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PII: S0308-8146(96)00163-X

Food Chemistry, Vol. 58, No. 3, pp. 255–258, 1997
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0308-8146/97 $17.00+0.00

Analytical, Nutritional and Clinical Methods Section

Effects of enzymatic treatments on carotenoid extraction from marigold flowers (*Tagetes erecta*)

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(Received 13 October 1995; accepted 30 January 1996)

Three techniques were used to quantitate total carotenoids extracted from fresh marigold flowers previously treated with a commercial enzyme; the shortest technique, based on carotenoid extraction with hexane, was selected to evaluate the efficiency of five commercial enzymes in the carotenoid extraction process. Fresh marigold treated with enzymes showed a higher susceptibility to pigment extraction than untreated samples, and the highest carotenoid yields were obtained using the enzyme ECONASE-CEP. This enzyme at 0.1% w/w increased extraction from 1.7 to 7.4 g/kg of marigold flower in dry weight; such treatments may enhance carotenoid extraction at the industrial level as well. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Marigold (*Tagetes erecta*) is a native plant to México and has been used in traditional Mexican medicine (Mendieta & Del-Amos, 1981; Neher, 1968), and in colouring poultry skin and eggs (Avila *et al.*, 1990; Hencken, 1992). The principal colouring component of marigold flower is lutein, a fat-soluble carotenoid [$C_{40}H_{56}O_2$,(3R,3'R,6'R)-β,ε-3,3'-diol] (Gau *et al.*, 1983; Quackenbush & Miller, 1972; Rivas, 1989).

Dietary carotenoids have been studied as agents of prevention and treatment of several illnesses such as cancer and photosensitivity diseases (Canfield *et al.*, 1993). Lutein has been identified in association with transthyretin, a protein implicated in the transport of thyroxine and retinol (Pettersson *et al.*, 1995).

Carotenoids, in agreement with their structure, are lipids. The AOAC method for the analytical determination of carotenoid content uses a mixture of polar (ethanol and acetone) and non-polar (hexane and toluene) solvents because of the range of carotenoid polarity, and the extraction time is about three hours (AOAC, 1984). On the other hand, the best solvents to extract carotenoids are carbon disulfide and chloride solvents, but volatility, flammability and toxicity limit their use (De-Ritter & Purcell, 1981). Briefly, the commercial extraction of marigold pigments consists of silage, pressing, drying, hexane extraction and saponification (Industrial Orgánica S.A., Monterrey, México, personal communication). The main drawbacks of the extraction process are the considerable loss of hexane, which is diffused into the air (environmental problem), health risks and economical losses (Dahlén & Lindn, 1983; Galvin & Kirwin, 1995). With these considerations, several researchers have looked for better extraction processes. Taking into account that lipids are enclosed within cells, and that cells walls are of complex composition, the use of cell-wall degrading enzymes with mixed activities (hemicellulase, cellulase, pectinase, proteinase, etc.) have resulted in significant oil-production increments (Dominguez *et al.*, 1994; Sosulski *et al.*, 1988).

To date, there have been no reports concerning the application of enzymes in the extraction of marigold pigments. On the other hand, the AOAC analytical conditions may not be adequate to monitor the effect of enzymatic treatments on extraction efficiency of such pigments. Thus, the objectives of the present project were to test the effectiveness of three analytical procedures in the assessment of carotenoids extracted with the aid of enzymes, and to evaluate the effect of enzymatic treatments, involving five commercial enzymes, on carotenoid extraction from fresh marigold samples.

MATERIALS AND METHODS

Marigold samples

Fresh marigold (*T. erecta*) flowers were supplied by Industrial Orgánica, S.A., Monterrey, México. Flowers were separated from receptacles and fresh and intact petals were processed. Moisture content was determined according to AOAC methodology (AOAC, 1984).

Enzymes and enzymatic treatments

ECONASE-CEP and PECTINASE-CEP were supplied by Enzyme Development Corporation (New York, NY). The manufacturer indicated that ECONASE-CEP showed glucosidase activity. CYTOLASE-0 and CYTOLASE-M129 were obtained from GENENCOR International (Rochester, NY) and RAPIDASE-PRESS from Gist Brocades (Seclin, France). The manufacturers reported that all of these enzymes were of fungal origin, and that they exhibited mixed activities (mainly cellulase, hemicellulase, pectinase). The enzyme concentrations and pH's utilized in this work were those recommended by the enzyme distributors.

