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Rapid analysis of 6-methoxymellein in carrots by boiling water extraction, solid phase extraction and HPLC

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Abstract

By modification and combination of existing methods we have developed a rapid method for quantification of 6-methoxymellein in carrot roots by means of boiling water and solid phase extraction, followed by high performance liquid chromatography (HPLC). The performance of the method was found to be acceptable, when tested by three consecutive boiling water extractions from carrot samples with different levels of 6-methoxymellein. The recovery was 96–99% after one extraction. With this method it was possible for one person to extract 20 samples during a working day and further quantify the amount of 6-methoxymellein by automatic HPLC the following night. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Carrots; *Daucus carota* L.; 3-Methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin; Extraction; HPLC

1. Introduction

The isocoumarin 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-methoxymellein, = 1H-2-Benzopyran-1-one, 3,4-dihydro-8-hydroxy-6-methoxy-3-methyl) is known to be involved in the development of bitter taste in carrots exposed to ethylene, fungus infection or other stress elicitors (Lafuente, Lopez-Galvarez, Cantwell & Yang, 1996; Marinelli, Ronchi & Salvadori, 1994; Mercier, Arul & Julien, 1993). Most of the extraction methods for 6-methoxymellein, based on organic solvent extraction (Hoffman & Heale, 1987; Howard, Griffin & Lee, 1994; Kurosaki, Amin & Nishim, 1986), are time consuming and therefore not convenient for routine analyses of a large number of samples. Mercier and Arul (1993), however, developed a simple method using boiling water extraction of this compound. In their experiments the amount of 6-methoxymellein extracted with boiling water, gave 18% higher yield than extraction with acetone. The water phase was partitioned with dichloromethane and dried on sodium sulphate before analysis with thin layer chromatography. Howard et al. evolved a convenient HPLC method for quantification of the compound. The aim of our work was to develop a rapid method for the extraction of 6-methoxymellein from carrots by improvement of the boiling water extraction method (Mercier & Arul), combined with solid phase extraction (SPE) and quantitative detection by HPLC (Howard et al.).

2. Materials and methods

2.1. Reagents

All reagents were of pro analysis quality. Acetone, chloroform, *n*-hexane, methanol for liquid chromatography, sodium hydroxide, hydrochloric acid and anhydrous sodium sulphate were purchased from Merck (Darmstadt, Germany).

2.2. Isolation and identification of 6-Methoxymellein standard

The method described by Kurosaki and Nishi (1983) was used with minor modifications to isolate 6-methoxymellein as standard for HPLC-analysis. Two kg carrots of cv. 'Yukon' that had previously been stored for 3 months at 0–1°C, were transferred to perforated plastic bags together with an equal amount of fully ripened

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'Golden delicious' apples and stored at 20°C for 1 week to accumulate 6-methoxymellein. The apples were used as source for ethylene. After storage the carrots were sliced (2 mm) and extracted three times (each of 24 h) with 4000 ml of acetone on a shaking machine with rotary motion (Model SM, Edmund Bühler, Tübringen, Germany) at 20°C. The extract was concentrated to 500 ml by use of a rotavapor (Büchi Laboratoriums-technic AG, CH-9230 Flawil, Schweiz) at 40°C. The concentrated extract was re-extracted three times with 500 ml of *n*-hexane. The upper phase (1500 ml) was then extracted two times with 500 ml 2 M sodium hydroxide. The aqueous phase was neutralised with 6 M hydrochloric acid to pH 7 at 0°C and re-extracted two times with 1000 ml *n*-hexane. The *n*-hexane phase was dried by anhydrous sodium sulphate and evaporated by rotavapor at 40°C to ca. 5 ml. The 6-methoxymellein in the extract was purified by silica gel column chromatography with a glass column (150 × 30 mm), manually packed with silica gel (60, 230–400 mesh, Merck, Darmstadt, Germany). Chloroform:hexane (50:50) were used as eluent. The solvent was evaporated by rotavapor (40°C) and the residue were resolved in 10 ml of methanol. The pure compound was identified by UV spectrophotometry (diode array spectrophotometer, 8452A, Hewlett-Packard, Palo Alto, CA) and gas chromatography-mass spectroscopy (Hewlett-Packard HP 5890 serie II and HP 5970 mass selective detector, Palo Alto, CA). The stability of 6-methoxymellein standard was measured during storage for up to 18 months storage at 4–5 and at −80°C.

