Metabolic Conjugates as Precursors for Characterizing Flavor Compounds in Ruminant Milks

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Metabolic conjugates (glucuronides, sulfates, and phosphates) and corresponding free compounds were isolated from cow's, sheep's, and goat's skim milks by adsorption on XAD-2 resin. Analysis of isolates by high-performance liquid chromatography before and after selected enzyme (β-D-glucuronidase, arylsulfatase, and acid phosphatase) hydrolysis indicated that phenols in sheep's skim milk were mostly bound as phosphate and sulfate conjugates with lesser amounts of glucuronides. Phenols in cow's and goat's skim milks were mostly bound as sulfates with smaller amounts of glucuronides, and none were bound as phosphates. Treatment of isolated conjugates with either thermal acidic hydrolysis (100 °C, pH 1.5, 15 min) or *N*-acylase resulted in the release of substantial amounts of *n*-chain free fatty acids from all three of the skim milks, indicating that these fatty acids were bound as amino acid conjugates. β-D-Glucuronidase released additional amounts of free fatty acids from isolates, indicating that some fatty acids were bound as 1-*O*-glycosidyl esters. Aromas of the volatiles isolated from the skim milks indicated that these conjugatable compounds were responsible for the cowy flavor of cow's milk and sheepy flavor of sheep's milk.

INTRODUCTION

Recently, several alkylphenols have been identified as key characterizing flavor compounds in certain varietal cheeses (Ha and Lindsay, 1991a,b), and have been reported to provide distinctive species-related flavors in ruminant meats (Ha and Lindsay, 1991c). Concentrations of alkylphenols have been observed to increase during cooking of mutton, which suggested that some alkylphenols occurred bound in precursors in meats (Brennand and Lindsay, 1992). Similarly, increases in free fatty acid concentrations were observed during cooking of mutton. The precursors for fatty acids were presumed to be acylglycerols, and the precursors for alkylphenols were not identified.

Since alkylphenols have been found to be important in the flavors of ruminant meats and milk products, information about the origin of these substances as flavor compounds is needed. Metabolic conjugation is a universally accepted means of detoxification and enhancement of aqueous solubility of foreign substances in mammals (Mulder, 1990). Conjugates are most actively formed by the liver and kidney, and they circulate in the bloodstream before elimination principally in the urine and bile. Conjugates of alkylphenols and a variety of other compounds have been found in milk by early researchers (Heyns et al., 1956; Brewington et al., 1973, 1974). However, the role of conjugates and free compounds in the flavor of milk has not been explored. Therefore, the purpose of this research was to investigate the nature of metabolic conjugates in relation to the flavor of cow's, sheep's, and goat's milks.

MATERIALS AND METHODS

Milk Samples. Pasteurized (72 °C for 16.5 s), winter, mixed-herd, cow's skim milk was obtained from the Dairy Plant of the Department of Food Science, University of Wisconsin—Madison. Frozen, raw, whole, fall, mixed-herd sheep's milk was obtained from La Paysanne Co. (Hinckley, MN); this milk had been frozen for approximately 90 days. Raw, whole, winter, mixed-herd goat's milk was obtained from Fantome Farms Inc. (Ridgeway, WI). The raw sheep's and goat's milks were pasteurized at 64 °C for 30 min and then were centrifuged (15300*g*) (Sorvall refrigerated centrifuge, Du Pont Instruments, Des Plaines, IL) for 15 min before visible cream layers were removed.

Method of Isolation of Conjugates. The procedure for the isolation of the conjugates was a modification of that described by Brewington et al. (1972). Batches of approximately 250 g of Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, PA) were slurried in water and poured into open glass chromatography columns (7 × 50 cm) fitted with Teflon stopcocks. Columns were prepared for use by washing each with 2 L of twice-distilled water, 2 L of distilled methanol (reagent grade, Fisher Scientific, Itasca, IL), and finally 2 L of distilled water. Eight-liter batches of pasteurized skim milk were passed through regenerated columns at a flow rate of 15 mL/min. After all of the batch of skim milk had penetrated the column bed, columns were rinsed with 2 L of water, and this volume resulted in a clear eluate. Substances adsorbed on each column were eluted using 4 L of distilled methanol. Each extract was then passed through a Whatman No. 1 filter paper and finally was evaporated to near dryness in a rotary vacuum evaporator (Model E50GD, Rinco Instrument Co., Greenville, IL) at speed 5 and 25 °C. Each extraction residue was dissolved in 5 mL of distilled water, and two aliquots were used for analyses.

