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Occurrence of 6-Methoxymellein in Fresh and Processed Carrots and Relevant Effect of Storage and Processing

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The occurrence of 6-methoxymellein (6-MM) in fresh and conventionally processed carrot products (for a total of 176 samples) marketed in European locations and the effect of *Alternaria* spp. infection and storage conditions on 6-MM accumulation were investigated. 6-MM was found in 78% of tested samples with levels ranging from 0.02 to 76.00 μg/g, with only 1 of 79 fresh carrots exceeding the "just noticeable difference" level for 6-MM. Storage of carrots at 1 °C was suitable to maintain low levels of 6-MM for a period of at least 17 weeks. No effect of *Alternaria* spp. infection was observed on 6-MM occurrence. The fate of 6-MM during carrot juice processing was also investigated by using different enzyme formulations for maceration and blanching procedures. Levels of 6-MM in blanched carrots obtained by boiling water or steam treatment were reduced by 69 or 33%, respectively, as compared to fresh carrots. No decrease in 6-MM levels was observed after maceration with pectinolytic enzyme preparations (Rapidase Carrot Juice and Ultrazym AFP-L). A reduction of 6-MM by 85 or 94% was obtained after the entire cycle of carrot juice processing, depending on the blanching procedure used.

KEYWORDS: Carrots; 6-methoxymellein; storage; processing; blanching; juice; pulp

INTRODUCTION

Carrot (*Daucus carota* L.) is a widely consumed vegetable with high nutritional value as it is an important source of carotene. It can be eaten either raw, minimally processed (canned, frozen carrots), or manufactured into a variety of products including juices, dehydrated soups, baby foods, and mixed vegetables. Environmental and growing conditions during production, or improper postharvest handling, minimal processing, and storage conditions can alter the functional characteristics of carrots by eliciting the synthesis of various phytoalexins, low molecular weight compounds, that serve to protect plant tissue from microbial and oxidative damage (*1*). *Alternaria alternata* and *Alternaria radicina* are the most common seedborne fungi of carrots that can also infect carrot roots during growing and storage. *A. alternata* is associated with leaf, stem, and fruit spots and produces a number of mycotoxins (*2*), whereas *A. radicina* is responsible for damping-off of carrot and lesions on carrot leaf petioles, crowns, and tap roots (*3*−*5*). The phytoalexin 8-hydroxy-3-methyl-6-methoxy-3,4-dihydroisocoumarin, also named 6-methoxymellein (6-MM), is associated with the bitterness in strained carrots and is in part responsible for the sensory quality of carrots.

The production of 6-MM has been reported in carrot roots and carrot cell suspensions in response to either infection by fungi (*Cerotocystis fimbriata*, *Chetomium globosum*, *Botrytis cinerea*, and *Thielaviopsis basicola*) or treatments with abiotic elicitors (metal salt and sulfhydryl reagents) (*6*, *7*). It has also been shown that 6-MM production is not a specific response to an attacking fungus as it is formed irrespective of the species of challenging fungi (*6*). Studies on 6-MM toxicity showed that 6-MM levels >0.1 mM inhibited growth of carrot cells and of fungi and bacteria, with *A. alternata* being the most sensitive fungus (*8*). Kurosaki et al. (*9*) showed that 6-MM accumulated in cultured cells upon addition of partial hydrolysates of carrot cells obtained by treatment with purified *endo*-polygalacturonase or *endo*-pectin lyase. Elicitation of 6-MM was also achieved in carrot cell suspension cultures by direct addition of a highly purified commercial preparation of pectinase and *endo*-polygalacturonase (*9*). Accumulation of 6-MM in carrots is also associated with exposure to ethylene gas, ultraviolet light, variations in temperature during handling, increasing temperature of storage, water stress, and oxygen levels (*10*). Steam treatments reduced 6-MM accumulation probably by retarding the activity of enzymes involved in the biosynthetic pathway (*11*).

Within conventionally processed carrots, carrot juice is considered an important product for the European market. The most important operations in juice processing are blanching (for the destruction of most microrganisms present in the product and inactivation of enzymes promoting browning and degradation), maceration with pectinolytic enzymes (for the production of healthy cloudy carrot juice), and pasteurization (for the shelf-life extension of the products) (*12*). The aims of this paper were

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(i) to evaluate the occurrence of 6-MM in fresh and conventionally processed carrots produced or marketed in different European locations, (ii) to investigate the effect of *Alternaria* spp. infection and storage conditions (temperature and duration of storage) on 6-MM accumulation in carrots, and (iii) to monitor the fate of 6-MM during carrot juice processing. In particular, the effects of enzymatic macerations and of different heat treatments (blanching in boiling water or under steam) were investigated.

