Immunoanalytical Method for Quality Control of Orange Juice Products

Agnes Sass-Kiss*,† and Miklós Sass‡

Department of Analytical Chemistry, Central Food Research Institute, Herman Ottó 15, 1022 Budapest, Hungary, and Department of Zoology, Eötvös Loránd University of Sciences, Puskin 3, 1088 Budapest, Hungary

The main goal of the present study is to develop an immunoanalytical method for the quality control of orange juice products. Peptides from various parts (juice, albedo, and flavedo) of citrus fruits (orange, mandarin, grapefruit, and lemon) were analyzed and isolated by SDS−PAGE. Antisera were developed in mice against the protein pool of orange juice and peel and tested by Western blot analysis. Using these antisera, some juice- and peel-specific peptides were detected. One of the antibodies in the antiserum developed against peel proteins recognized a single peel-specific peptide with a molecular mass of 28 kDa in 10000-fold dilution. It did not give any positive reactions against the sample prepared from the juice. The 24 and 27 kDa juice-specific peptides were isolated in electrophoretically pure form, and polyclonal antibodies were developed against them in mice. The anti-27 kDa antibody reacted with a 29 kDa protein in the peel sample, and it gave a positive reaction against the 27 kDa peptide of the juice. The antibodies developed in the course of the present work seem to be useful for determining the juice content in commercial citrus beverages and for evaluating the peel contamination in them.

Keywords: *Orange; juice; albedo; flavedo; peptides; gel electrophoresis; Western blot*

INTRODUCTION

The quality control of citrus juice beverages is of crucial importance as these products can be easily falsified. Increasing numbers of papers related to the adulteration of fruit juice beverages have been published, indicating the significance of this relevant and yet unresolved problem. Adulteration can be based on a simple dilution with water or substitution of cheaper artificial ingredients (sugars, acids, and colorants).

Developing analytical methods and broadening our knowledge on the constituents of the fruits have resulted in the increasingly effective detection of adulteration. Numerous compounds have been investigated for their suitability for quality control of citrus juices, such as minerals (Royo and Giménez, 1974; McHard et al., 1979), organic acids (Primo et al., 1969; Lang, 1972), and sugar components (Benk, 1968; Low and Swallow, 1991). Flavanon-glycosides as characteristic compounds of citrus species (naringin in grapefruit and hesperidin in orange) were studied by many authors (Mears and Shenton, 1973; Grandi et al., 1994; Hermann, 1994). Several publications are available on the free amino acid composition of citrus fruits (Mears and Shenton, 1973; Zamorani et al., 1973; Ooghe and Waele, 1982; Navarro et al., 1984). Determination of the formol number is an indirect way of quantifying the free amino acid content in fruit juices and drinks. It allows the estimation of the possible dilution of beverages.

Due to increasing information on the chemical composition of fruits, more refined methods of adulteration are now used. Most of these methods are based on supplementation of citrus juice with pulp and peel extracts or with some cheaper fruit juice (Mears and Shenton, 1973). None of the analytical methods mentioned above is specific enough to establish the presence and concentration of substances originating from waste materials (peel and pulp wash) or other citrus species.

On the basis of the species and tissue specificity of the proteins, it might be possible to identify the presence of various components of citrus fruits in industrial products. Differences in protein composition of the exo-, meso-, and endocarp of citrus fruits (Clements, 1966) and species-specific immunogenicity of orange and lemon juice were reported (Cantagalli et al., 1972) earlier. A method based on immunodiffusion has been worked out by Firon et al. (1979) for determining the juice content in commercial products using an antiserum developed against the total protein content of orange juice. Although these early results using antisera and immunodiffusion were quite promising, they were not specific enough to determine and quantify the proteins originating from various parts of citrus fruits.

Therefore, our goal was to identify tissue- and species-specific peptides in citrus fruits, to develop antibodies against these particular peptides from orange juice and peel, and to test their suitability for quality control of commercial orange juice products using the Western blot technique.