Hydrolysis of Conjugates. The water-soluble materials in the extracts of each skim milk sample were dissolved in 0.2 M acetate buffer (pH 4.5), and samples (0.3 mL) of each of the concentrated isolates were subjected to enzymic hydrolysis. These included exposure to 2000 units of β-D-glucuronidase (EC 3.2.1.31) from *Escherichia coli* 9 (Type VII; Sigma Chemical Co., St. Louis, MO), 250 units of arylsulfatase (EC 3.1.6.1) from *Helix pomatia* (Type H-5; Sigma), 250 units of acid phosphatase (EC 3.1.3.2) from wheat germ (Sigma), and 2000 units of *N*-acylase (EC 3.5.1.14) from porcine kidney (Grade 1; Sigma). Saccharic acid 1:4-lactone (20 mM) (Aldrich Chemical Co., Milwaukee, WI) was used to inhibit accompanying glucuronidase activity that was present in the arylsulfatase and acid phosphatase preparations (Okhubo and Sano, 1974). Enzymes were incubated with substrates for 24 h at 38 °C. Progress of hydrolysis and specificity of enzymes were monitored by carrying out parallel treatments of solutions of authentic conjugates (phenyl glucuronide, phenyl phosphate, and naphthyl sulfate).

Mild thermal acidic hydrolysis was carried out by acidifying samples (0.3 mL) to pH 1 with sulfuric acid (Mallinkrodt, Rochester, NY) and heating at 100 °C for 15 min. Total hydrolysis of conjugates was achieved by sequential mild thermal acidic

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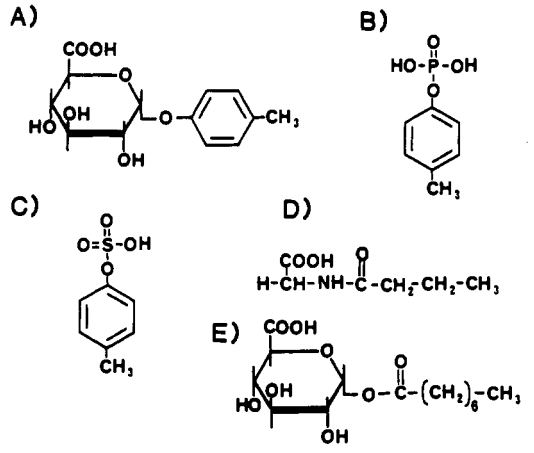

Figure 1. Classes of metabolic conjugates found in ruminant milks. Examples for classes are (A) *p*-cresyl glucuronide, (B), *p*-cresyl phosphate, (C) *p*-cresyl sulfate, (D) glycyl butyrate, and (E) glucuronyl 1-*O*-octanoate.

hydrolysis followed by enzymic hydrolysis. After thermal hydrolysis and subsequent pH adjustment of the samples to 4.5, a mixture of arylsulfatase, acid phosphatase, and β-glucuronidase whose activities were each the same as described for hydrolysis by individual enzymes was added to samples of isolates. The reaction for each sample was allowed to progress for 24 h at 38 °C.

HPLC Analysis of Conjugates and Phenols. Potassium phenyl glucuronide, potassium phenyl phosphate, potassium naphthyl phosphate, and potassium naphthyl sulfate were purchased from Sigma. Phenol and *p*-cresol were from Aldrich. Tetrabutylammonium hydrogen sulfate (TBAHS, HPLC grade), which was used as an HPLC ion pair reagent, was from Eastman Kodak Co. (Rochester, NY). Methanol (HPLC grade) was obtained from Fisher. An aqueous solution composed of phenol, *p*-cresol, potassium phenyl glucuronide, potassium phenyl phosphate, potassium naphthyl phosphate, and potassium naphthyl sulfate standards (100 ng/μL each) was used to characterize the HPLC elution profile of free phenols and conjugates.