Enzymatic slurries containing 0.0, 0.01 or 0.1% w/w of enzyme were prepared with Tween 80 (0.01% v/v) and sodium azide (0.01% w/v) in deionized water, pH 5.0. Each enzymatic slurry was prepared in a separate flask. Fresh intact petals (15% d.w.) were mixed with enzymatic slurries. Sample flasks were covered with aluminium foil, stored at room temperature and mixed daily. The reaction was monitored at 2, 5, 10, 24, 48, 72, 96 and 120 h of reaction. The possibility of a synergistic effect was tested with two of the enzymes.

Analytical procedures

In the selection of an analytical method for monitoring the enzymatic reaction, only ECONASE-CEP was tested because of its glucosidase activity as reported by the manufacturer. Total carotenoids were measured in samples by using three techniques. 1) Abbreviated method: hydrolyzed samples of fresh marigold (2 g) were milled with 20 ml of hexane in a mortar, and residues were recovered with three portions of hexane (10 ml/each) and 5 ml of acetone. Samples were refluxed (56°C/10 min), maintained at room temperature (5 min) and transferred to 100 ml volumetric flask; the volume was made up with 10% anhydrous sodium-sulphate aqueous solution. Then flasks were mixed (1 min), let stand in dark 5 min, a 5 ml aliquot taken from epiphase, and 20 ml of hexane added. Immediately the samples were mixed (1 min), and total carotenoids evaluated at 474 nm by using a Beckman DU 640 UV vis spectrophotometer (Beckman Instruments, Berkeley, CA, USA). 2)AOAC method: total carotenoids of fresh samples were determined according to the AOAC method (AOAC, 1984); here petals were milled in a mortar with 20 ml of HEAT (hexane:ethanol:acetone: toluene, 10:6:7:7) and the residues recovered with 10 ml of HEAT. And 3) AOAC-H_2O: total carotenoids were determined according to the AOAC procedure (AOAC, 1984), but after enzymatic treatment water-soluble substances of samples (20 g) were eliminated by adding deionized water to reach 1% d.w. of sample, agitated by 2 h with magnetic stirrer, and centrifuged (16274 g/4°C/ 15 min); the pellet was subjected to this procedure twice. The pellet was recovered, dried in a vacuum oven (Forma Scientific, OH, USA) at 60°C to 10% of moisture content, and analyzed immediately or stored in black bags at 4°C under nitrogen until use. The supernatant was extracted with hexane and total carotenoids evaluated by reading at 474 nm.

The same procedures were used for control samples (treatments without enzyme).

Statistical analysis

All data were evaluated using analysis of variance procedures with Fisher's PLSD multiple comparison tests (Statview software: Abacus Concepts, 1991).

RESULTS AND DISCUSSION

Comparison of methods for total carotenoid quantitation

Carotenoid content evaluated in fresh material by the abbreviated method showed significant differences between control (without enzyme) and enzymatically treated samples (Table 1), whereas AOAC and AOAC-H_2O methods did not show statistical differences between such samples. This could be explained because the extraction conditions were stronger in the latter two techniques than in the former. Considering that lutein, the principal carotenoid in marigold (Rivas, 1989), is a polar carotenoid (xanthophyll), strong polar solvents in the extraction process may mask the effect of the enzymatic treatments, whereas the use of hexane in the abbreviated method, which is a milder solvent used in the industrial process, illustrates the positive effect of the enzymatic treatment (Table 1). This enzymatic effect agrees with results obtained for oil extraction in other crops (Dominguez *et al.*, 1994). In the AOAC–H_2O technique, the carotenoid content was evaluated in the discarded water containing water-soluble substances and less than 1% of the total carotenoids were found in such samples. Hence, we continued the evaluation of the effect of enzymatic degradation of fresh marigold on carotenoid extraction using the abbreviated method.

