclear,

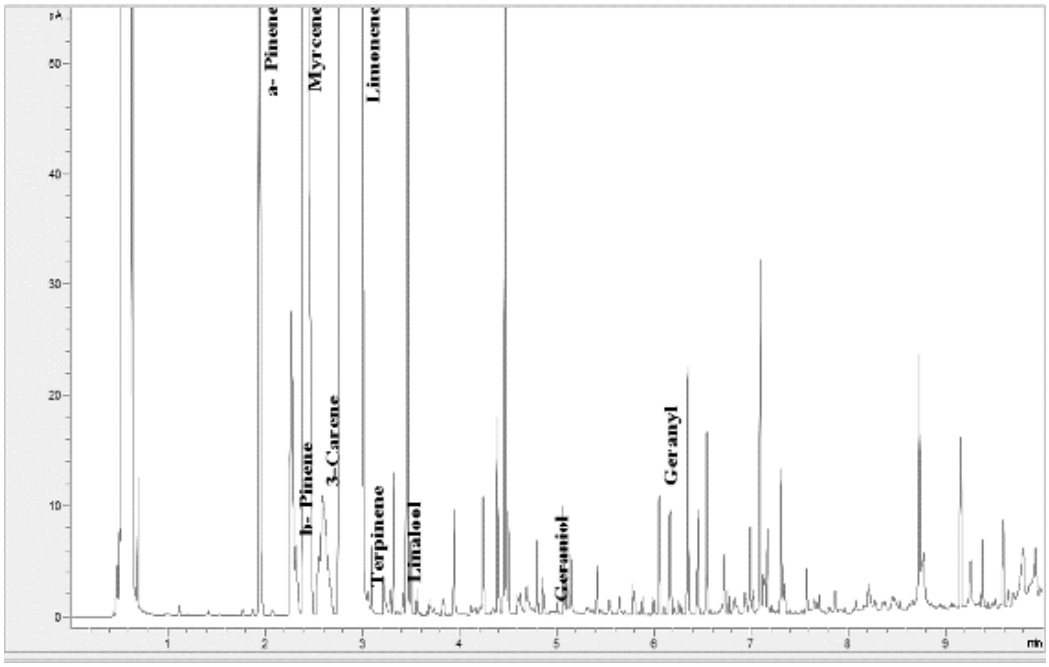
because the results depend on the vegetal species, the type and the concentration of enzyme, temperature, pH and time. For example, the use of cellulase for extraction of essential oil from *Forsythia fruit* considerably decreased the amount of α-thujene, α-pinene, camphene, sabinene, β-pinene, β-myrcene, α-phellandrene, α-terpinene, β-ocimene and p-cymen-8-ol, while oxygenated monoterpenes such as linalool, camphor, terpinen-4-ol, α-terpineol trans-carveol y trans-nerolidol were increased (Hosni *et al*., 2013).

In this contribution, the profile of compounds in both extracts (from OP and LP) was analyzed after enzymatic maceration and subsequent recuperation, and blank (Figures 3 and 4). It was found that for both citrus the variety of compounds was higher when the enzymatic complex was employed and that blank resulted in fewer compounds as compared to the enzymatic treatments. It is noteworthy that a larger variety of compounds, and in a higher concentration, could be extracted from OP as compared to LP. On OP, nearly 40 compounds were detected, from which the following were identified: Limonene, -pinene, -pinene, myrcene, 3-carene, limonene, terpinene, linalool, geraniol and geranyl. In LP around 15 compounds were detected and the same type of compounds as in OP were identified.

Table 2. Methods for extraction of limonene and their yields from orange and lime peels.