MATERIALS AND METHODS

Chemicals. Sodium dihydrogen phosphate and *Alternaria* toxins (radicinin, altertoxin-I, alternariol, and alternariol methyl ether) were supplied by Sigma (Sigma-Aldrich, Milan, Italy) and had a purity of >95%; solvents were of HPLC grade (Mallinckrodt Baker, Milan, Italy). *epi*-Radicinol was produced and isolated in our laboratory from cultures of *A. radicina* cultured on carrot slices according to the procedure described by Solfrizzo et al. (*13*); radicinol was kindly provided by Dr. H. Nakajima (Department of Agricultural Chemistry, Tottori University, Koyama, Tottori, Japan). Oasis HLB (3 mL capacity, containing 60 mg of polymeric reversed phase sorbent) and C_{18} (10 mL capacity, containing 500 mg of C_{18} sorbent) columns were purchased from Waters (Milford, MA).

Enzymes. Rapidase Carrot Juice (DSM Food Specialties S.p.A., Milan, Italy) and Ultrazym AFP-L (Novozymes A/S, Bagsvaerd, Denmark), both containing enzymes with pectinase and hemicellulase activity (80000 AVJP/g, AVJP = viscosimetric activity on apple juice), were used for enzymatic macerations.

Samples. *Fresh Carrots.* Two kilograms of fresh carrots was purchased from a vegetable market in Bari, Italy, and used for the isolation and purification of 6-MM; another 144 fresh carrots roots of similar size were purchased in the same place and were used for storage experiments to evaluate the influence of *Alternaria* spp. inoculation and storage conditions (increasing temperatures from 1 to 20 °C for a total of 25 weeks of storage) on 6-MM accumulation in carrots. An additional 79 batches, each containing 30 carrot roots, were sampled soon after the harvest period or purchased from different European vegetable markets and analyzed for 6-MM content. In particular, 22 batches from Italy, 16 from France, 23 from The Netherlands, and 18 from Denmark were collected. Five roots from each batch were blended, and 20 g was analyzed for 6-MM content. Six kilograms of fresh carrots purchased from a vegetable market in Conversano, Bari, Italy, was analyzed for 6-MM content and then used for carrot juice production on a small scale.

Processed Carrots. Carrot-based products were purchased from European markets (14 from Italy, 10 from France, 22 from The Netherlands, 4 from Denmark, and 37 from Poland) and were analyzed for 6-MM content. According to their typologies the 87 carrot products were classified into 10 categories such as fruit−carrot juice blends containing up to 30% of carrot extract; nectars containing 50−100% of carrot extract; soups containing a mix of precooked vegetables (carrots, potatoes, peas, zucchini, parsley, broccoli, cauliflower, tomato, honey, artichokes, spinaches); vegetable sauces; carrot puree (thick smooth carrot sauce); whole canned baby carrots; vacuum-packed sliced carrots; pudding; baby foods (including carrot purees, vegetable soups, and blended carrots); and baby juices.

Isolation and Structure Characterization of 6-MM from Carrots. A 1 kg batch of carrot roots was washed under tap water, peeled, sliced into disks of ~1 cm thickness, and disinfected with a 1.5% sodium hypochlorite solution for 3 min. Carrot slices were transferred to sterile Petri dishes containing a sterile filter paper moistened with 2 mL of sterile water containing 0.5 mg/mL of streptomycin sulfate and chlortetracycline−HCl to avoid microbial contamination. Carrot slices were incubated for 14 days at 28 °C in the darkness, then dried at 40 °C, and finely ground. The ground carrots (37.80 g) were blended by Sterilmixer blender (International Pbi, Milan, Italy) and extracted four times with 100 mL of a mixture of acetonitrile/methanol/water (45:10:45, v/v/v) at pH 3 (HCl) by shaking for 30 min. After filtration through filter paper (Whatman no. 4), the extracts were pooled and submitted three times to liquid−liquid extraction with 60 mL of dichloromethane in a separatory funnel. The dichloromethane extracts were combined, dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum. The residue was reconstituted with 10 mL of chloroform and purified by preparative silica gel column chromatography on a glass column (270 × 22 mm) manually packed with silica gel 60 (particle size = 0.063−0.200 mm, 70−230 mesh ASTM, E. Merck, Darmstadt, Germany) in chloroform. The column was eluted with a gradient of chloroform/acetic acid/methanol (100 mL of chloroform/acetic acid, 99:1, v/v; 100 mL of chloroform/acetic acid/methanol, 98.5:1:0.5, v/v/v; 100 mL of chloroform/acetic acid/methanol, 98:1:1, v/v/v; 100 mL of chloroform/acetic acid/methanol, 97:1:2, v/v/v). Twenty-six fractions of ~15 mL each were collected; an aliquot of each fraction was dried, dissolved in methanol, and analyzed by reversed phase liquid chromatography with ultraviolet diode array detection (LC-UV/DAD). Fractions 6−9, containing a compound with a UV spectrum similar to that of 6-MM, with absorbance maxima at 267 and 302 nm, were pooled together, evaporated to dryness, reconstituted with the minimum volume of methanol, and fractionated by two semipreparative precoated thin-layer chromatography (TLC) plates (silica gel 60 F-254, layer thickness = 0.5 cm, 20 × 20 cm, E. Merck) using chloroform/hexane (9:1, v/v) as the mobile phase. The band of 6-MM detected under UV light at 254 nm at R_f 0.65 was scraped off, and the compound was eluted from the silica powder with 9 mL of methylene chloride/methanol (9:1, v/v). The purified extract was dried under nitrogen stream to give 50 mg of pale yellow crystals, identified as 3-methyl-6-methoxy-8-hydroxy-3,4-dihydro-isocoumarin (6-MM), with a final purity of >99%. Identification and structure characterization was performed by GC-MS and NMR analysis based on the literature data reported by Czepa and Hofmann (*14*): ^{1}H NMR (360 MHz, $CDCl_3$) δ 1.51 (3H, d, 6.6 Hz), 2.87 (2H, d, 6.6 Hz), 3.85 (3H, s), 4.67 (1H, m), 6.25 (1H, d, 2.2 Hz), 6.37 (1H, d, 2.7 Hz), 11.25 (1H, s); ^{13}C NMR (360 MHz in $CDCl_3$) δ 20.6 [CH_3, C(10)], 34.8 [CH_2, C(8)], 55.5 [CH_3, C(11)], 75.4 [CH, C(9)], 99.4 [CH, C(6)], 101.5 [CH, C(4)], 106.1 [C, C(2)], 140.8 [C, C(7)], 164.5 [C, C(3)], 165.7 [C, C(5)], 169.8 [CO, C(1)]; GC-MS, *m*/*z* 164 (100), 208 (82), 190 (38), 165 (27), 178 (12), 78 (11), 209 (10), 119 (8), 69 (8), 162 (6).