2.3. Determination of 6-methoxymellein in carrot samples

The 6-methoxymellein was extracted as described by Mercier and Arul (1993) with some modifications. Carrots were washed, cut into 10 mm cubes, pulverised in liquid nitrogen (IKA-Universalmühle M20, Janke & Kunkel GmbH & Co KG, Staufen, Germany) and stored at −80°C until analysis. Five grams of frozen carrot powder were extracted with 40 ml of boiling water and boiled for 10 min. The extract was passed through a filter (Black ribbon ashless, no. 300 009, Schleicher & Schuell GmbH, Dassel, Germany) wetted with boiling water before use. The filter with contents was then rinsed with approximately 20 ml of boiling water. The water filtrates were kept at 40°C on a water bath before solid phase extraction (SPE) using C_{18} silica gel column (Sep-Pak® cartridge, Part no. 51910, volume 0.85 ml/filled cartridge, Waters, Millford, MA) was carried out. Before use, the column was conditioned with 4 ml of methanol, followed by 10 ml of water. The filtrate was pressed through the cartridge by use of a 50 ml plastic syringe with luer fitting (Plastipak®, Becton Dickinson S.A., Madrid, Spain). The column was then washed by 5 ml water followed by drying with 10 ml air. 6-Methoxymellein was then eluted with 1.9 ml methanol into a 2 ml graduated flask. The trapped solvent was forced out of the column by means of 10 ml air. The sample volume was adjusted to 2 ml with methanol. Samples were kept at −20°C for maximum 14 days if not analysed by HPLC immediately. Prior to HPLC analysis samples were filtered through 0.50 μm filters (Millex®-FH, Millipore corp., Bedford, MA). The HPLC (HP 1050, Hewlett-Packard, Avondale, PA) was equipped with a silica gel column (C_{18}, 4 μm, 3.9 × 150 mm, Part. no. WAT086344, Nova-Pak®, Waters, Milford, MA). Samples were analysed according to Howard et al. (1994) using flow 1 ml min^{-1} with a linear gradient: 40 to 60% methanol in water in 10 min, and 60 to 80% in 15 min. Detection of 6-methoxymellein was performed by a HP 1050 series diode-array detector at 267 nm. Injection volume was 10 μl. The 6-methoxymellein previously isolated was used as external standard at concentrations from 0.1 to 500 μg ml^{-1} in methanol (concentrations determined by UV spectrophotometer at 267 nm).

2.4. Extraction tests

Extraction tests were performed in three replicates of carrots with low (< 1 μg g^{-1} carrot), medium (about 40 μg g^{-1} carrot) and high (about 100 μg g^{-1} carrot) contents of 6-methoxymellein. Samples were extracted three times with 40 ml of boiling water each. The SPE-column was tested for any possible residue of 6-methoxymellein after the elution with 1.9 ml methanol, by eluting with two more volumes of methanol. The recovery of extracted 6-methoxymellein was measured in the same way by mixing known amounts of 6-methoxymellein with carrot samples without detectable amount of this compound; 10 ml of a methanolic 6-methoxymellein solution (13.4 μg ml^{-1}) was transferred to a Erlenmeyer flask and 2 ml water was added. The methanol was evaporated by flushing with N_2 flow at 40°C. Frozen carrot powder was then added, the mixture was extracted and the 6-methoxymellein recovery calculated. Standard deviations were calculated for the three replicates.

3. Results

3.1. Isolation and identification of 6-methoxymellein

The absorbance (A) of the isolated 6-methoxymellein (diluted to 11.8 μg ml^{-1}) was 0.84 at 267 nm and 0.34 at 302 nm giving a peak height ratio (A 267/A 302) of 2.47 (Fig. 1) equivalent to that of the characteristic ultraviolet absorption spectra of 6-methoxymellein (A 302 nm; ε 6000/A 267 nm; 14 800) obtained by Sondheimer (1957).