MATERIALS AND METHODS

Orange (*Citrus sinensis*), mandarin (*Citrus reticulata*), grapefruit (*Citrus paradisi*), and lemon (*Citrus limon*) originating from Spain were purchased on the market. After the fruits had been peeled, the juice was obtained using a fruit press. The peel (albedo plus flavedo) was comminuted, or albedo and flavedo were handled separately before cutting.

* Author to whom correspondence should be addressed (telephone +36-1-3558838; fax +36-1-2741005; e-mail a.sass@cfri.hu, h9747sas@ella.hu).

† Central Food Research Institute.

‡ Eötvös Loránd University of Sciences.

10.1021/jf9903962 CCC: $19.00
Published on Web 08/12/2000

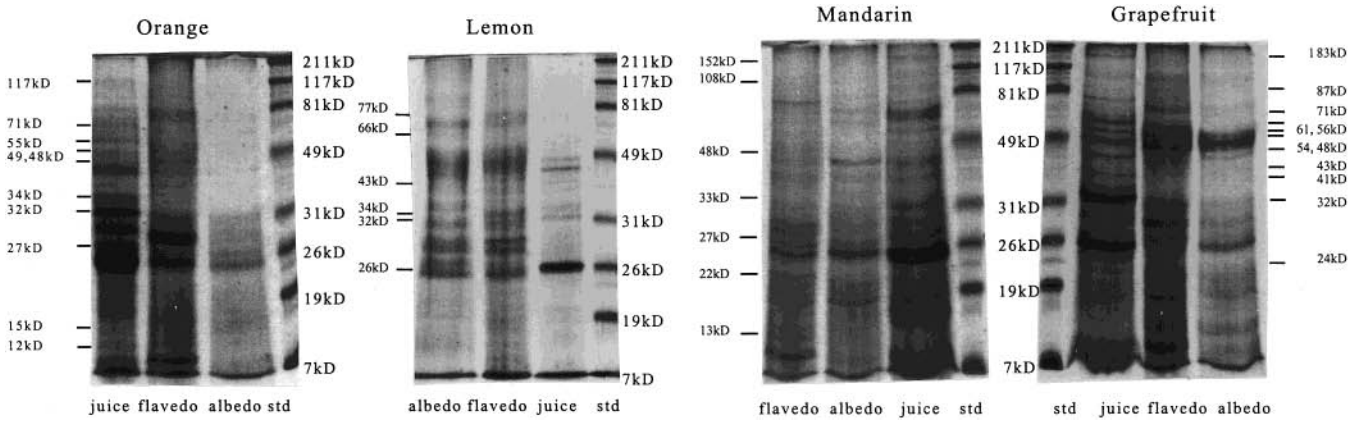

Figure 1. Gel electrophoretograms of peptide samples prepared from different parts of citrus fruits (std = molecular mass standard).

Isolation of Proteins from Juice, Albedo, and Flavedo of Citrus Fruits. Ten grams of albedo and 10 g of flavedo (or in some experiments the whole peel) were suspended in 50 mL of phosphate buffer (0.04 M $Na_2HPO_4 \cdot 12H_2O$, 0.005 M KH_2PO_4, 0.685 M NaCl, 0.025 M KCl) and homogenized in an Ultra-turrax device. Fifty grams of juice or homogenate of peel samples was centrifuged at 12000*g* for 30 min. The sediments were resuspended in 50 mL of phosphate buffer and centrifuged at 12000*g* for 30 min again. The first and second supernatants were pooled. Proteins were precipitated with trichloroacetic acid (final concentration of 10%) at 4 °C overnight. The precipitates of juice or peel samples were centrifuged at 16000*g* for 20 min, washed three times with 1 mL of distilled water to reach pH 3.0, and then resuspended in 500 μL of phosphate buffer and lyophilized. These protein samples were directly used to develop antisera against them or to isolate some tissue-specific peptides using preparative gel electrophoresis.

Sample Preparation for Gel Electrophoresis. Approximately 1 mg of protein was dissolved in 50 μL of Tris-HCl (pH 8.3) buffer and mixed with an equal volume of Laemmli's sample buffer (0.125 M Tris-HCl, 2.72 M glycerol, 0.28 M SDS, 0.40 mM bromophenol blue, 0.71 M β-mercaptoethanol). The samples were boiled for 3 min.