The HPLC employed was an ISCO (Lincoln, NE) system which consisted of a pump (Model 2350) and a gradient programmer (Model 2360). A 10-μL sample loop was used for sample introduction. The system was equipped with a variable-wavelength absorbance detector ISCO V^4 set at 210 nm, and the chromatograms were recorded on a Spectra-Physics Model 4100 computing integrator (Spectra-Physics, San Jose, CA). Separations were achieved using a C_{18} reversed-phase column (Zorbax ODS 4.6 mm i.d. × 25 cm, Du Pont Co., Wilmington, DE) operated under gradient elution conditions. The flow rate was 1 mL/min and the chad speed 1 cm/min. The elution solvent was a gradient composed of solvent A, which contained 10 mM TBAHS in 10% methanol in water, and solvent B, which was 10 mM TBAHS in 50% methanol in water. The gradient conditions selected for analysis were (a) 0–5-min isocratic with 100% solvent A, (b) 5–25-min linear gradient from 0 to 50% solvent B, and (c) 25–65 min linear gradient from 50 to 100% solvent B. Compounds were located in chromatograms by coincidence of retention times between unknowns and authentic compounds. All samples were passed through a 0.45-μm filter (Alltech, Deerfield, IL) before injection. Calibration curves for the standard compounds were constructed by plotting peak area vs concentration from analyses of the undiluted solutions of standard compounds as well as from analyses of serial dilutions to yield 50, 25, 12, and 6 ng/mL of each component.

Gas Chromatographic (GC) and Mass Spectrometric Analysis of Volatile Compounds. For GC analysis of volatile compounds in XAD-2 flavor isolate concentrates and subsequent hydrolyzed samples, 1 μg of internal standard 2,4,6-trimethylphenol (Aldrich) was added to each, and each sample was successively extracted five times with 1-mL portions of freshly prepared ether/pentane (2:1). The extracts from each sample were combined before doing overexcess anhydrous sodium sulfate (Aldrich). The solvent was removed from each sample under a slow stream of nitrogen until the volume was about 20 μL, and 1-μL injections were gas chromatographically analyzed. The chromatograph employed was a Varian 20 Model 3410 GC (Varian Associates, Inc., Sunnyvale, CA) equipped with a flame ionization detector (FID) and with a Supelcowax-10 capillary column (60 m × 0.32 mm i.d., 0.25 m coating thickness; Supelco, Inc., Bellefonte, PA). Helium was used as the carrier gas (head pressure, 10 psi). The column temperature was programmed from 50 to 210 °C at 4 °C/min after a 1-min hold at 50 °C.

Table I. Recovery of Conjugates and Free Phenol from an Aqueous Standard Solution Using XAD-2 Isolation and Analysis by HPLC

compound	% recovery	compound	% recovery
phenyl glucuronide	68	phenyl sulfate	65
phenyl phosphate	75	phenol	70

Phenols in extracts also were analyzed by selected ion monitoring gas chromatography/mass spectrometry (SIM-GC/MS) using a Finnigan 4021 GC/MS (Finnigan Instruments, Sunnyvale, CA). The ion source was maintained at 250 °C and a ionizing voltage of 70 eV. Ions selected for monitoring were the same as those previously used by Ha and Lindsay (1991c): (thiophenol, *m/z* 110/109, I_E 8.72; thiocresol, *m/z* 91/124, I_E 9.88; *o*-cresol, *m/z* 108/107, I_E 13.08; *m*-cresol, *m/z* 108/107, I_E 14.00; *p*-cresol, *m/z* 108/107, I_E 13.94; phenol, *m/z* 94/66, I_E 13.50; 2-ethylphenol, *m/z* 107/122, I_E 13.17; 3/4-ethylphenol, *m/z* 107/122, I_E 14.73; 2,4-dimethylphenol, *m/z* 122/107, I_E 13.83; 2,3/3,5-dimethylphenol, *m/z* 122/107, I_E 14.43; 2-isopropylphenol, *m/z* 121/136, I_E 14.11; 3/4-isopropylphenol, *m/z* 121/136, I_E 15.06; thymol, *m/z* 135/150, I_E 14.69; carvacrol, *m/z* 135/150, I_E 14.97; 2,6-diisopropylphenol, *m/z* 163/178, I_E 13.02; 2,5-diisopropylphenol, *m/z* 163/178, I_E 15.80; 3,5-diisopropylphenol, *m/z* 163/178, I_E 16.51). Peaks were identified by comparison of retention times and by comparing primary to secondary ion count ratios for authentic phenols contained in a standard prepared at a total concentration of 50 ppm. A quantitative mixture of standard compounds and the internal standard (2,4,6-trimethylphenol, *m/z* 121/136) was carried through GC/MS analysis, and correction factors for responses relative to the standard were calculated using these data (Heil and Lindsay, 1988). An additional correction factor for efficiency of recovery of compounds from samples was also employed for quantitative estimates.

