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Analysis of sweet orange (*Citrus sinensis* (L.) Osbeck) callus cultures for volatile compounds by gas chromatography with mass selective detector *

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Abstract

Volatile constituents of embryogenic and nonembryogenic sweet orange (*Citrus sinensis* (L.) Osbeck) callus cultures were analyzed by gas chromatography-mass spectrometry to determine if sweet orange flavor essences were produced. Fifteen compounds were identified from the embryogenic callus methylene chloride extracts, with 10 previously reported as volatile constituents of orange juice or peel essential oil, 3 are known fermentation products, 2 have no reported aroma, and 2 were unknown. No volatile compounds were detected from nonembryogenic callus methylene chloride extracts.

Abbreviations: eV – electron volt; GC-MS – gas chromatography-mass spectrometry

Introduction

The development of frozen concentrated orange juice (FCOJ) by USDA and Florida Citrus Commission researchers in 1946 dramatically increased the demand for orange juice. One of the key discoveries was the use of 'cut-back' juice to restore the fresh orange flavor to juice concentrate lost during the concentration process. When fresh juice was added back to highly concentrated juice samples, a fresh orange flavor was restored to the entire sample, which could then be stored frozen for long periods with little decrease in quality (Berry and Veldhuis, 1977; MacDowell et al., 1948). The concurrent development of home refrigerators and freezers during the 1940s permitted the widespread use and acceptance of FCOJ.

It was later found that the peel oil and aqueous fractions of volatile compounds that make up the aroma of fresh orange juice could further enhance the quality of frozen concentrate or even replace the use of 'cut-back' juice (Shaw, 1977a; Wolford and Attaway, 1967). Research to determine what compound(s) were responsible for orange flavor concluded that orange flavor is not the result of one or a few compounds, but rather an interaction of many compounds (Dougherty and Ahmed, 1973; Shaw, 1977b), although some compounds in certain combinations significantly enhance the flavor of processed orange juice.

Embryogenic and nonembryogenic cell lines from sweet orange (*C. sinensis*) have been developed in our laboratory (Niedz, 1993; Osswald et al., 1992). One difference between these lines, apart from their embryogenic capacity is a sweet, fruity aroma emitted from the embryogenic lines. The objective of this study was to determine what compounds were responsible for the characteristic aroma of the embryogenic lines.

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Materials and methods

Plant material

Embryogenic cell lines of *C. sinensis* (L.) Osbeck cvs. Hamlin (H89) and Valencia (V90) were initiated and maintained as previously described for Hamlin cell line H89 (Niedz, 1993). These embryogenic cell lines were cultured in 100 × 15 mm ($\phi \times$ h) polystyrene culture dishes sealed with Parafilm tape (Sigma Chemical Corp., St. Louis, MO, USA). Also, a nonembryogenic cell line of Valencia (Val88-1) derived from immature juice vesicles was initiated and maintained as previously described (Osswald et al., 1992). This nonembryogenic cell line was cultured in polycarbonate GA-7 vessels with a polypropylene closure (Magenta Corp., Chicago, IL, USA)

Tissue samples and blanks

Embryogenic and nonembryogenic cell lines were grown for 21 days and then analyzed for volatile constituents. Blanks were prepared by incubating the culture medium in the appropriate plastic culture containers or glass 100 × 15 mm ($\phi \times$ h) culture dishes under the same conditions for 21 days. The blanks allowed for the identification of compounds not produced by the cell lines, but produced by the culture medium or plastic containers used.

Extraction procedures

Embryogenic tissue cultures from Valencia orange, V90, grown on 20 petri dishes (samples 10–89 V and 10–90 V) were removed with a spatula, combined and extracted with 200 ml of methylene chloride (Capillary GC/GC-MS Solvent, Burdick and Jackson, Muskegon, MI) by stirring the mixture for 30 min, filtering and drying the filtrate over powdered magnesium sulfate. The extract was filtered and concentrated on a rotary evaporator under reduced pressure (38 mm Hg) at < 40 ° to a small volume (250 μl) for analysis by gas chromatography (GC) and GC-mass spectrometry (GC-MS). Embryogenic tissue cultures from Hamlin orange, H89, were similarly extracted and concentrated for GC and GC-MS analysis as well.

The concentration of each constituent in Table 1 is μg g^{-1} tissue was calculated from the GC peak area percent value for that constituent in 250 μl of methylene chloride extract obtained from 6 g dry weight (60 g wet weight) of tissue cultures collected from 20 petri dishes. The solvent concentration procedure may cause some loss of volatiles with boiling points lower than that of the solvent (e.g. acetaldehyde) but it has been used by several workers to afford quantitative estimates of volatile constituents in citrus juices (Marsili et al., 1989; Schreier, 1981).

Several blanks were extracted also with methylene chloride and treated as above. The blanks included agar gel culture medium (embryogenic or nonembryogenic) samples from 5 polystyrene and 5 glass culture dishes, and nonembryogenic tissue from Valencia orange grown on agar gels. These concentrated extracts were similarly analyzed by GC and GC-MS.