Extraction Method	**D-Limonene (mg/g peel)**		**Reference**
	Orange	Lime	
Blank	0.24 ± 0.08	0.27 ± 0.02	This study
Hydrodistillation	4.2 ± 0.12	4.9 ± 0.05	This study
Macerex	4.0 ± 0.20	4.7 ± 0.47	This study
Hydrodistillation	4.9		Mamma *et al*., (2014)
Hydrodistillation	1.9		Choi *et al*., (2015)
Enzymatic	1.8		Choi *et al*., (2015)
Methanol	4.8		Davidowski and DiMarco, (2009)
Steam explosion	5		Boluda and López (2013)

a)



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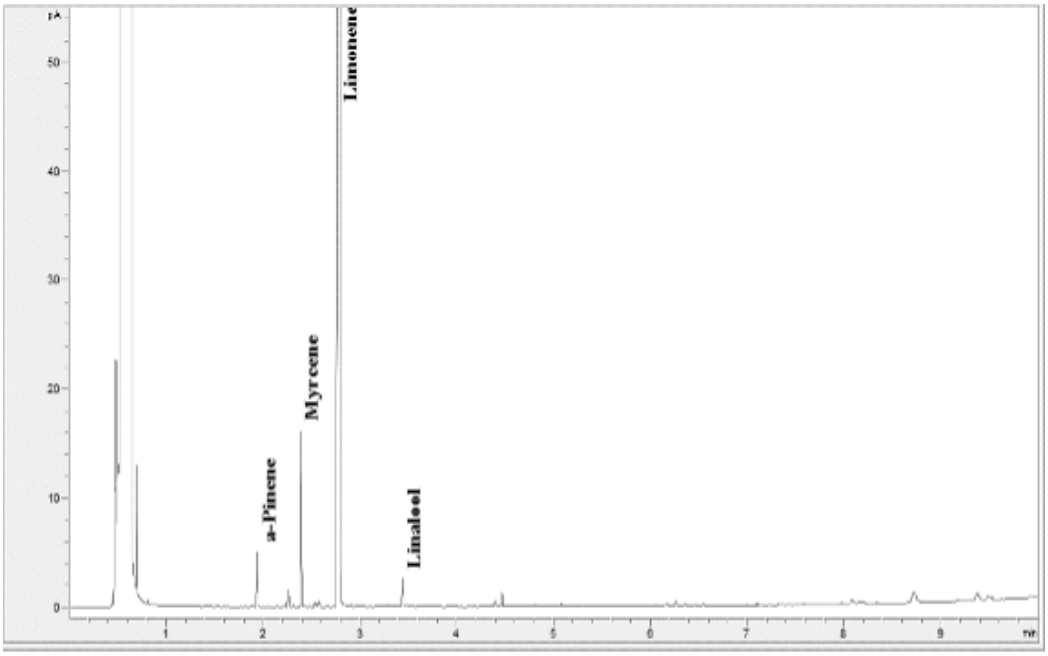
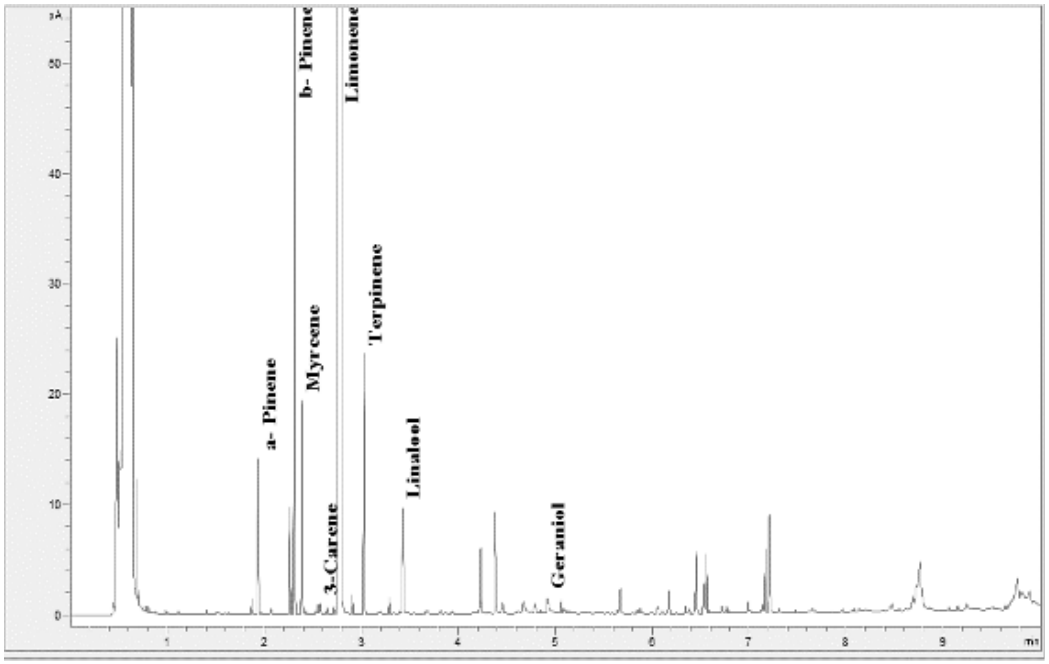
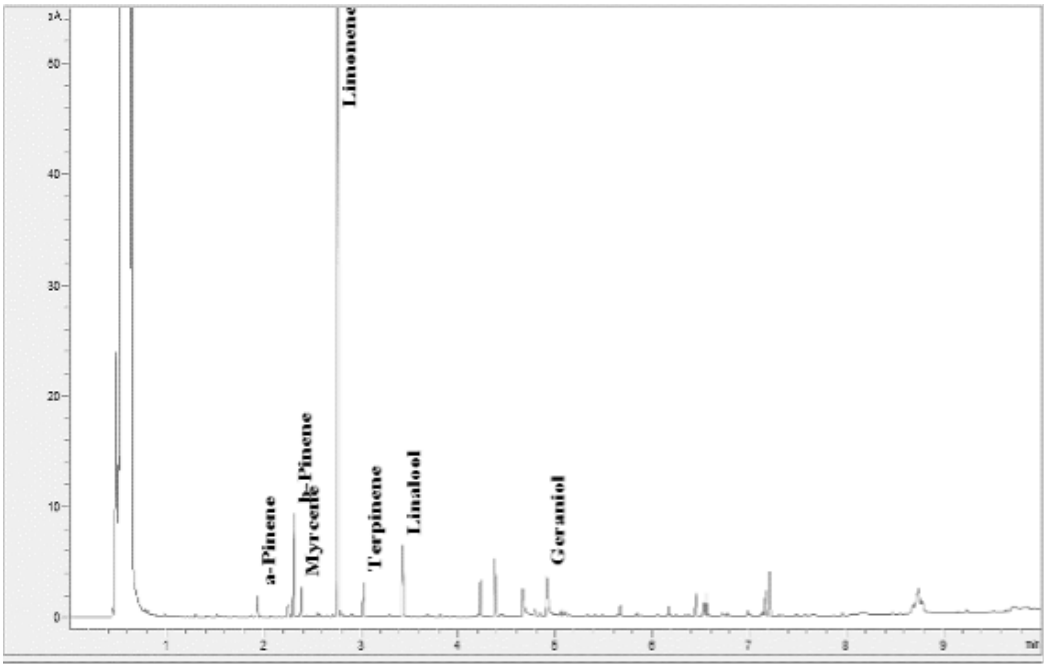