Carvacrol (2-methyl-5-isopropylphenol) and thymol (5-methyl-2-isopropylphenol) were obtained from Fluka (Hauppauge, NY). All other phenols (*o*-cresol, *m*-cresol, *p*-cresol, phenol, 2-ethylphenol, 3-ethylphenol, 4-ethylphenol, 2,4-dimethylphenol, 2,3-dimethylphenol, 3,5-dimethylphenol, 3,4-dimethylphenol, 2-isopropylphenol, 3-isopropylphenol, 4-isopropylphenol, 2,4-diisopropylphenol, 2,5-diisopropylphenol, 3,5-diisopropylphenol, thiophenol, and thiocresol) were obtained from Aldrich.

Aroma assessments of samples were made by the authors.

RESULTS AND DISCUSSION

The compounds sought in the milks were all quite polar and included both free flavor compounds and those bound as metabolic conjugates (Figure 1). Initially, attempts were made to isolate the free flavor compounds and conjugates from whole milk because it would be useful to understand the behavior of these substances in fat-containing milk products. Methods attempted for isolation of conjugates and phenols included anion-exchange chromatography (Anderson and Warren, 1951; Bush and Gale, 1957; Hahnel, 1962), alumina adsorption (Barlow, 1957), and silica adsorption (Kushinsky et al., 1960), but phenolic conjugates did not elute easily from these columns and the recovery was low. The XAD-2 adsorption method (Brewington et al., 1972) also did not perform adequately with whole milk, but it was found to give satisfactory recovery with skim milk. Since attempts to overcome problems with separations caused by coatings of fat on adsorbants were unsuccessful, studies were continued with skim milk samples.

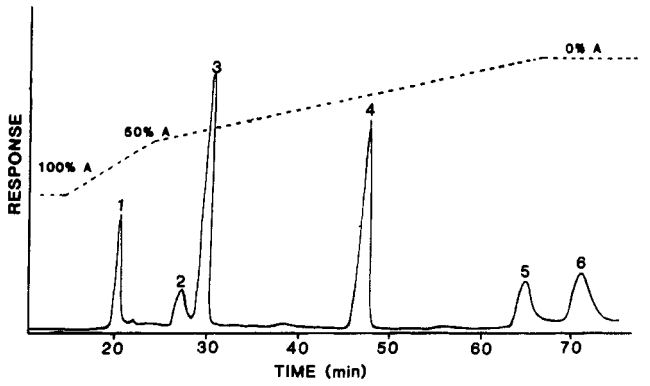

Figure 2. HPLC chromatogram of a standard mixture of free and conjugated phenols (100 ng/μL each). Peaks: (1) potassium phenyl glucuronide; (2) potassium phenyl phosphate; (3) phenol; (4) *p*-cresol; (5) potassium naphthyl sulfate; (6) potassium naphthyl phosphate. Conditions: solvent A, 10 mM TBAHS in 10% methanol in water; solvent B, 10 mM TBAHS in 50% methanol in water; flow rate, 1 mL/min; detector wavelength, 210 nm.

The efficiency of the XAD-2 column for recovering authentic phenols and selected conjugates was examined using HPLC. Data in Table I showed that recoveries of about 70% of both conjugates and phenol (100 μg/mL each) were realized when an aqueous solution of a standard mixture was carried through the entire recovery and HPLC analysis process. Therefore, quantitative values for conjugates and free phenols were corrected using this figure for recovery.

The concentrated isolates obtained from the XAD-2 adsorption procedure possessed extremely characteristic aromas generally reflecting the species from which they were obtained. These aromas were potentiated many-fold after enzymic hydrolysis with a mixture of acid phosphatase, β-D-glucuronidase, and arylsulfatase. Sheep's milk samples possessed very pronounced typical sheep-like aromas, and cow's milk isolates exhibited very cowy, barny aromas. Previously, *p*-cresol and *p*-ethylphenol have been identified as important components in cow's urine aroma (Suemitsu et al., 1965; Ushijima, 1964; Heyns et al., 1956). Goat's milk samples had aromas that resembled the cow's milk isolates and did not exhibit the pronounced goaty aroma provided by 4-methyloctanoic and 4-methylnonanoic acids (Brennand and Lindsay, 1982; Wong et al., 1975). Successive dilution of the samples caused a lessening of the distinctive species-related aroma notes, and each became reminiscent of the flavor and aroma of warm milk.