Carrot Inoculation with *Alternaria* Cultures and Storage at Different Temperatures. Two sets of 16 PDA dishes were inoculated in the center with a toxigenic strain of *A. alternata* (isolated from carrot seed in France) and *A. radicina* (isolated from carrot seed in The Netherlands), respectively. The strains were deposited in the culture collection of the Institute of Sciences of Food Production, Bari, Italy (ITEM, http://www.ispa.cnr.it/Collection) with the accession numbers ITEM 4745 *A. alternata* and ITEM 4218 *A. radicina*. The inoculated dishes were sealed with transparent film and left at room temperature for 24 h and then incubated at 26 °C for 1 week under UV light in order to induce production of conidia. After the incubation period, the agar of each PDA dish with fungal colonies of *Alternaria* strain was washed with 6 mL of sterile water. The washing water from each group of PDA cultures was collected, and the concentration of spore suspension obtained was determined by using a Thoma counting chamber (HGB Henneberg-Sander GmbH, Lutzellinden, Germany). After appropriate dilutions with sterile water, the following spore suspensions were prepared: 750 mL of ITEM 4218 with a final concentration of ~70000 spores/mL and 300 mL of ITEM 4745 with a final concentration of 35000 spores/mL.

Carrots roots, previously analyzed for 6-MM content, were washed with tap water, air-dried for 2 h, and subdivided into three groups of 48 carrots; in particular, 48 carrots were inoculated with *A. alternata* and 48 with *A. radicina* by immersion for 30 s in the corresponding spore suspensions kept under agitation by magnetic stirring. The remaining 48 carrots having the natural mycoflora were used as naturally contaminated control samples. The carrots were stored in plastic boxes and incubated in a climatic cabinet (WTB Binder Labortech GmbH, Tuttlingen, Germany) to maintain constant conditions for 17 weeks at 1 °C, followed by 4 weeks at 10 °C and another 4 weeks at 20 °C for a total of 25 weeks. For each treatment (control and *A. alternata* and *A. radicina* inoculated carrots) the sampling was

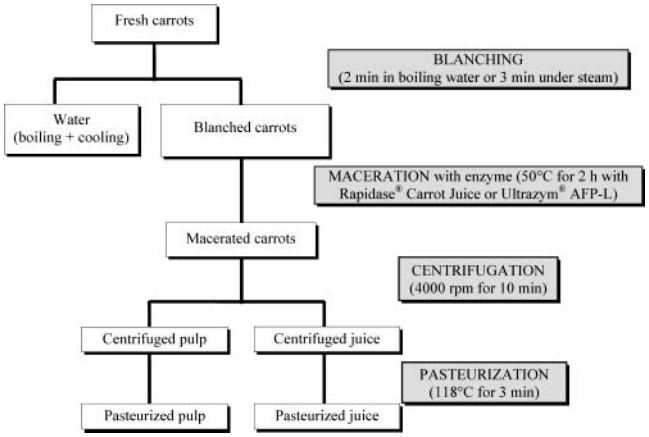

Figure 1. Flow diagram of carrot juice production.

performed by collecting six carrots per time after 9, 17, 19, 20, 21, 23, 24, and 25 weeks, obtaining a total of 24 (3 treatments × 8 samplings) samples. Each sample of carrots was blended, and a test portion size of 60 g was analyzed for 6-MM content by performing triplicate measurements (20 g per analysis) for a total of 72 analyses.