Analytical Gel Electrophoresis. SDS−PAGE was carried out according to the method of Laemmli (1970) on a 13.6% w/v separation gel overlaid by 3% stacking gel in a Bio-Rad mini gel chamber. Gels were stained with Coomassie BBR.

Separation of Peptides by Preparative Gel Electrophoresis. Peptides dissolved in sample buffer were applied on and separated in a preparative gel electrophoretic chamber (Bio-Rad, model 492). SDS−acrylamide of 14% was used to prepare the column according to the manufacturer's instructions. This column was suitable to isolate peptides in the 24 and 30 kDa range. The separation of peptides was performed at 30 mA and 15 °C, in 0.1 M Tris-HCl (pH 8.3) buffer. The speed of the eluent was adjusted to 0.7 mL/min. Four hundred fractions, 1.5 mL each, were collected. The purity of the fractions was tested by analytical SDS−PAGE. Fractions containing only a single particular peptide were pooled. The samples were dialyzed and concentrated in a Speed-Vac (Juan).

Preparation of Antibodies. Isolated peptides (25−30 μg) in 100 μL of PBS were injected with an equal volume of Freund's complete adjuvant into the skin of male BALB/C mice. The animals were boosted 3 weeks later with the same amount of protein emulsified in 100 μL of Freund's incomplete adjuvant. The titer of antibodies was tested in blood samples collected from the tip of the tail. The third boost was sufficient to achieve a useful titer of antibodies against our peptides. After completing immunization, the animals were bled and the IgG fraction was separated from the sera by ammonium sulfate precipitation.

Immunoblotting. The peptides were transferred from SDS−polyacrylamide gel to a sheet of nitrocellulose filter (Bio-Rad, 0.2 μm pore size) by electroblotting according to the procedure of Towbin et al. (1979). Nonspecific binding sites were blocked by 5% Carnation nonfat dry milk powder in TBS buffer (0.15 M Tris-HCl, 0.5 M NaCl, pH 7.0). After three washings with TTBS buffer (TBS buffer containing 0.05% Tween 20), the nitrocellulose sheet was incubated overnight at 4 °C in the presence of the first antibody diluted in TBS. The blot was washed three times in TTBS and incubated with the second antibody (alkaline phosphatase conjugated anti-mouse antibody, Bio-Rad) dissolved in TBS at 1:1000 for 1 h at room temperature. After the final washes, the blot was developed in the solution of freshly prepared substrate (BCIP and NBT, Bio-Rad). The molecular masses of the positive bands were determined using Bio-Rad prestained standards (myosin 211 kDa, β-galactosidase 117 kDa, bovine serum albumin 81 kDa, ovalbumin 49 kDa, carbonic anhydrase 31 kDa, soybean trypsin inhibitor 26 kDa, lysozyme 19 kDa, aprotinin 7 kDa).

Sample Preparation for Testing the Contamination of Orange Juice with Peel. *Filtered Orange Juice.* Ten milliliters of juice was filtered through folded filter paper. The filtrate was centrifuged at 15000*g* for 20 min. The sediment was resuspended in 0.5 mL of 0.5 M Tris-HCl (pH 8.3) buffer and mixed with an equal volume of Laemmli's sample buffer. The solution was boiled for 3 min.

Peel Extract. One gram of peel was extracted with 10 mL of water (10% peel extract) and filtered through folded filter paper. The filtrate was handled as filtered orange juice.

Juice Samples Contaminated with Peel Extract. Peel extracts (1, 2, and 5%) were prepared by diluting 10% peel extract with filtered juice. These samples were handled according to the above.

RESULTS AND DISCUSSION

Tissue- and Species-Specific Peptides. Protein samples prepared from juice, albedo, and flavedo of various citrus fruits were separated by SDS−PAGE. The peptide patterns of the samples (orange, grapefruit, mandarin, and lemon) can be seen in Figure 1. The molecular masses of the peptides were determined in a densitometer (Biotec Fisher). All of these analytical data are summarized in Table 1.