HPLC Analysis of Concentrated Isolates. Analysis of metabolic conjugates has proven to be quite difficult because of their wide range of polarities and occurrence in complex biological materials. Most early analyses of conjugates utilized acid or enzyme hydrolysis followed by gas chromatographic/mass spectrometric analysis of aglycons (Kaufman et al., 1976; Horning et al., 1967). Liquid and paper chromatographic techniques have been somewhat useful but generally lack resolution and sensitivity required for detection of metabolic conjugates in complex samples. Recently, more modern techniques have been employed for the analysis of metabolic conjugates with variable success; these include countercurrent liquid–liquid chromatography (Assandri and Perazi, 1974), ion-exchange chromatography (Brown, 1989; Anders, 1971), ion exclusion partition chromatography (Languardt, 1979), reversed-phase chromatography (Knox, 1977), and ion pair chromatography (Walhund, 1975; Frausson et al., 1976; Karakaya and Carter, 1979; Sawa, 1988; Ragan and Mackinnon, 1979). High-performance liquid chromatography (HPLC) offers a potentially direct and rapid approach to analysis of conjugates.

Table II. Retention Times and Molar Absorptivities for Some Free and Conjugated Phenols When Analyzed by Ion Pair Reversed-Phase HPLC with Gradient Elution

compound	retention time, min	molar absorptivity at 210 nm
phenyl glucuronide	23	1660
phenyl phosphate	27	690
phenol	29	5230
p-cresol	48	4160
naphthyl sulfate	64	1550
naphthyl phosphate	72	1660

The HPLC method adapted in this study used ion pair, reversed-phase conditions and gradient elution. The chromatogram shown in Figure 2 illustrates the separation achieved for a standard mixture of phenols and conjugates. Although authentic phenyl sulfate was not available, it was presumed that it would elute in advance of phenyl phosphate in a manner similar to that observed for the naphthyl sulfate and naphthyl phosphate pair of compounds. It was also observed that the HPLC approach could not separate the *o*-, *m*-, or *p*-cresol isomers. Initial studies employing isocratic elution with 10 mM TBAHS dissolved in 50:50 methanol/water yielded very low resolution and rapid elution from the column, and gradient elution conditions greatly improved resolution for selected compounds.

The standard mixture shown in Figure 2 contained equimolar amounts of each of the conjugates and the aglycon phenolic compounds. Phenol (peak 3) and *p*-cresol (peak 4) gave much higher molar absorptivities (Table II) than any of the phenolic conjugates, and therefore the method was much less sensitive for bound than for free forms of phenols. This somewhat low sensitivity for conjugates detracts from application of UV detection for conjugates. Initial studies were carried out using dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride] derivatives of phenols which were formed under alkaline conditions to yield fluorescent dansylphenols (Lawrence, 1979; Frei-Hausler and Frei, 1973; Cassidy and LeGay, 1974). The reaction provided very good results for free phenols, but the reagent did not react with conjugates. Thus, direct fluorescence detection of conjugates is not possible, but indirect measurements of phenols following hydrolysis might be practical.

When concentrated isolates from cow's, sheep's and goat's skim milk were analyzed by HPLC using UV absorption at 210 nm for detection of compounds, very complex chromatograms were obtained for each of the skim milk extracts (Figure 3). Some similarities in overall profiles of compounds isolated by the XAD-2 column were observed, but the goat's milk isolate was particularly more complex than those from cow's and sheep's skim milks. Assessment of the retention times for authentic compounds (Figure 2; Table II) indicated that the expected conjugated compounds probably were present in the XAD-2 isolates, but co-eluting contaminants were also likely.

To determine which HPLC peaks contained hydrolyzable compounds, samples from concentrated XAD-2 isolates obtained from the skim milks of each species were acidified (pH 1) and heated 100 °C for 15 min before analysis. This treatment revealed that many of the major compounds observed in the unheated control samples were removed or decreased in the profiles, and this is illustrated in Figure 4 (compare A and B), which shows the profiles for a sheep's milk isolate. The peaks in the regions where