Carrot Processing for Juice Production. Carrot juice production was performed as reported below, based on the technological flow sheets reported in the FAO guidelines for vegetable processors (*12*, *15*). The principal steps were sample preparation, blanching, maceration with pectinase, juice extraction (pressing), and pasteurization. Two blanching procedures were used, either boiling water for 2 min or steam for 3 min. Two macerating enzymes were used for each blanched carrot, Rapidase Carrot Juice and Ultrazym AFP-L. The flow diagram of carrot juice production is reported in **Figure 1**.

Sample Preparation. Sorted carrots roots (6.2 kg) were washed under tap water, surface sterilized in a 0.01% NaOCl solution for 2 min, rinsed with distilled water, and dried at room temperature for 30 min on filter paper. A representative sample of 10 raw carrots was homogenized (Dito Sama F23200, Aubusson, France), and quadruplicate measurements of 6-MM were performed by analyzing aliquots of 20 g. The remaining carrots roots were divided into two portions that were used for the two different blanching procedures, using boiling water or steam.

Blanching. Blanching can be carried out by cooking carrots in hot (90 °C) or boiling water for 2−5 min or under steam for ∼3 min depending on carrot sizes (*12*, *15*). Carrots to be blanched in boiling water were homogeneously cut into 2 cm slices, placed in a wire basket, and blanched for 2 min in 10 L of boiling water. Blanching was timed from the moment the water started to boil again (10 min) after the wire basket was dipped in the pan (*16*). Blanched carrots were then cooled in 5 L of cold water until the core temperature of the sliced carrots had reached 50 °C.

Carrots to be blanched under steam were ground and placed in a beaker under fluent steam (100 °C) in an autoclave (Fedegari SpA, Abuzzano, Italy) for 3 min. Blanched carrots were then cooled to 50 °C in 5 L of cold water.

Enzyme Treatment. The two batches of blanched carrots were weighed and divided each into two equal portions to be macerated with two different enzymes, for a total of four macerations. Before maceration, each portion was acidified to pH <5 with 10% (w/w) of 0.4% citric acid solution to avoid chemical oxidation. Acidified carrots were then treated with 400 μg/g of a 5% enzyme solution and incubated in a climatic cabinet set at 50 °C for 2 h, which produced a crushed carrot mass.

Juice Extraction. The macerated carrots were pressed with a juice extractor (Micromax Simac, Bergazzo con Fogliaro, Como, Italy) consisting of a centrifugal basket and a pulp-retaining container; the obtained juices were clarified by centrifugation (Beckman J2-21, Beckman Instruments, Inc., Palo Alto, CA) at 4500 rpm (4195.8*g*) for 10 min.

Pasteurization. Carrot juice and pulp were pasteurized by autoclaving at 118 °C for 3 min.

Representative samples (60 g of carrots or pulp or 50 mL of juice or water solution) were collected during the entire process and analyzed in duplicates to determine the fate of 6-MM during carrot juice production. Another aliquot of 10 g of solid sample was dried at 80 °C to determine its water content. The following samples were collected: sliced carrots blanched in boiling water, ground carrots blanched under steam, boiling water, cooling water, carrots macerated with Rapidase Carrot Juice enzyme, carrots macerated with Ultrazym AFP-L enzyme, centrifuged juices, centrifuged pulps, pasteurized juices, and pasteurized pulps.

Extraction Procedure. *Fresh and Stored Carrots.* The method described by Solfrizzo et al. (*17*) for determination of *Alternaria* toxins in carrots proved to be applicable to the analysis of 6-MM in carrot samples.

Processed Carrot Products. For the analysis of carrot-based products (baby foods, carrot purees, puddings, sauces, and vegetable soups) a test portion size of 20 g was placed in a centrifuge tube with 20 mL of distilled water, vortexed for 1 min, centrifuged (Jouan CR4-22, Z. I. de Brais, Saint-Nazaire, Cedex, France) at 4500 rpm for 10 min, and filtered through a filter paper (Whatman no. 4). For the analysis of carrot juice and water solutions from processing (boiling and cooling water), 10 mL of carrot juice was clarified by centrifugation at 4500 rpm for 10 min and filtered through a filter paper (Whatman no. 4). An aliquot of 4 mL of clarified carrot products or 10 mL of water solutions from processing was purified through a C_{18} SPE column previously conditioned with 5 mL of methanol and 10 mL of distilled water. Columns were washed with 5 mL of distilled water followed by drying with air for a few seconds, and then 6-MM was eluted with 2 mL of methanol into a 4 mL vial. The purified extract was dried under a nitrogen stream at 60 °C, reconstituted in 500 μL of acetonitrile/water (25:75, v/v), and stored at ∼4 °C until LC analysis.