Chromatographic procedures

Analyses of the extracts were performed using a Hewlett-Packard Model 5890 instrument equipped with a flame ionization detector, a 50 m x 0.32 mm i.d. x 1.0 μm fused silica capillary column of cross-linked 5% phenylmethyl silicone (Hewlett-Packard, Avondale, PA), and a capillary inlet system fitted with a split line that allowed the helium carrier gas flow to be split at 50:1. Helium flow through the column was 1.5ml/min^{-1}. Injection port temperature was 2500 °C and detector temperature was 2750 °C. The column temperature was held at 600 °C for 6 min and then programmed to 2100 °C at 60 °C/min and held there for 45 min. The threshold was set at 1, peak width at 0.02, and chart speed at 1 cm min^{-1}. Samples of extracts (1.0 μl each) were injected manually. Quantitative estimates were determined with average values of triplicate runs for each peak.

Mass Spectra

Identification of individual constituents was made using GC-MS. A Hewlett-Packard Model 5970B, MSD instrument was used with a 50 m × 0.32 mm i.d. × 1.0 μm fused silica column of cross-linked 5% phenylmethyl silicone. The initial oven temperature was held at 550 °C for 9 min and then programmed at 7.50 °C/min to 2200 °C, and held there for 30 min. Injection port and ionizing source were kept at 2500 °C and the transfer line was kept at 2800 °C. Mass units were monitored from 25 to 350 at 70eV. Mass spectral matches were made by comparison of mass spectra and retention times with those of authentic compounds purchased from commercial sources. The two isomers of 2,3-butanediol were purchased from Aldrich, Milwaukee, WI.

Table 1. Constituents produced by embryogenic Valencia and Hamlin tissue culture samples.

Constituent	R.T.[a]	Concn., μg g^{-1} tissue[b]		Aroma characteristics[g]
		Valencia	Hamlin	
Acetaldehyde[c]	4.33	15	45	pungent aroma, major component of orange flavor
Ethyl acetate[c]	6.96	63	33	fruity aroma
3-Methyl-2-butanone	8.44	28	20	
2-Pentanone[c]	9.27	33	16	pleasant aroma
3-Hydroxy-2-butanone[d]	10.22	3,610	3,690	pleasant aroma
1,1-Diethoxyethane[c]	10.76	10	5	pleasant ethereal aroma
2,3-Butanediol[d,e]	12.53	17	242	mild yeasty aroma
Diethyl carbonate[c]	12.77	3.3	n.d.[f]	pleasant ethereal aroma
meso-2,3-Butanediol[d]	12.91	62	1250	
Unknown	13.74	26	12	
Unknown	14.13	41	24	
Ethyl 2-methylbutanoate[c]	15.20	2.8	1.9	fruity aroma
γ-Butyrolactone	17.62	34	19	
Ethyl 3-hydroxybutanoate[c]	18.13	29	13	fruity aroma
p-Cymene[c]	21.26	5.6	2.5	strong medicinal aroma, important in tangerines and limes
Limonene[c]	21.41	207	74	pleasant lemon-like aroma
1,8-Cineole[c]	21.72	2.7	2.7	oil of eucalyptus, camphor/spice aroma, major component of lime essence.

[a] R.T. indicates GC retention times on a nonpolar capillary column (stationary phase) under conditions described above.
[b] Dry weight based on 0.3 g dry tissue per plate.
[c] Previously reported as volatile constituent in orange juice or peel oil (Maarse & Visscher, 1989; The Merck Index, 1983).
[d] Known product of fermentation (Davidek et al., 1990).
[e] Optically active form of 2,3-butanediol. The isomer used as the authentic sample was (2R,3R)-(-)-2,3-butanediol obtained from Aldrich.
[f] n.d. = not detected.
[g] Descriptions from The Merck Index (1983).

Results and discussion

Embryogenic cell lines derived from nucellar tissue were previously developed for sweet orange (Niedz, 1993). Plants could be regenerated from these lines via somatic embryogenesis under a variety of conditions, including the substitution of glycerol for sucrose as the primary carbon source. A characteristic of these embryogenic cell lines is the emission of a pleasant, sweet, fruity aroma. This aroma seems associated with rapidly growing and 'healthy' cultures. It is not present in the nonembryogenic Val88-1, and disappears soon after the embryogenic cells are transferred to the glycerol medium used to stimulate embryogenesis. The aroma also disappears when the embryogenic lines are cultured for extended periods without subculturing. Though the development and use of embryogenic cell lines have been extensively reported for sweet orange (Spiegal-Roy and Vardi, 1984; Vardi and Galun, 1989), we are not aware of any reports that have identified the volatile components, or discuss the distinctive aroma characteristic of these cells.