Fig. 3. Compound profile from orange peels obtained with: a) Commercial enzymes, b) blank.

The increased variety and concentration of unidentified compounds might have a significant effect on the biological activity of such extracts. Hosni *et al*. (2013) found that employing cellulase, hemicellulase or both, induced changes on the concentration of the components from extracts of rosemary and thyme, which were associated to the increased antimicrobial property of such extracts. Also, Ferhat *et al*. (2006) obtained lemon extracts using cold pressing, hydrodistillation and microwave distillation and found that the variety and concentration of compounds were different, which affected also their antimicrobial activity.

a)





b)

Fig. 4. Compound profile from lime peels obtained with: a) Macerex enzymes, b) blank.

Table 3. Central composite design for extraction of limonin from orange and lime seeds.

Temperature (ºC)	**Time** (h)	**Enzymes (%)**	**Limonin (mg/g-seed)**	
			Orange	Lime
40	2	60	1.42	1.35
60	2	60	1.4	1.24
40	8	60	1.29	1.3
60	8	60	0.51	1.4
40	2	80	3.07	3.06
60	2	80	3.29	2.4
40	8	80	3.52	**3.12**
60	8	80	**3.74**	1
50	5	70	2.42	1.53
50	5	70	2.27	1.55
50	5	70	2.31	2.15
50	5	70	2.33	1.63

The increased variety and concentration of unidentified compounds might have a significant effect on the biological activity of such extracts. Hosni *et al*. (2013) found that employing cellulase, hemicellulase or both, induced changes on the concentration of the components from extracts of rosemary and thyme, which were associated to the increased antimicrobial property of such extracts. Also, Ferhat *et al*. (2006) obtained lemon extracts using cold pressing, hydrodistillation and microwave distillation and found that the variety and concentration of compounds were different, which affected also their antimicrobial activity.

3.3 Limonin extraction from orange and lime seeds

Seeds contain limonoid as limonin; however, limonin are specific for each species (Mahato, *et al*., 2019). Limonin content depends upon the cultivar and part of the fruit, and a higher content of limonoids has been found in the seeds (Rouseff and Nagy, 1982;

Chinapongtitiwat *et al*., 2013).

The extraction of limonin from orange and lime seeds was evaluated using a commercial enzymes (Macerex®) by employing a central composite experimental design for testing enzyme load (with respect to the weight of seeds), temperature and maceration time (Table 3).

The highest amount of extracted limonin from orange seeds was 3.74 mg/g-seeds at 8 h, with a ratio 80/20 (enzymatic solution/seed) and 60 ºC; however, slightly less amount of limonin (3.52 mg/g-seeds) was extracted at 40 ºC at the same enzymatic solution/seeds ratio and maceration time (8 h). Evidently, a lower temperature has a commercial advantage by reducing the energy cost with similar yields of limonin. With lime seeds the yield was 3.12 mg/g-seeds at the same conditions as above at 40 ºC. Interestingly, a similar amount of limonin was extracted (3.06 mg/g-seeds) at 2 h, 40 ºC and 80% enzymes (Table 3), decreasing thus considerably the time of maceration. It was found that the significant factors on the extraction of limonin from orange seeds were time and the enzyme load, as well as the double interactions of the three factors and the quadratic coefficient of time ($P<0.05$). For the enzymatic extraction of limonin from lime seeds, the significant factors were temperature, the enzyme load and their interactions.

Figure 5 shows the surface response for the yield of limonin extracted from orange seeds. It can be seen that the highest yield is obtained at both high-enzyme load and long maceration time. It has been reported that increasing the enzyme load and the maceration time has a positive effect on the process of extraction in a range of temperatures between 35 to 45 ºC and 1-8 h of extraction with an enzyme load up to 70 % with respect to the treated vegetal material (Dominguez *et al*., 1994).

Regarding the lime seeds, the behavior on the extraction of limonin was different, since the surface response (Figure 6) showed that when the load of enzyme was 60 %, the temperature had no effect; however, increasing the enzyme load to 80% at 40 ºC the highest yield of limonin was obtained. This fact suggests that the composition of seeds from lime differs from the composition of the orange seeds; however it is necessary to determine the chemical composition of both orange and lime seeds to corroborate this hypothesis.

Limonin was also extracted with water from both orange and lime seeds as blank; the limonin obtained was 1.78 ± 0.03 mg/g-orange seeds and