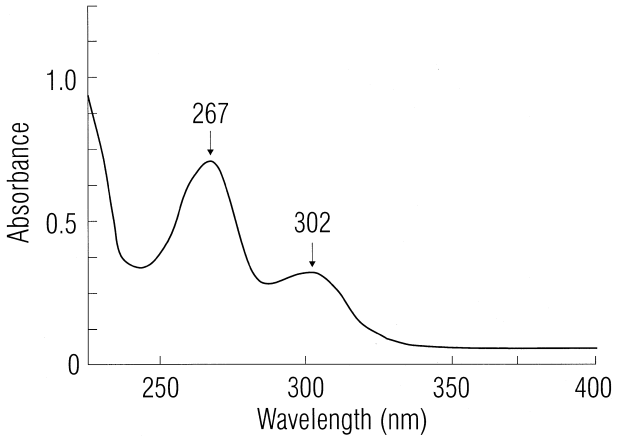

Fig. 1. Ultraviolet absorption spectrum for 6-methoxymellein (11.9 μg ml^{-1} methanol) isolated from carrots showing the characteristic peaks at 267 and 302 nm.

The peak height ratio was not altered during two weeks storage at 4–5 or at −80°C. After 18 months' storage, the peak height ratio increased to 2.62 by storage at 4–5°C and to 2.56 by storage at −80°C. Totally 40 mg of 6-methoxymellein was obtained from the 2 kg of carrots exposed to ethylene.

The HPLC calibration curve for 6-methoxymellein was linear within the concentration range applied (0.1 to 500 μg ml^{-1}). The retention time for the 6-methoxymellein standard was about 10 min (Fig. 2a). A typical chromatogram of a sample extracted from carrots is shown in Fig. 2b. The detection limit of 6-methoxymellein (peak height at least 10 times the noise level) was set to 0.1 g ml^{-1} extract injected which corresponds to 0.02 μg g^{-1} fresh carrot.

3.2. The recovery of 6-methoxymellein from spiked carrot samples

The recovery of 6-methoxymellein from the three spiked carrot samples was 97.4 ± 1.4% after one water extraction (Table 1). A second extraction gave only a slight increase (0.5 ± 0.2%) in the 6-methoxymellein recovery. The amount of 6-methoxymellein eluted from the SPE-column with a second volume (1.9 ml) of methanol was 0.4 ± 0.1% in the first water extraction. No 6-methoxymellein was detected in a third methanol eluate.

3.3. Efficiency of water extraction of 6-methoxymellein from carrot samples

Analysis of the three carrot samples with low, medium and high contents of 6-methoxymellein (Table 2) gave similar results as the recovery test of spiked carrot samples. Total amount of 6-methoxymellein in these samples was set to the total amount of detectable substance extracted by three extractions. For carrots with