Blanks were prepared to distinguish compounds produced by the cells from those produced by the culture container or medium. Blanks included the plastic containers (polystyrene dishes and GA-7 vessels) containing the culture medium used for culturing the cells, and glass containers with embryogenic or nonembryogenic culture medium. The glass containers permitted the identification of compounds produced by either culture medium alone. Blanks were incubated under the same conditions and for the same length of time as the cell lines.

Volatile constituents of embryogenic Valencia and Hamlin orange tissue, and associated with a sweet fruity aroma at the time of analysis, are listed in Table

1. These volatiles were not present in the nonembryogenic Val88-1 samples. Val88-1 tissue samples were the same as the blanks. Fifteen of the seventeen compounds were identified by comparison of GC retention times and mass spectra with those for authentic samples. Ten of the identified constituents had been reported as volatile constituents of orange juice or peel essential oil, as indicated by footnote c in Table 1. Three known products of fermentation, 3-hydroxy-2-butanone and two 2,3-butanediol isomers, were identified as well (Davidek et al., 1990). Two unknown volatile constituents present in moderate amounts were found, and are also listed in Table 1.

The aromas emitted by the embryogenic tissue samples from Valencia and Hamlin orange were both sweet and somewhat fruity in character, but that from Valencia had a stronger overall intensity. This was reflected in the GC patterns for extracts from the two samples. Most of the same constituents were found in both extracts, as shown by the constituents quantified (Table 1). Of the seventeen compounds listed, all except one, diethyl carbonate, were found in both samples. Thirteen were found in Valencia tissue at levels equal to or higher than those found in Hamlin tissue, and include nine of the ten identified constituents previously reported in orange flavor fractions. 3-hydroxy-2-butanone and the 2,3-butanediol isomers were stronger in the Hamlin tissue sample, and have been identified in orange juice (Parish et al., 1990) and other plants (Hansen et al., 1993; Jennings, 1977; Shaw and Wilson, 1982). These compounds do not have fruity aromas and were probably metabolites from acetaldehyde. Their presence indicates that some anaerobic metabolism was occurring in the cell cultures. Volatile yields were considerably less than what is found in fruit. For example, volatile yield from Valencia (Table 1) was 0.42 mg volatiles/g (fresh weight) tissue. The average yield of fruit is 6.8 mg peel oil/g. However, total yield over time would be expected to be higher for cell cultures as it takes 1 year for a Valencia fruit to ripen, but only 21 days for one cycle of cell culture growth.

Not shown in Table 1 were the several unknown components and dibutyl phthalate, which were present in all the blanks as well as in the nonembryogenic tissue. Dibutyl phthalate is an artifact extracted from plastic which often is present in extracted samples from fruit. A small amount was present even in the extract of agar medium prepared in glass plates.

Madhavi et al. (1991) initiated callus, presumably nonembryogenic, from albedo and juice vesicles of the Coorg mandarin (*C. reticulata* Blanco). GC analysis of methylene dichloride extracts identified 7 compounds in albedo-derived callus (limonene, *alpha*-terpenene, linalool, nerol, geraneol, geranyl acetate, and linalyl acetate) and three in juice vesicle-derived callus (limonene, geranyl acetate, and linalyl acetate). Callus from both sources emitted a fruity odor, unlike the nonembryogenic callus Val88-1 used in this study. Val88-1 was also initiated from juice vesicles but is an established 8 year old cell line. The Coorg mandarin calluses used were newly initiated from albedo or juice vesicle tissues, and probably did not reflect the secondary metabolite expression of an established undifferentiated cell line. Limonene was the only compound identified in Coorg mandarin callus that was also identified in the Valencia embryogenic callus extract used in this study. Agrawal et al. (1991) reported a study with lime (*C. aurantifolia* Christm.) similar to the Coorg mandarin study. Callus was initiated from lime flavedo, subcultured three times, and methylene chloride extracts were analyzed by GC-MS. Four aromatic compounds were identified and imparted a lime-like aroma to the callus - citral a (=geranial), citral b (=neral), terpinyl acetate, and dodecanal. None of these compounds were identified in the Valencia embryogenic callus extracts. Reil and Berger (1996) reported that heterotrophically-initiated cells of grapefruit, lemon, or lime did not produce volatiles. Our results with the nonembryogenic, heterotrophic, Val88-1 cell line confirm these observations.

The *in vitro* production of volatile constituents important in the flavor and aroma of orange could be useful in the further characterization of their underlying enzymology. Fruit is only available on a seasonal basis in citrus, making a tissue culture system particularly useful in researching orange essence biosynthesis. With a higher metabolic rate than fruit, tissue cultures produce secondary metabolites rapidly, making detailed biochemical studies of these metabolic pathways possible (Dörnenburg and Knorr, 1995).

In conclusion, both the Valencia and Hamlin orange embryogenic tissues were capable of producing a mixture of constituents which possessed a sweet, fruity aroma. Most of the fifteen constituents identified have been identified in orange juice aqueous essence or peel oil. The nonembryogenic tissue did not produce these constituents, and they were not present in any of the agar plates not containing the embryogenic tissue samples.

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