Mean recovery of three replicates of 6-MM spiked on fresh carrot samples at 5.0 μg/g was 65% with a laboratory repeatability (RSD_r) of 3%. Mean recoveries of three replicates of fortified carrot juice and carrot purees at 6-MM spiking levels of 0.040−0.50 μg/g were 60−95% with an RSD_r of 17−24% for juices and 66−86% with an RSD_r of 24−39% for carrot purees.

All analyses of 6-MM were performed by correcting results for water content ranging from 85% in raw carrots to 94% in blanched carrot samples.

High-Performance Liquid Chromatography (HPLC). The separation of 6-MM was performed using a Thermo Separation Products liquid chromatograph (ThermoQuest Inc., San Jose, CA) equipped with a quaternary gradient pump capable of delivering 1 mL/min constant flow rate (Spectraseries gradient pump P4000), a vacuum membrane degasser (SCM 1000), an autosampler injection system with a 50 μL loop (AS 3000), a column oven set at 30 °C, a diode array detector (DAD, UV 6000 LP detector), and a chromatography data system for Windows 2000 (ChromQuest version 2.53). The reversed phase column used was a SymmetryShield C_{18} (150 × 4.6 mm i.d., 5 μm particle size, Waters), preceded by a guard filter (3 mm diameter, 0.5 μm pore size). The mobile phase consisted of a gradient of acetonitrile (eluent A) in water (eluent B) from 10 to 30% for 16 min followed by 50% A and 50% B for 19 min at a flow rate of 1.0 mL/min. The injection volume was 20 μL, equivalent to 15 mg, 80 μL, and 400 μL of carrot, juice, and water (boiling or cooling), respectively. Chromatograms were recorded at 267 nm (bandwidth = 11 nm, scan rate = 10 Hz).

Gas Chromatography−Mass Spectrometry (GC-MS). The GC-MS analysis was performed with an Agilent 6890 series GC system (Agilent Technologies, Inc., Palo Alto, CA) coupled with a 5973N mass selective detector and equipped with a GC-MSD ChemStation (G1701A version C.00.00) and a capillary HP-5MS 5% phenyl methyl siloxane column (30 m × 0.25 mm i.d., film thickness = 0.25 μm). The injection volume of 1 μL was made in split mode with an injector temperature set at 250 °C and a carrier gas (helium) flow of 2.9 mL/min. The column temperature program was 70 °C for 2 min, ramped at 25 °C/min to 150 °C, at 3 °C/min to 200 °C, and at 8 °C/min to 280 °C, and held at 280 °C for 10 min. Mass chromatography in the electron-impact mode (MS/EI) was performed at 70 eV.

Nuclear Magnetic Resonance Spectroscopy (NMR). Proton (^1H) and carbon nuclear magnetic resonances (^{13}C) were recorded on a DRX500 Advance Bruker instrument (Rheinstetten, Germany) with a

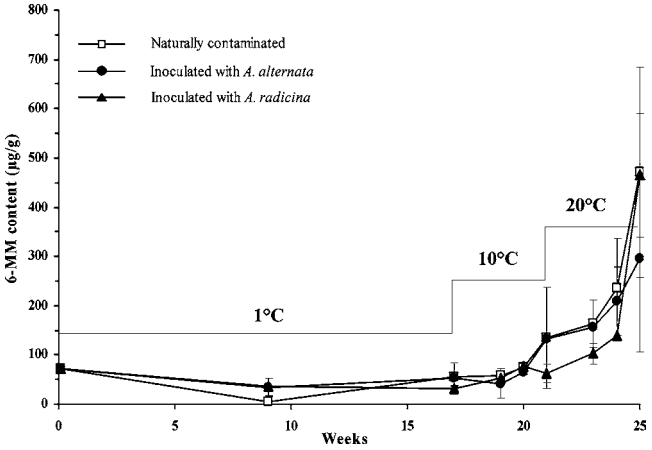

Figure 2. Mean concentration of 6-methoxymellein (6-MM) in naturally contaminated and *Alternaria* spp. inoculated carrots stored for 17 weeks at 1 °C followed by 4 weeks at 10 °C and 4 weeks at 20 °C. Each value, corresponding to the mean of three analytical measurements ± 1 standard error of the mean (SEM), is reported on a fresh weight basis.

Table 1. Distribution of 6-Methoxymellein in Fresh and Processed Carrots Produced or Marketed in Europe

country	no. of samples	no. of positive samples/total	mean of positive samples[a] (μg/g)	range of positive samples[a] (μg/g)
		Fresh Carrots		
Italy[b]	22	22/22	13.97	0.02–76
Denmark[c]	18	11/18	5.64	0.11–34.46
The Netherlands[c]	23	16/23	3.44	0.15–18.45
France[c]	16	5/16	1.03	0.26–2.96
		Processed Carrots		
Italy	14	4/14	0.63	0.36–1.37
Denmark	4	4/4	5.57	0.11–15.64
The Netherlands	22	19/22	1.18	0.11–6.11
France	10	10/10	0.20	0.04–0.62
Poland	47	47/47	0.94	0.04–12.04

[a] Levels are reported on a fresh weight basis. Detection limits were 0.020 and 0.010 μg/g for fresh and processed carrots, respectively. [b] From the market. [c] Fresh carrot roots produced in the relevant country.

reverse probe. Deuteriochloroform was used as the solvent in the NMR experiments. Residual ^{1}H and ^{13}C peaks of the solvent (δ_H 7.26, δ_C 77.0) were used as internal standards to calculate chemical shifts referred to tetramethylsilane.