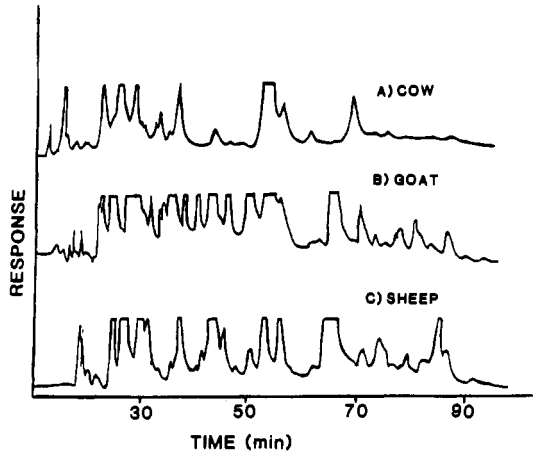

Figure 3. HPLC chromatograms of concentrated XAD-2 isolates from (A) cow's, (B) goat's, and (C) sheep's skim milks.

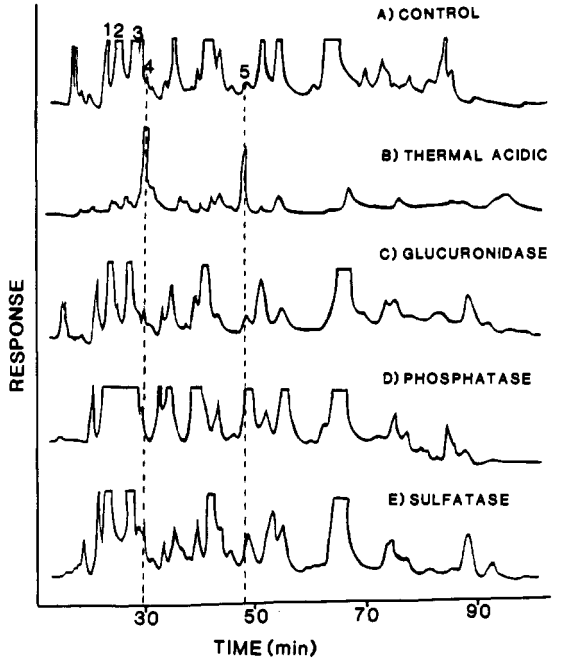

Figure 4. HPLC chromatograms of concentrated XAD-2 isolates from sheep's skim milk: (A) control before hydrolysis; (B) after thermal acidic hydrolysis; (C) after β-D-glucuronidase hydrolysis; (D) after acid phosphatase hydrolysis; (E) after arylsulfatase hydrolysis. Conditions: solvent A, 10 mM TBAHS in 10% methanol in water; solvent B, 10 mM TBAHS in 50% methanol in water; flow rate, 1 mL/min. Numbers over peaks indicate regions of elution for (1) phenolic glucuronide conjugates, (2) phenolic sulfate conjugates, (3) phenolic phosphate conjugates, (4) phenol, and (5) *p*-cresol.

authentic phenyl glucuronide, phenyl phosphate, and possibly phenyl sulfate standards eluted (Figure 4, regions 1–3; retention times, 20–30 min) were greatly diminished. Peaks corresponding to phenol (retention time, 29 min; peak 4) and *p*-cresol (retention time, 48 min; peak 5) were generally quite large relative to corresponding peaks in the other chromatograms.

However, the large reduction in the size of the peaks in the region where the phenyl conjugates elute (20–30 min, Figure 4A) cannot be attributed substantially to the hydrolysis of phenolic conjugates because some of these conjugates are quite resistant to the conditions of mild thermal acidic hydrolysis employed here (Lopez and Lindsay, 1993). Additionally, the large peaks eluting in the 20–30-min region of the control sample should have produced much larger peaks for phenol and *p*-cresol in the acid hydrolysate if they have been caused by the phenolic conjugates, which exhibit relatively low molar absorptivities compared to these for free phenols (Table II). Nevertheless, the acid hydrolysis revealed a distinct increase in the relative sizes of the phenol and *p*-cresol peaks.

Table III. Concentrations (Parts per Billion) of Total Volatile Phenols Found in XAD-2 Isolates from Cow's, Sheep's, and Goat's Skim Milks after Sequential Mild Thermal Acidic and Enzymic Hydrolysis

compound	concn in isolates[a] cow	sheep	goat
thiophenol	*b*	230	*b*
phenol	860	1890	6600
o-cresol	70	50	60
p-cresol	310	6150	2140
m-cresol	70	3610	610
2-ethylphenol	20	650	*b*
3(*and/or*)4-ethylphenol[c]	6	70	*b*
3,4-dimethylphenol	*b*	1580	8
2-isopropylphenol	*b*	60	60
3(*and/or*)4-isopropylphenol[c]	*b*	520	*b*
thymol	4	10	20
carvacrol	14	80	220

[a] Calculation of concentrations: $(Q_i A_x / W_t A_i)$CF, where Q_i is the quantity of internal standard added, A_x is the area of the peak, A_i is the area of the internal standard, W_t is the sample weight, and CF is a correction factor for relative response of compound to the internal standard. [b] Not detected. [c] Isomers not separated on Supelcowax 10 capillary column.

