Changes in protein, on a fresh weight basis, were noted with increased fruit maturity. The number of isozymes of each enzyme system varied among stages and no trend was observed, except for peroxidase. Disc gel electrophoresis revealed minor differences between peroxidase enzyme patterns of Chico III (firm) and Homestead-24 (soft) tomato cultivars. During fruit development, wide peroxidase bands with low electrophoretic mobilities were replaced by thinner higher mobility bands in both cultivars. At the color-turning stage of fruit development, three peroxidase forms from Homestead-24 extracts exhibited higher mobilities than those of Chico III.

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Registry No. Peroxidase, 9003-99-0; esterase, 9013-79-0; MDH, 9001-64-3; ADH, 9031-72-5; acid phosphatase, 9001-77-8; LAP, 9054-63-1.

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Toluene as an Alternative to Benzene in the Woessner Determination of Hydroxyproline

Toluene extraction is a suitable alternative to benzene extraction in the Woessner procedure for the chemical quantitation of 4-hydroxyproline.

Intramuscular collagen content is usually estimated through the chemical determination of 4-hydroxyproline, an amino acid confined essentially to connective tissue proteins. The Woessner (1961) modification of the Stegemann (1958) procedure is the most frequently used method for the determination of hydroxyproline in meat (Etherington and Sims, 1981). This approach involves extractions with benzene, a solvent known to be chronically toxic and carcinogenic (Sax, 1975), even at concentrations much too low to detect through the sense of smell. Its use is either prohibited, discouraged, or permitted with extreme caution. Although the much less commonly used Prockop–Udenfriend (1960) method makes use of extractions with toluene instead of with benzene, the purpose is to extract the reaction intermediates, pyrrole-2-carboxylic acid and pyrrole, from the impurities. On the other hand, the Woessner (1961) procedure makes use of benzene to extract impurities from the chromaphore that is to be analyzed spectrophotometrically. The present study tests the use of toluene as an alternative to benzene in the Woessner (1961) procedure.

MATERIALS AND METHODS

Ten 75% or 100% Simmental or Limousin steers were slaughtered at approximately 15 months of age and 450 kg live weight. One steak was removed from the longissimus dorsi muscle of the left side of the carcass 6 days postslaughter. A thin slice (2 mm thick) was removed from each steak, freeze-dried under vacuum for approximately 72 h, and then broken up by blending with a Virtis homogenizer. A 0.5-g portion was subjected to the salt extraction procedure of Hill (1966) to yield salt-soluble and salt-insoluble fractions. These fractions were hydrolyzed in 6 N HCl at 113 °C for 18 h, neutralized, and made to a volume of 150 mL. The hydrolysate of the salt-insoluble fraction was diluted 5-fold with distilled water before analysis. All hydrolysates were then analyzed for hydroxyproline content using "method II" of Woessner (1961). This analysis includes (1) oxidation by Chloramine T, (2) perchloric acid treatment, (3) incubation with *p*-(dimethylamino)benzaldehyde at 60 °C followed by cooling, (4) extraction with benzene, and (5) measurement of the absorbance at 557 nm before and after peroxide treatment. Each muscle sample was analyzed in triplicate and each standard was analyzed in quadruplicate. The volumes of all sample and reagent solutions added prior to benzene extraction were increased 3-fold over those used by Woessner (1961). Following step 3, two 5-mL aliquots were removed from each reaction mixture. One was extracted with benzene in the normal fashion and the other

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Table I. Means and Standard Errors of Absorbance (557 nm) in the Presence of Known Quantities of Hydroxyproline (μg) after Extraction with either Benzene or Toluene

hydroxyproline	absorbance[a]	extraction solvent: benzene mean	benzene SE	toluene mean	toluene SE	benzene-toluene mean	benzene-toluene SE
0	*A*	0.004	0.0002	0.003	0.0003	0.001	0.0003
	B	0.004	0.0003	0.004	0.0005	0.000	0.0007
	A − *B*	0.000	0.0003	−0.001	0.0006	0.000	0.0008
1.0	*A*	0.095	0.0005	0.097	0.0006	−0.002	0.0011
	B	0.003	0.0004	0.008	0.0004	−0.005	0.0006[b]
	A − *B*	0.092	0.0007	0.088	0.0003	0.004	0.0009[b]
2.5	*A*	0.230	0.0008	0.232	0.0005	−0.002	0.0003[b]
	B	0.005	0.0002	0.010	0.0002	−0.005	0.0002[b]
	A − *B*	0.225	0.0007	0.222	0.0005	0.003	0.0002[b]
5.0	*A*	0.451	0.0011	0.458	0.0017	−0.007	0.0016[b]
	B	0.010	0.0003	0.014	0.0003	−0.004	0.0003[b]
	A − *B*	0.441	0.0010	0.444	0.0019	−0.003	0.0014

[a] A and B = before and after the addition of hydrogen peroxide, respectively. [b] Mean difference between benzene and toluene extractions differs ($P < 0.05$) from 0.

Table II. Mean and Standard Error of Absorbance (557 nm) and Hydroxyproline Content (HP, μg/g of Dry Meat) of 10 Meat Samples after Extraction with either Benzene or Toluene

salt solubility	variable[a]	extraction solvent: benzene mean	benzene SE	toluene mean	toluene SE	benzene-toluene mean	benzene-toluene SE
soluble	*A*	0.100	0.009	0.102	0.009	−0.002	0.0003[b]
	B	0.015	0.001	0.001	0.001	−0.004	0.0004[b]
	A − 1.12*B*	0.084	0.008	0.081	0.008	0.003	0.0005[b]
	HP	273	28	274	28	0.6	1.4
insoluble	*A*	0.178	0.006	0.182	0.006	−0.004	0.0006[b]
	B	0.075	0.001	0.081	0.001	−0.005	0.0007[b]
	A − 1.12*B*	0.094	0.006	0.091	0.006	0.002	0.0006[b]
	HP	1533	93	1545	98	14	9

[a] *A* and *B* = before and after the addition of hydrogen peroxide, respectively. [b] Mean difference between benzene and toluene extraction differs ($P < 0.05$) from 0.

was extracted with toluene. The corrected absorbance was calculated according to Woessner (1961) as $A - B$ for the standards and $A - 1.12B$ for unknown samples, where A and B are the absorbances before and after peroxide treatment, respectively. The absorbance of the water blank after the addition of the hydrogen peroxide (C) was omitted from the expression since its value equaled zero.

RESULTS AND DISCUSSION

The absorbance values resulting with the use of known (standards) and unknown (meat samples) quantities of hydroxyproline are presented in Tables I and II, respectively. Absorbance values for both standards and meat samples were significantly ($P < 0.05$) higher with toluene extraction than with benzene extraction both before (A) and after (B) adding hydrogen peroxide. The corrected absorbance was slightly higher for the 1.0- and 2.5-μg standards and for muscle samples with a benzene extraction than with toluene extraction. Although the standard curves appeared similar for the two extraction methods, closer examination of the data indicate slight curvature of that obtained with benzene extraction. The hydroxyproline content of the meat samples, obtained by using the appropriate standard curve, ranged from 114 to 381 and from 1080 to 1939 μg/g of dry muscle for the salt-soluble and salt-insoluble fractions, respectively. The mean difference between the hydroxyproline contents resulting from the two extraction procedures (Table II) was small and nonsignificant ($P > 0.05$). These data suggest that toluene is an acceptable alternative to benzene for the extraction of interfering substances in the Woessner (1961) procedure for quantitation of intramuscular hydroxyproline.

Registry No. 4-Hydroxyproline, 51-35-4; toluene, 108-88-3.

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