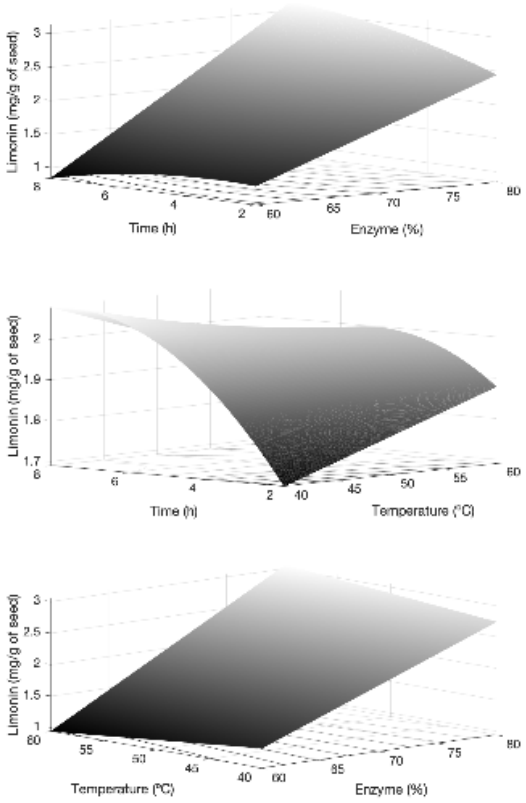


Fig. 5. Surface-response for the yield of extraction of limonin from seeds of orange.

1.80 ± 0.06 mg/g-lime seeds, respectively. Extraction of limonin with ethanol 70 % vol. reached 1.84 mg/g-orange seeds and 2.2 mg/g- lime seeds.

Extraction of limonin with hexane and ethanol has ranged from 0.65 to 1.1 mg limonin/g sour-orange seeds (Vikram *et al*., 2007; Dandekar *et al*., 2008). Extraction of limonin from orange with ethanol and ultrasound was 1.84 ± 0.06 mg/g-seeds; using lime seeds, limonin was extracted at 2.20 0.16 mg/g-seeds (Liu *et al*., 2012). Using super-critic fluids 0.38 mg limonin/g-seeds OP were obtained (Farias, 2013). Also, the limonin extracted in hexane from *Citrus limon* and *Citrus sinensis* reached 1.44 and 0.51 mg limonin/g of dry seeds, respectively (Mahato *et al*., 2019). The results obtained in this study represent at least twice the amount of limonin obtained from other reports. This fact can be explained by the hydrolytic enzymatic action on the cell walls, which is mainly composed of cellulose, hemicellulose and lignin, besides pectins. The role of the hydrolytic enzymes (cellulase, hemicellulase and pectinase, which make up the commercial enzymatic complex) is to breakdown the

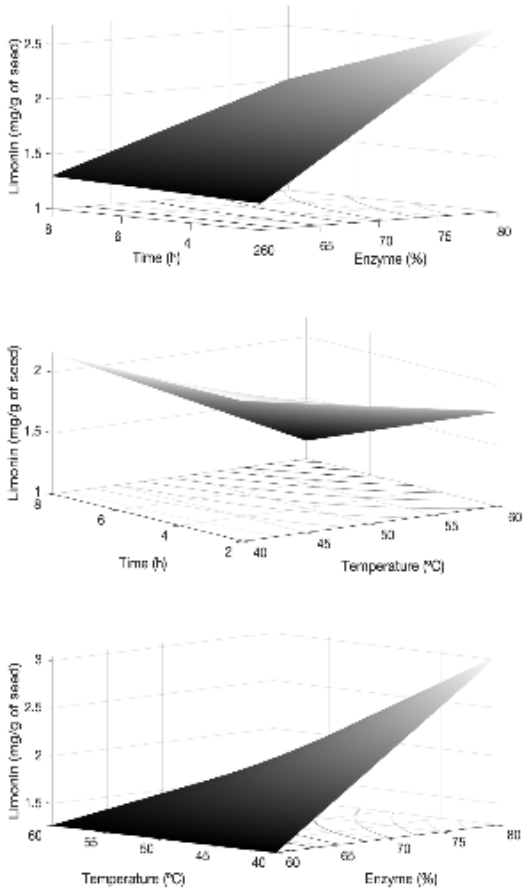


Fig. 6. Surface-response for the yield of extraction of limonin from seeds of lime.

structural cell walls of the cotyledon, resulting in more permeable cell wall structures (Arriola-Guevara *et al*., 2006; Kalia *et al*., 2001; Rosenthal *et al*., 1996). Such permeability allows for a higher release of limonin as compared to the extraction with solvents, in which limonin gets trapped in the cells tissues. To the best of our knowledge this is the first report on the enzymatic extraction of limonin fron *Citrus sinensis* and *Citrus aurantiifolia*.