Several peptides were found to occur exclusively in the albedo, in the flavedo, or in the juice of the fruits studied.

Orange. Twenty-two peptides from the juice, 15 peptides from the flavedo, and 10 peptides from the albedo were separated on gels. Some of them were tissue-specific, that is, characteristic of one particular part of the orange fruit. Seven of them occurred exclusively in the juice and one of them exclusively in the flavedo. In

Table 1. Molecular Mass of Tissue- and Species-Specific Peptides of Citrus Fruits

	orange SDS−PAGE				Western blot	grapefruit SDS−PAGE				lemon SDS−PAGE				mandarin SDS−PAGE			
peptides	juice	flavedo	albedo	peel	juice	juice	flavedo	albedo	peel	juice	flavedo	albedo	peel	juice	flavedo	albedo	peel
tissue-specific						183								152			
														108			
	71				**71**	87	71				77		70				
						61						66					62
	55				**55**	56	54										
	49	48			42	41	48	43		43					48		42
	34				**34**				31	32	34		30			33	
	32					32											
	27			28	**27**	24			28	26			29	27	22		28
					24								28				26
	12		15	16			11		13						13		
species-specific	**36**	12				183	61	54	43	47	35	26	66	152	62	42	

the albedo one peptide was identified, which did not appear in the flavedo or juice.

Grapefruit. Twenty peptides from juice, 15 peptides from flavedo, and 15 peptides from albedo could be separated. Seven peptides in juice, four peptides in flavedo, and a single one in albedo were identified as tissue-specific, that is, not present in the other two parts of the fruit.

Lemon juice contained a relatively small number (eight) of peptides. Fifteen peptides could be separated from the flavedo and 10 from the albedo of lemon. Three specific peptides in the juice, one in the albedo, and two in the flavedo appeared that could not be observed in the other tissues of the fruit.

Mandarin juice was very rich in peptides. It contained 20 peptides; 15 peptides could be separated from the flavedo and 10 peptides from the albedo. Juice and albedo contained three characteristic peptides each, whereas albedo contained but one.

According to the gel electrophoretograms all of the proteins present in the albedo appeared in the flavedo too, but only some of them occurred in the juice samples. Only a single very weak, pale albedo-specific band was identified. It is of practical significance that there are several peptides in all four fruits investigated characterizing exclusively the albedo plus flavedo parts (categorized as peel peptides), but they are not present in the juice samples; some juice-specific peptides were found as well.

From a comparison of the peptide patterns of the three different parts of various fruits, it is clear that some of them show species specificity too. The molecular masses of these peptides are listed in Table 1.

On the whole, several peptides separated from the exo-, meso-, and endocarp of the four citrus fruits studied show tissue and species specificity.

Antiserum was developed in mice against total proteins, isolated peptides of orange juice (27 and 24 kDa), and total proteins of orange peel, and it was tested by Western blot analysis.

Figure 2 shows the Western blot of juice and peel samples using nonimmune mouse serum as negative control.

In Figure 3 Western blot patterns of orange samples prepared from juice, albedo, and flavedo are shown using antiserum developed against total proteins of orange juice. Using this antiserum in 2000-fold dilution, six juice-specific peptides could be detected. The 71, 55, 42, 34, 27, and 24 kDa peptides were immunologically specific for orange juice. The 71, 55, 34, and 27 kDa peptides were also detected as orange juice-specific ones

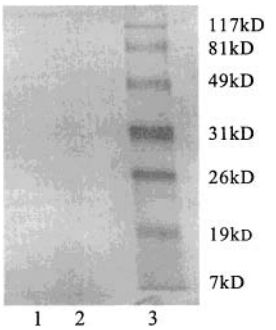

Figure 2. Western blot with nonimmune mice serum: (1) orange juice; (2) orange peel; (3) molecular mass standard (Bio-Rad).