Statistical Analysis. Statistical analysis was performed by using the GraphPad Instat software (Instat, San Diego, CA). Data were subjected to the Mann−Whitney test (nonparametric test, two-tailed *P* value) to determine the statistical differences when two groups of data were compared. The Kruskal−Wallis post test (nonparametric test, ordinary ANOVA) was used when three groups of data were compared.

RESULTS AND DISCUSSION

Occurrence of 6-MM in Fresh and Processed Carrots. Results of 6-MM analyses in fresh and processed carrots are reported in **Table 1**. Levels of 6-MM in positive samples ranged from 0.02 to 76 μg/g in fresh carrots and from 0.04 to 15.64 μg/g in processed carrot products. This random variability of 6-MM levels may be attributable to the different carrot genotypes, carrot maturity, and environmental and growing conditions (*18*).

With respect to processed carrots, 6-MM was found in 100% of samples from Denmark, Poland, and France, in 86% from The Netherlands, and in 29% from Italy. Levels of 6-MM in carrot products from Italy and France were significantly lower ($p < 0.01$) than those in carrot products from The Netherlands and Poland. A significant difference ($p < 0.05$) was also observed between 6-MM content in carrot products from Italy and Denmark. The distribution of 6-MM in the 10 categories of carrot products is reported in **Table 2**. The highest levels of 6-MM were found in fruit−carrot juice blends (15.64 μg/g), carrot baby foods (12.04 μg/g), carrot baby juices (7.08 μg/g), and carrot nectar (6.11 μg/g), all with mean levels >1.0 μg/g.

In previous studies, sensory analyses of strained carrots to evaluate the minimum levels of phenolic compounds that impart bitter flavor in carrots showed that the threshold concentration for 6-MM is 94 μg/g (*1*); however, the perception of bitterness may occur at a much lower concentration due to the effects of additional phytoalexins occurring simultaneously with 6-MM. In our investigation, of 79 samples of fresh carrot roots, only 1 sample from Italy exceeded (76 μg/g) the level of the "just noticeable difference" (JND) of 6-MM that was established by Talcott et al. (*1*) for strained carrots within the range of 48−71 μg/g.

Accumulation of 6-MM in *Alternaria*-Contaminated Carrots during Storage at Different Temperatures. The content of 6-MM in carrots naturally contaminated and artificially inoculated with *A. radicina* or *A. alternata*, over a storage period of 25 weeks, is shown in the graph in **Figure 2**. The starting level of 6-MM in fresh carrots ranged from 65 to 77 μg/g, whereas it ranged from 5 to 56 μg/g, from 42 to 135 μg/g, and from 146 to 672 μg/g during storage at 1, 10, and 20 °C, respectively. No significant difference ($p > 0.05$) was observed at each sampling time between 6-MM levels in noninoculated carrots and *A. alternata* or *A. radicina* artificially inoculated carrots. All samples were also analyzed for *Alternaria* toxins content, but no *A. alternata* toxins were found, whereas with respect to *A. radicina* toxins, 38% of samples contained radicinin (from 0.43 to 33 μg/g), 30% contained radicinol (from 0.4 to 7.7 μg/g), and 49% contained *epi*-radicinol (from 0.4 to 128 μg/g). More details on *A. radicina* accumulation in carrots during storage have been reported elsewhere by Solfrizzo et al. (*13*). Recently, Fan et al. (*19*) tested several fungal pathogens and found that *A. radicina* was the most effective elicitor of 6-MM accumulation in carrot disks, followed by *Penicillium expansum* and *Alternaria brassicola*. Results of our investigation do not confirm the elicitor role of fungal contamination and, in particular, of *A. radicina* to produce 6-MM in carrots. Although in this experiment we did not use carrots without *Alternaria* contamination, the inoculum present on carrots artificially inoculated with *Alternaria* spp. was much higher than that present on naturally infected carrots, which reflects the situation of marketable carrots. Another result of this experiment was that a storage temperature of 1 °C for 17 weeks does not increase 6-MM level in carrots, and this is in agreement with the estimated storage life (12 weeks) and the best storage temperature (0 °C) reported for carrots by FAO (*16*), whereas storage at 20 °C increased 6-MM content up to 6.5-fold in both naturally and artificially *Alternaria* spp. inoculated carrots. The effect of temperature was amplified by the length of storage and the physiological state of carrots, which became withered and soft during storage.