Table IV. Relative Increases in Amounts of Selected Phenols in XAD-2 Isolates from Cow's, Sheep's, and Goat's Skim Milks after Hydrolysis with either β-D-Glucuronidase, Arylsulfatase, or Acid Phosphatase

enzymic treatment of XAD-2 isolate	phenolic compound[a] phenol	*p*-cresol	*m*-cresol
	Cow		
β-D-glucuronidase	+	+	–
arylsulfatase	++	++	–
acid phosphatase	–	–	–
	Sheep		
β-D-glucuronidase	+	+	+
arylsulfatase	++	++	+
acid phosphatase	+++	+++	+
	Goat		
β-D-glucuronidase	+	+	–
arylsulfatase	++	++	+
acid phosphatase	–	–	–

[a] – = no change; + = small increase; ++ = notable increase; +++ = large increase in peak size compared to untreated control.

Individual samples of XAD-2 extracts from the skim milks from each species were also hydrolyzed with either β-D-glucuronidase, acid phosphatase, or sulfatase, and the HPLC profiles for the sheep's skim milk extract are shown in parts C, D, and E, respectively, of Figure 4. Only hydrolysis with acid phosphatase resulted in substantial increases in the phenol and *p*-cresol peaks (Figure 4D), which supports the study of Kao et al. (1979), who found that sheep produce mainly phosphate metabolic conjugates. For sheep's skim milk, it appeared that relatively lesser amounts of phenol or *p*-cresol were released by sulfatase (Figure 4E) and β-D-glucuronidase (Figure 4C).

Hydrolytic treatment of cow's and goat's skim milk extracts revealed generally similar information (data not shown), except that *p*-cresol and phenol were released only by β-D-glucuronidase and sulfatase, which indicated that these species do not contain phosphate conjugates to any extent in the milk. Naphthol conjugate standards were incorporated into the model system to determine their

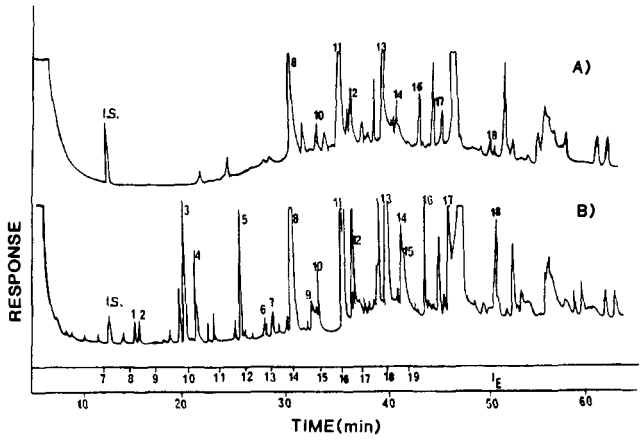

Figure 5. Capillary column (Supelcowax 10) gas chromatograms of goat's skim milk extracts from (A) untreated XAD-2 isolate and (B) XAD-2 isolate after acid hydrolysis (100 °C, 15 min, pH 1). See Table V for peak identities.

HPLC behavior, but evidence for the occurrence of this group of compounds was not found.

Exposure of concentrated XAD-2 isolates to either acidic or enzymic hydrolysis resulted in marked increases in species-associated aroma intensities. This observation indicated that the full range of odorous phenols (Ha and Lindsay, 1991c) was released. However, because of the complexity of the HPLC profiles and the relative insensitivity of this technique, information about the less abundant conjugates and free flavor compounds could not be extracted from the data. Further purification and concentration of the desired components will be required before HPLC can be extended to this application.