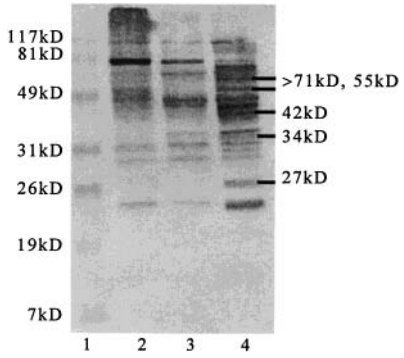

Figure 3. Western blot with antiserum developed by all of the peptides of orange juice: (1) molecular mass standard (Bio-Rad); (2) orange flavedo; (3) orange albedo; (4) orange juice.

by analytical SDS−PAGE. The molecular masses of these juice-specific peptides are shown in boldface type in Table 1.

The blot of orange, mandarin, grapefruit, and lemon juice samples using the antiserum developed against orange juice proteins in 2000-fold dilution is shown in Figure 4. According to this figure the peptides of orange juice were immunologically more similar to the peptides from mandarin than to those of grapefruit or lemon. In mandarin juice, the peptides found in the largest number gave a positive reaction with antiserum against orange juice. Only the 36 kDa peptide could not be detected in the juice of the other three citrus species. This result confirmed the results obtained with of SDS−PAGE.

Figure 5 shows the Western blot of various orange juice beverages from the market, indicating that the antiserum developed gives positive reaction with the peptides of processed (pasteurized) orange juices too.

On the basis of these results, the 24 and 27 kDa

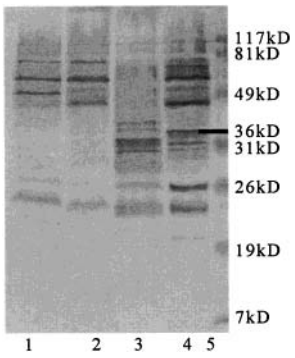

Figure 4. Western blot with antiserum (2000-fold dilution) developed by all of the peptides of orange juice: (1) lemon juice; (2) grapefruit juice; (3) mandarin juice; (4) orange juice; (5) molecular mass standard (Bio-Rad).

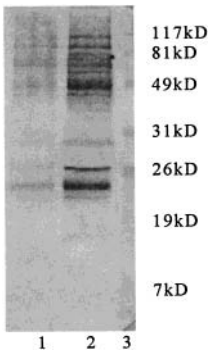

Figure 5. Western blot with antiserum (2000-fold dilution) developed by orange juice peptides: (1) orange juice beverage; (2) orange juice beverage; (3) molecular mass standard (Bio-Rad).

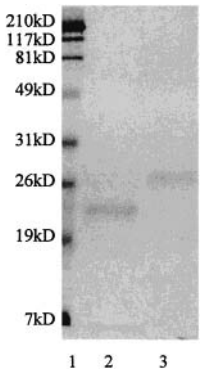

Figure 6. Gel electrophoretogram of peptides, 24 and 27 kDa isolated: (1) molecular mass standard (Bio-Rad); (2) 24 kDa peptide; (3) 27 kDa peptide.

peptides of orange juice were selected for isolation by preparative gel electrophoresis because they occurred exclusively in the juice of orange and were not found in any other part of the fruit. The gel electrophoretogram of the isolated and purified 24 and 27 kDa peptides is shown in Figure 6.

A polyclonal antibody was developed against the 24 and 27 kDa peptides, respectively. The specificity and titer of the antibodies were tested on Western blots. Figure 7 demonstrates the positive reaction of the antibody developed against the 24 kDa peptide. It reacted in 500-fold dilution exclusively with a single band of the sample containing all of the peptides of the orange juice; however, it did not give any positive reaction with the peptides of the peel extract. Therefore, this antibody seems to be useful for the quantitative

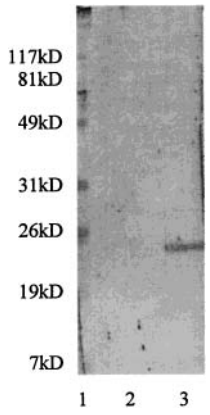

Figure 7. Western blot with antibody (500-fold dilution) developed against 24 kDa juice peptide: (1) molecular mass standard (Bio-Rad); (2) orange peel sample; (3) orange juice sample.