Fate of 6-MM during Carrot Juice Processing. Distribution of 6-MM in samples obtained during carrot processing using different blanching procedures or enzymatic macerations are

Table 2. Occurrence of 6-Methoxymellein in Different Carrot Products Marketed in Europe

sample	no. of samples	carrot content (%)	no. of positive samples/total	range of positive samples[a] (μg/g)	overall mean[a] (μg/g)
carrot nectar	4	50–100	4/4	0.36–6.11	3.38
baby juice	7		7/7	0.04–7.08	1.48
fruit–carrot juice blends	30	3–30	21/30	0.05–15.64	1.10
baby foods	18	9–85	16/18	0.09–12.04	1.09
vegetable soups	16	12–50	15/16	0.02–1.05	0.33
canned whole baby carrots	2	100	2/2	0.19–0.30	0.25
carrot puree	4	25–100	4/4	0.07–0.40	0.23
pudding	1	39	1/1	0.17	0.17
vacuum-packed sliced carrots	3	100	3/3	0.04–0.32	0.14
vegetable sauce	2	up to 34	1/2	0.25	0.13

[a] Levels are reported on a fresh weight basis. Detection limits were 0.020 and 0.010 μg/g for fresh and processed carrots, respectively.

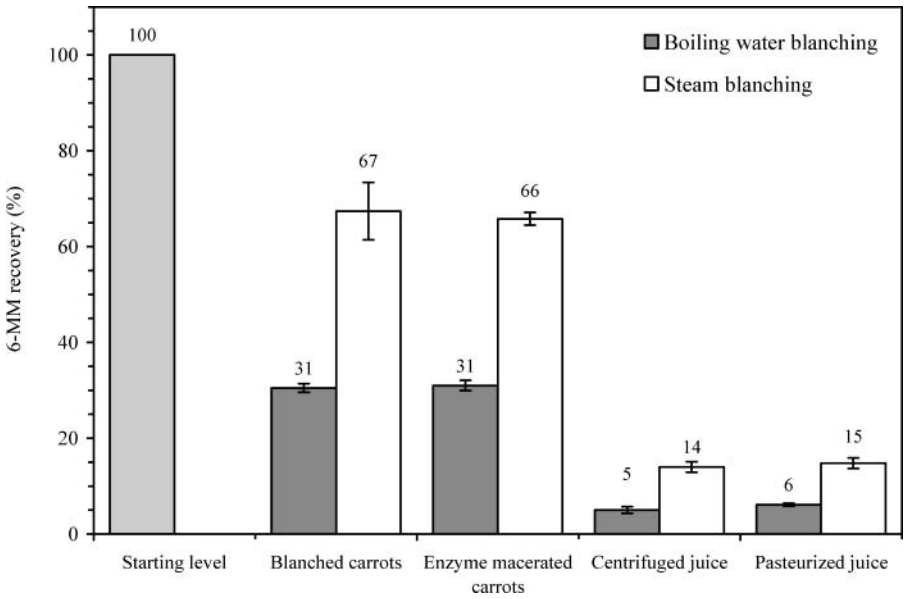

Figure 3. Fate of 6-methoxymellein (6-MM) during carrot juice processing using blanching with boiling water or steam treatment. Bars represent mean recoveries ± 1 SEM of two (blanched carrots) or four analytical measurements.

reported in **Table 3**. Blanching reduced 6-MM content in carrots to different extents depending on the procedure used; in particular, 6-MM levels dropped from 13.50 μg/g in fresh carrots to 4.23 and 8.97 μg/g in blanched carrots with boiling water or steam treatment, respectively. With respect to blanching, maceration with pectinolytic enzymes from either Rapidase Carrot Juice or Ultrazym AFP-L preparation did not influence 6-MM levels; this is in disagreement with previous reports of 6-MM elicitation in carrot cell suspension cultures by direct addition of pectinolytic enzymes (*7*, *9*, *20*). Data obtained with the two enzyme preparations were pooled together for calculation of the absolute amount of 6-MM recovered in the different steps of carrot juice processing (**Table 4**). Carrots blanched with boiling water contained an average absolute amount of 12.34 mg of 6-MM corresponding to 31% of the original amount present in fresh carrots. 6-MM was also recovered in boiling and cooling water that retained 51 and 2% of the original amount of 6-MM, respectively. Blanching of fresh carrots in boiling water or by steam treatment resulted in reductions of 6-MM by 69 and 33%, respectively, in carrot tissues. However, the total amount of 6-MM recovered in blanched carrots and water was similar with both procedures, accounting for ~84% of the original 6-MM. The loss of the remaining 16% 6-MM could be attributed to degradation due to heat treatment. This finding is in agreement with a previous investigation reporting mainly a leaching of 6-MM into the boiling water after carrot blanching for 15 min (*21*).