3.4 Correlation of carbohydrates and extraction of bioactive compounds

During maceration of the vegetal tissue enzymes breakdown polysaccharides (hemicellulose and cellulose) to monosaccharides than can be quantified to provide information on the release of compounds from citrus peels and seeds. Therefore, the amount of limonene and limonin extracted by enzymatic means was correlated to the concentration of sugars released during hydrolysis.

Table 4 shows the amount of sugars released from orange and lime seeds and peels, P-value and the Spearman correlation coefficient (r_s). It can be seen that glucose was found in higher concentration than others carbohydrates. Peels have more glucose than seeds, and that oranges have more glucose than limes. The presence of glucose is attributed to degradation of cellulose. Arabinose was found second in concentration after glucose, and the amount found was similar in both seeds and peels. Arabinose originates from hemicellulose. Cellobiose and xylose were found in a lower amount than glucose and arabinose but the behavior regarding the amount was similar to glucose, i.e. peels have a higher amount of cellobiose and xylose than the seeds, and oranges have a higher amount of cellobiose and xylose than limes. Cellobiose and xylose are products of the degradation of cellulose and hemicellulose respectively.

The values of r_s associated to the release of sugars from peels and seeds were both positive and negative and some were close to zero. Glucose and xylose released from peels showed a positive and significant correlation with the extraction of limonene ($P < 0.05$) because the enzymes degraded cellulose and hemicelluloses that form the glands or vesicles of oil that contain limonene (Mahato et al., 2019). Limonin from orange correlated only with glucose, as the highest amount of recovered limonin corresponds to the highest amount of glucose released, suggesting that cellulose was efficiently hydrolyzed. In lime seeds, a negative correlation was found for xylose and limonin ($P < 0.05$) indicating thus that the lower the xylose released the lower the limonin obtained. It worth pointing out that the concentration of xylose was low indicating that this carbohydrate is uncommon in seeds. It is likely that sugars derived from the hydrolysis of lime seeds were unidentified and non-quantified and therefore the correlation was inexistent. Depending on the biological origin of plants, different hemicellulose structures can be found, which contain a diversity of carbohydrates (xylose, mannose, arabinose, glucose, galactose, ramhnose, and fucose) (Gírio *et al*., 2010); it is convenient to precisely characterize the chemical composition of lime seeds, since the information is restricted only to the percentage of crude fiber (Arriola-Guevara *et al*., 2006).

Table 4. Correlation of carbohydrates and extraction of bioactive compounds from citrus byproducts.

	Limonene						**Limonin**					
Parameter	**Orange peels**			**Lime peels**			**Orange seeds**			**Lime seeds**		
	mg/g peels	*rs*	*P-value*	*mg/g peels*	*rs*	*P-value*	*mg/g seed*	*rs*	*P-value*	*mg/g seed*	*rs*	*P-value*
Glucose	45.5	0.706	0	25.2	0.2611	0.003	20.8	0.537	0.0017	14.5	-0.1248	0.4602
Celobiose	7.2	-0.109	0.218	6.5	-0.1433	0.106	1.5	0.1903	0.267	2	0.1546	0.3604
Xylose	4.8	0.491	0	2.4	0.22	0.013	1.8	0.1375	0.4228	1.7	-0.3424	0.0428
Arabinose	18.1	0.403	0	12.5	-0.0323	0.716	12.5	0.2647	0.1227	15.1	-0.1054	0.5329

Conclusions

The potential of enzymatic extraction of limonene and limonin from citrus by-products has been demonstrated; the multienzyme preparation resulted in a significantly higher number of active compounds from citrus in comparison to blank. The enzymatic extraction of limonene and limonin from citrus by-products could substitute or complement other extraction methods such as cold pressing or mechanical rupture (milling) increasing the yield and variety of active compounds such as α-pinene, β-pinene, myrcene, 3-carene, limonene, terpinene, linalool, geraniol and geranyl which must positively influence their biological activity.

Acknowledgements

JRRI is grateful to CONACyT for funding his Ph.D. studies under scholarship # 234650. CIATEJ is acknowledged for allowing the use of their facilities for the conduction of this work.

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