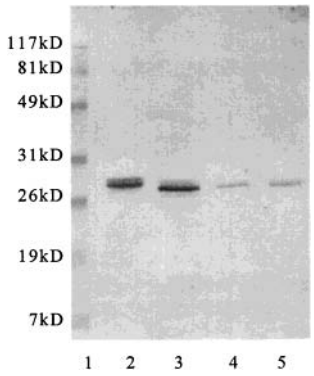

Figure 8. Western blot with antibody (1000-fold dilution) developed against 27 kDa juice peptide: (1) molecular mass standard (Bio-Rad); (2) orange peel sample; (3) orange juice with pulp sample; (4) filtered orange juice sample; (5) commercial orange juice beverage.

determination of the natural juice content in orange juice products.

Figure 8 shows the blot of the antibody developed against the 27 kDa peptide. It reacted with a single band of the juice sample but gave an unspecific cross-reaction with the 29 kDa peptide of the peel sample. It can be clearly observed in this figure that the pulp sample is relatively rich in this particular peptide. The antibody reacted also with a single 27 kDa band of the sample prepared from a commercial processed fruit juice beverage, which means that this antibody might be useful for the quantitative determination of the juice content in commercial orange juice products, too.

The juice samples were experimentally contaminated with different quantities of peel. Western blot patterns of these samples are shown in Figure 9. On the blot of uncontaminated orange juice only the 27 kDa peptide appeared; however, two bands with molecular masses of 27 and 29 kDa, respectively, could be seen in the juice samples contaminated with peel extract. The intensity of the 29 kDa band showed good correlation with peel concentration. This antiserum might be useful for the estimation of the peel contamination in juice products.

An antiserum against the total protein content of peel samples was developed in mice. One of the antibodies in this antiserum recognized a single, peel-specific peptide with a molecular mass of 28 kDa in 10000-fold dilution. It does not give any positive reaction against proteins of filtered orange juice.

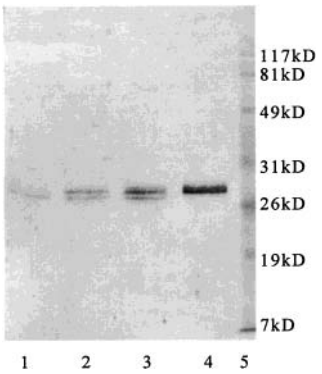

Figure 9. Western blot with antibody (1000-fold dilution) developed against 27 kDa juice peptide: (1) filtered orange juice; (2) orange juice contaminated with 1% peel; (3) orange juice contaminated with 2% peel; (4) orange juice contaminated with 5% peel; (5) molecular mass standard (Bio-Rad).

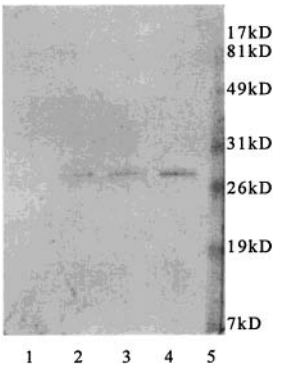

Figure 10. Western blot with antiserum (10000-fold dilution) developed by peel peptides: (1) filtered orange juice; (2) orange juice contaminated with 1% peel; (3) orange juice contaminated with 2% peel; (4) orange juice contaminated with 5% peel; (5) molecular mass standard (Bio-Rad).

Juice samples were experimentally contaminated with graded quantities of peel as described above. The Western blot of these samples is shown in Figure 10. The peptide band with a molecular mass of 28 kDa did not appear in the filtered juice samples, but it occurred in juice samples contaminated with peel extract in various concentrations. The intensities of the bands show a direct correlation with the peel content of the samples.

It can be concluded that tissue- and species-specific peptides occur in various citrus fruits. Antibodies developed against these characteristic peptides may be useful for detecting peel contamination in juice products. Our objective is, in the future, to develop a quantitative (ELISA) method for determining the juice content and the ratio of possible peel contamination in commercial citrus juice beverages using antibodies raised against these characteristic peptides.

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Received for review March 24, 1999. Revised manuscript received February 2, 2000. Accepted June 30, 2000. This work has been supported by the National Scientific Research Fund (OTKA) under Grant T019872.

JF9903962