The centrifugation step did not alter, but simply distributed, the levels of 6-MM between juice and pulp by concentrating the phytoalexin in the pulp by ~3-5times. The amount of 6-MM found in the blanched carrots was fully retained in the mass of macerated carrots (**Table 4**). Considering the pasteurization step, a reduction of 6-MM was observed only for pulp and not for juice. In particular, with respect to centrifuged pulp, the amount of 6-MM dropped from about 10 to 2 mg and from 18 to 7 mg in pasteurized pulp originated from boiling water and steam blanching, respectively (**Table 4**). As for blanching treatment, this loss of 6-MM could be attributed to thermal degradation occurring in solid carrot tissue. The lack of 6-MM degradation in juice after pasteurization may be tentatively explained by a higher resistance of free 6-MM in solution to heat treatment. Similar results were obtained by Mercier et al. (*21*), who boiled for 15 min a 6-MM water solution obtained by boiling UV-treated carrot slices. The fate of 6-MM during carrot juice processing is graphically shown in **Figure 3**. It clearly shows a higher reduction of 6-MM with boiling water blanching with respect to steam blanching (69 vs 33%). Moreover, centrifugation produced a 6-MM decrease (of ~80%) in the juice. The amount of 6-MM recovered in the final product (pasteurized juice) obtained

Table 3. Distribution of 6-Methoxymellein (6-MM) in Blanched Carrots Macerated with Two Different Enzyme Preparations (Rapidase Carrot Juice and Ultrazym AFP-L Enzymes)

sample	6-MM[a] (μg/g)	
	Ultrazym AFP-L[b]	Rapidase Carrot Juice[b]
fresh carrots	40.50 ± 1.18	40.50 ± 1.18
blanching in boiling water for 2 min		
blanched carrots	4.23 ± 0.13	4.23 ± 0.13
macerated carrot mass	4.40 ± 0.01	4.05 ± 0.27
centrifuged juice	1.62 ± 0.08	1.39 ± 0.03
centrifuged pulp	9.69 ± 0.9	7.97 ± 0.33
pasteurized juice	1.74 ± 0.15	1.85 ± 0.01
pasteurized pulp	2.11 ± 0.09	1.88 ± 0.13
blanching under steam for 3 min		
blanched carrots	8.97 ± 0.80	8.97 ± 0.80
macerated carrot mass	8.80 ± 0.30	8.46 ± 0.19
centrifuged juice	4.48 ± 0.58	5.17 ± 0.06
centrifuged pulp	12.77 ± 0.06	10.17 ± 0.02
pasteurized juice	5.13 ± 0.06	5.57 ± 0.13
pasteurized pulp	4.73 ± 0.26	4.85 ± 0.31

[a] Data refer to mean values of two analytical measurements ± standard error mean (SEM). Levels are reported on a fresh weight basis. [b] Statistical analysis of the data showed no significant difference ($p > 0.05$) between the two enzyme preparations for each row.

Table 4. Absolute Amount of 6-Methoxymellein (6-MM) in Fractions Obtained during Carrot Processing by Using Two Different Blanching Procedures

sample	blanching in boiling water		blanching under steam	
	6-MM (mg)	wt (kg)	6-MM (mg)	wt (kg)
fresh carrots[a]	40.50 ± 1.18	3.00	40.50 ± 1.18	3.00
blanched carrots[a]	12.34 ± 0.37	2.92	27.28 ± 1.40	3.04
boiling water[a]	20.77 ± 0.18	8.80		
cooling water[a]	0.97 ± 0.02	4.50	6.83 ± 0.88	3.50
macerated carrot mass[b]	12.49 ± 0.44	2.96	26.80 ± 0.59	3.10
centrifuged juice[b]	2.11 ± 0.11	1.41	5.45 ± 0.40	1.13
centrifuged pulp[b]	9.89 ± 0.71	1.12	17.89 ± 1.17	1.56
pasteurized juice[b]	2.53 ± 0.09	1.41	6.04 ± 0.16	1.13
pasteurized pulp[b]	2.24 ± 0.11	1.12	7.47 ± 0.26	1.56
total recovered amount (pasteurized juice + pulp + water)	26.51 (65.5%)		20.34 (50.2%)	

[a] Data refer to mean values of two analytical measurements ± standard error mean. [b] Data refer to mean values of four analytical measurements (duplicate determinations for each enzyme) ± standard error mean.

by using boiling water or steam blanching accounted for 6 and 15%, respectively, of the original amount present in fresh carrots.

Conclusions. Levels of 6-MM in fresh carrots and carrot-based food products in Europe are generally below the JND level, thus not representing a problem for the consumer in terms of sensory quality. Moreover, 6-MM accumulation in fresh and stored carrots does not seem to be affected by one of the major biotic stress factors, fungal attack by *Alternaria* species. The level of 6-MM in carrots is stable at low temperature for quite long periods. A strong decrease of the 6-MM level occurs during carrot juice processing, with blanching and centrifugation representing the steps during which the major decrease occurs. Blanching with boiling water is more effective than steam treatment in reducing 6-MM levels, leading to final products (pasteurized juice) containing 6 and 15% of the initial 6-MM content, respectively.

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