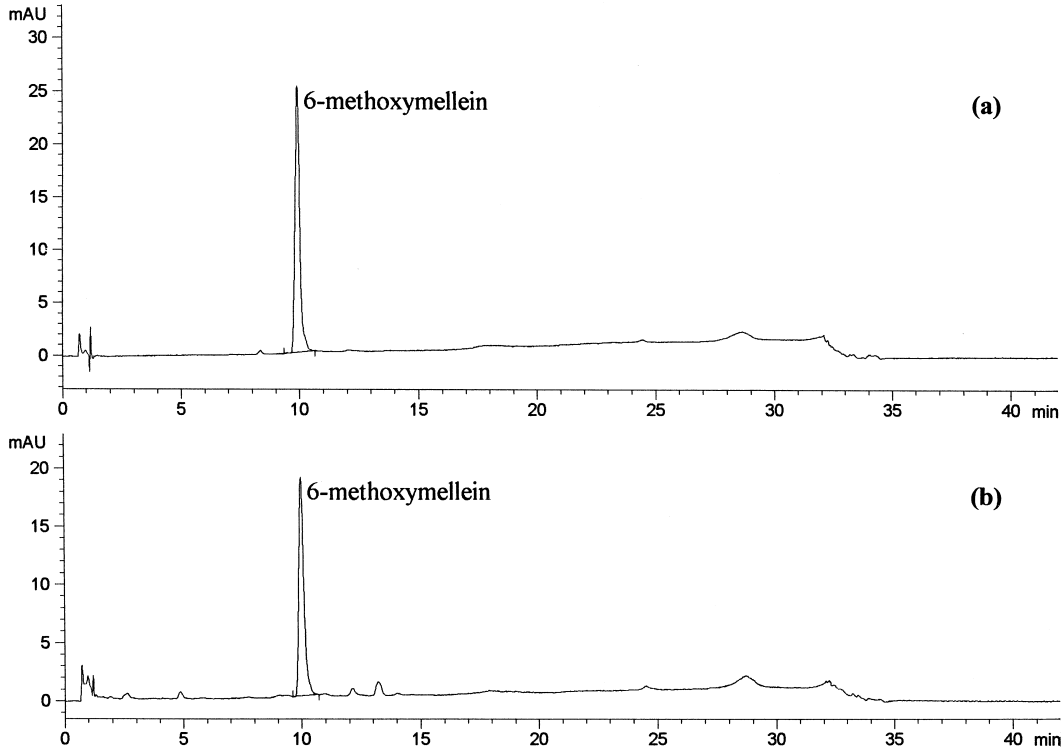

Fig. 2. HPLC chromatogram of (a) isolated 6-methoxymellein (8.9 μg ml^{-1}) and (b) a carrot sample extracted by the present method giving 7.1 μg ml^{-1} of 6-methoxymellein. Injection volume was 10 μl.

Table 1
Recovery of 6-methoxymellein from a spiked carrot sample (initial content below detection limit)[a]

Carrot sample	Amount 6-methoxymellein added (μ g^{-1})	Recovery of 6-methoxymellein							
		Water extraction 1				Water extraction 2		Total amount extracted	
		Methanol elution 1 (μg g^{-1})	(%)	Methanol elution 2 (μg g^{-1})	(%)	Methanol elution 1 (μg g^{-1})	(%)	(μg g^{-1})	(%)
I	26.4	26.1	99.0	0.06	0.3	0.08	0.3	26.2	99.5
II	25.7	24.7	96.3	0.10	0.4	0.16	0.6	25.0	97.3
III	25.4	24.6	96.8	0.14	0.5	0.12	0.5	24.9	97.9
Mean ± S.D.[b]	25.8 ± 0.5	25.1 ± 0.8	97.4 ± 1.4	0.10 ± 0.04	0.4 ± 0.1	0.12 ± 0.04	0.5 ± 0.2	25.4 ± 0.8	98.2 ± 1.1

[a] Three consecutive water extractions were performed. During solid phase extraction (SPE), three consecutive methanol elusions were carried out from each column. Data of 6-methoxymellein levels for the third extraction and the second and third SPE elution that were below the limit of detection (0.02 μg g^{-1} fresh carrot) are not shown in the table.
[b] Standard deviation.

low content of 6-methoxymellein all detectable amounts were extracted in the first water extraction and recovered in the first SPE-column elution. Correspondingly, 97.9 ± 0.9% of the detectable amount of compound were extracted by the first water extraction and the first elution from SPE column for carrots with medium level of 6-methoxymellein. By the second extraction an additional 1.3 ± 0.8% of the total amount was isolated. Only trace amounts of 6-methoxymellein were recovered in the second methanol elution from the SPE-column (0.6 and 0.1% for the first and second water extraction, respectively). No 6-methoxymellein was detected in the third water extraction of these samples. Carrots with high content of 6-methoxymellein led to 96.3 ± 1.9% of 6-methoxymellein extracted by the first water extraction and the first elution from SPE column, and 3.1 ± 1.9% in the second water extraction. By a third water extraction, 0.3 ± 0.1% of the total amount extracted was obtained. Only trace amounts of 6-methoxymellein were obtained by the second methanol elution from SPE-column (0.4 and 0.07% for the first and second water extraction, respectively).

Table 2
Efficiency of water extraction from carrot samples with different levels of 6-methoxymellein[a]