Table 1. Levels of extracted carotenoids with three different methods using non- and enzyme- treated fresh marigold

	Total carotenoids (g/kg d.w.)[a]		
Treatment	Abbreviated	AOAC	AOAC–H_2O[b]
Control	1.2 ± 0.2 y	12.3 ± 0.8 y	13.3 ± 0.2 y
Enzymatic[c]	7.4 ± 0.4 z	11.8 ± 0.5 y	12.7 ± 0.2 y

[a]Means of three determinations ± standard error. Different letters in the same column indicate significant differences between means ($\alpha = 0.05$).
[b]Soluble substances eliminated before AOAC determination (see Materials and Methods).
[c]ECONASE-CEP 0.1% w/w.

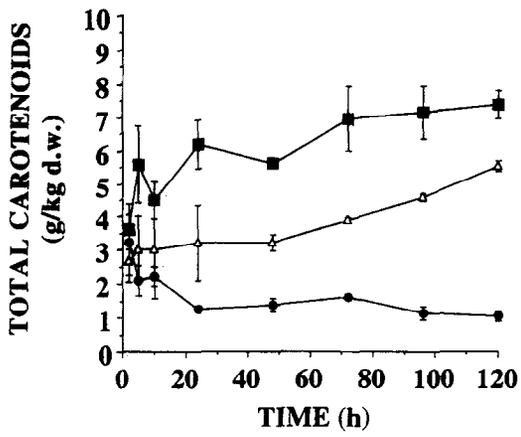

Fig. 1. The effect of ECONASE-CEP concentration and hydrolysis time on total carotenoids of marigold fresh petals (data represent the mean of three determinations ± standard error). Enzyme concentrations were 0.0 [●], 0.01 [△] and 0.1% w/w [■].

Enzymatic treatments

Figure 1 shows the effect of ECONASE-CEP concentration and hydrolysis time on extracted carotenoids of marigold fresh material. It could be observed that both enzyme concentrations, 0.01 and 0.1% w/w, showed a high extraction of carotenoids related to control but especially the latter, and that 120 h of reaction time was enough to reach a maximum plateau. This behavior was similar for the other commercial enzymes and RAPIDASE-PRESS was the only product that did not degrade fresh marigold at any of the enzyme concentrations tested (data not shown). Table 2 shows the extracted carotenoids at 120 h of reaction time for each enzyme. With 0.01% w/w, ECONASE-CEP gave a value significantly higher than any other single enzyme tested; this also happened at 0.1% w/w. Thus ECONASE-CEP was ranked as the best of the enzymatic products. The synergistic test, carried out with the best enzymes at the two concentration conditions, gave values at 0.01% that were not statistically different from the best of the single enzymes and at 0.1% the extracted carotenoids were higher but not higher than the addition of the values generated by each individual enzyme (Table 2). Thus, a synergistic effect was not observed (Henderson, 1979).

Table 2. Carotenoid content of marigold fresh material treated with commercial enzymes after 120 h of hydrolysis

Treatment	Total carotenoids (g/kg d.w.)
Control	1.7 ± 0.1 a
0.01% w/w	
ECONASE-CEP	5.5 ± 0.2 de
PECTINASE-CEP	2.0 ± 0 ab
RAPIDASE-PRESS	1.6 ± 0.1 a
CYTOLASE-O	2.3 ± 0 ab
CYTOLASE-M129	2.7 ± 0.5 b
ECONASE-CEP 0.01% + CYTOLASE-O 0.01%	5.3 ± 0.6 d
0.1% w/w	
ECONASE-CEP	7.4 ± 0.4 f
PECTINASE-CEP	4.2 ± 0.5 c
RAPIDASE-PRESS	1.7 ± 0.1 a
CYTOLASE-O	6.3 ± 0.2 e
CYTOLASE-M129	5.6 ± 0 de
ECONASE-CEP 0.01% + CYTOLASE-O 0.01%	8.5 ± 0.2 g

Means ± standard error of at least duplicates.
Different letters in column show significant differences ($\alpha = 0.05$).

As mentioned above, the results for fresh flowers indicated a high availability of carotenoids to hexane extraction. This could be important because the industrial processors only use hexane, and enzymatic treatment could be applied at the silage stage.

ACKNOWLEDGEMENTS

Financial support by Consejo Nacional de Ciencia y Tecnología-México is acknowledged, as well as the manuscript revision by Mr. Horacio Guzmán-Maldonado (CINVESTAV-Irapuato), and the enzyme samples provided by Enzyme Development Corporation, GENENCOR International and Gist Brocades.

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