Level of 6-methoxymellein in samples[d]	Amount of 6-methoxymellein extracted										
	Water extraction 1				Water extraction 2				Water extraction 3		Total
	Methanol elution 1 (μg g^{-1})[e]	(%)[f]	Methanol elution 2 (μg g^{-1})	(%)	Methanol elution 1 (μg g^{-1})	(%)	Methanol elution 2 (μg g^{-1})	(%)	Methanol elution 1 (μg g^{-1})	(%)	(μg g^{-1})
Low I	0.18	100	n.d.[c]		n.d.		n.d.		n.d.		0.18
II	0.16	100	n.d.		n.d.		n.d.		n.d.		0.16
III	0.20	100	n.d.		n.d.		n.d.		n.d.		0.20
Mean ± S.D.[b]	0.18 ± 0.02	100									0.18 ± 0.02
Medium I	41.3	98.2	0.38	0.9	0.33	0.8	0.03	0.7	n.d.		42.1
II	41.1	98.5	0.25	0.6	0.37	0.9	0.04	0.1	n.d.		41.8
III	41.8	97.2	0.13	0.3	1.00	2.3	0.08	0.2	n.d.		43.0
Mean ± S.D.	41.4 ± 0.4	97.9 ± 0.9	0.25 ± 0.12	0.6 ± 0.3	0.57 ± 0.37	1.3 ± 0.8	0.05 ± 0.02	0.3 ± 0.3			42.3 ± 0.7
High I	105.0	97.0	0.27	0.2	2.83	2.6	0.08	0.07	0.30	0.27	108.5
II	105.6	97.8	0.41	0.4	1.76	1.6	0.06	0.06	0.20	0.19	108.0
III	114.2	94.2	0.54	0.4	5.96	5.3	0.10	0.08	0.39	0.32	121.2
Mean ± S.D.	108.3 ± 5.2	96.3 ± 1.9	0.40 ± 0.13	0.4 ± 0.1	3.52 ± 2.18	3.1 ± 1.9	0.08 ± 0.02	0.07 ± 0.01	0.3 ± 0.1	0.3 ± 0.1	112.6 ± 7.5

[a] Three consecutive water extraction steps were performed. During solid phase extraction (SPE), three consecutive methanol elusions were carried out from each column. Data of 6-methoxymellein levels for the second and third elution that were below the limit of detection are not shown in the table.
[b] Standard deviation.
[c] Below detection limit of 0.02 μg g^{-1} fresh carrot.
[d] Three parallel extractions (I, II and III) were carried out from three samples with different amounts of 6-methoxymellein.
[e] μg g^{-1} fresh carrot.
[f] Percentage of total extracted amount of substance above detection limit.

4. Discussion

The results show that only one water extraction is sufficient for extraction of 6-methoxymellein from carrots. Elution of SPE-columns with a single portion of 1.9 ml methanol seems to be sufficient for all the samples tested. The last step with pressing out the solvent residue from the cartridge with air led to reduced loss of 6-methoxymellein in the SPE column. Thermostating of samples at 40°C prior to SPE purification was necessary to avoid clogging of the column at a lower temperature. The average yield of 6-methoxymellein obtained by one extraction of three carrot samples with approximately 40 μg g^{-1} of the substance, was higher with our method, 97.9%, than by acetone extraction or by the previous boiling water extraction method, 80.5 and 89.9% respectively (Kurosaki et al., 1986; Mercier & Arul 1993). By our method the standard deviation of three carrot samples was lower than by the previous water extraction method and the standard solvent extraction method (0.9% versus 5.7 and 5.3% respectively). The beneficial properties of boiling water for extracting this compound compared with organic solvent extraction, has already been discussed (Mercier & Arul). One reason for the relatively high yield and the low standard deviation of our method could be the efficient homogenisation before extraction. Another reason could be reduced loss due to fewer purification steps. Furthermore, moistening of the filter before filtration of the boiled extract, and washing of the residue in the filter with boiling water, would reduce the binding of 6-methoxymellein to the filter and the carrot residue, and thus contribute to the higher yield.

Time for extraction and purification of one sample was on an average 25 min, enabling processing of 20 samples in an 8 h working day, which makes this method convenient for larger number of samples.

In the present study, the pure 6-methoxymellein was prepared before the final extraction procedure was developed and the acetone extraction as described by Kurosaki and Nishi (1983) was used. Pure 6-methoxymellein could probably be obtained more rapidly by using our boiling water extraction method followed by purification with the same silica gel column chromatography procedure (Kurosaki & Nishi).

5. Conclusions

The present method is a rapid and environmentally safer way to analyse 6-methoxymellein in carrots. The yield of 6-methoxymellein was higher with the present method compared with both traditional acetone extraction and the boiling water extraction method described by Mercier and Arul (1993). Purification of the water extract by solid phase extraction provides a rapid removal of water and a concentrated methanol extract with 6-methoxymellein ready for the analysis on HPLC.

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