GC Analysis of Hydrolysates of Conjugates. Total, free, and bound phenolic compounds present in XAD-2 isolates from skim milks were quantitatively determined by SIM-GC/MS of compounds obtained by sequential analyses of each sample. The hydrolysis of samples was first carried out under mild thermal acidic conditions, which was then followed by enzymic hydrolysis with a mixture of β-D-glucuronidase, arylsulfatase, and acid phosphatase. Both acidic and multiple enzymic treatments were employed to effect complete hydrolysis because mild acid hydrolysis was not adequate for release of volatiles from phenolic conjugates (Lopez and Lindsay, 1993). A summary of relative quantitative data obtained for the volatile phenols in XAD-2 isolates from the skim milks is shown in Table III. The XAD-2 isolates were each obtained from 8 L of skim milk, but quantitative extrapolation to native concentration in skim milks seemed imprudent because recovery values have not been determined for individual phenols and their various conjugates. However, relative abundances of total phenolic compounds found in the three milks (Table III) revealed that sheep's skim milk contained the greatest variety and quantity of phenols. Similar observations were made in comparisons of phenolic compounds found in adipose tissue of these species of animals (Ha and Lindsay, 1991c). The concentrations of phenols in the XAD-2 isolates confirmed that the pronounced aromas of these samples were the direct effect of the low threshold and distinctive aroma properties of the alkylphenols (Ha and Lindsay, 1991c).

Sheep's skim milk provided the only sample that yielded large concentrations of thiophenol, 3(*and/or*)4-ethylphenol, 3,4-dimethylphenol, and 3(*and/or*)4-isopropylphenol. These alkylphenols have been reported to provide characterizing sheepy, animal-like flavors, and thiophenol provides sulfur and meaty flavor characteristics (Ha and Lindsay, 1991c). It is likely, however, that thiophenol arises from a thermally mediated reaction occurring during the periods of elevated temperatures encountered in the acid hydrolysis of conjugates (unpublished data). The relative amounts of *p*-cresol and *m*-cresol are also higher in sheep's skim milk than in the goat's and cow's skim milks, but goat's skim milk contained an exceptionally high concentration of phenol. Thymol and carvacrol were present in each of the skim milk samples, and these compounds have been reported to be excreted as conjugates of glucuronic and sulfuric acids by humans, dogs, and rabbits (Baumann and Herter, 1977; Takao, 1923).

Table V. Identities of Peaks in Supelcowax 10 FID Gas Chromatograms of XAD-2 Isolates from Goat's Skim Milk

peak	I_E[a]	compound
I.S.	7.0	ethyl hexanoate (internal standard)
1	8.2	furaldehyde
2	8.6	benzaldehyde
3	9.9	butanoic acid
4	10.7	pentanoic acid
5	11.8	hexanoic acid
6	12.9	benzothiazole
7	13.2	phenylacetic acid
8	13.9	octanoic acid
9	14.9	furoic acid
10	15.1	nonanoic acid
11	16.0	decanoic acid
12	17.0	undecanoic acid
13	18.0	dodecanoic acid
14	19.0	tetradecanoic acid
15	19.2	acetovanillone
16	>19	pentadecanoic acid
17	>19	hexadecanoic acid
18	>19	octadecanoic acid

[a] Retention index relative to ethyl ester standards (Van den dool and Kratz, 1963).

Additional information about the classes of phenolic conjugates present in the skim milks was obtained from direct FID gas chromatographic analysis of the most abundant phenols in extracts from preparations obtained by incubation of samples of XAD-2 isolates with individual enzymes. These data (Table IV) are reported as relative increases in peak sizes because absolute quantification was precluded by a partial obscuring of the internal standard. Only the sheep sample contained phenols that were liberated by acid phosphatase, and this was in agreement with the results of direct analysis of enzymic hydrolysates by HPLC (Figure 4) and those reported by Kao et al. (1979). Each of the species appeared to conjugate substantial amounts of the phenols as sulfates (Table IV), and each also appeared to conjugate some of the phenols as glucuronides.

Phenol and *p*-cresol have been found previously as conjugates in cow's skim milks (Brewington et al., 1973, 1974). Also, Suemitsu et al. (1970) found that several aromatic acids and phenols were excreted as glucuronides in the urine of dairy cows as part of the detoxification metabolism. Sulfate conjugation appeared to occur to a greater degree than glucuronide conjugation in all three species (Table IV). The sulfation reaction has a high affinity but low capacity for conjugation of phenols, whereas glucuronidation has low affinity for phenols but a much higher capacity for conjugation of these compounds. As a result, sulfation predominates in the presence of low concentrations of substrate and glucuronidation predominates at high concentrations of substrate (Weitering et al., 1979; Oehme et al., 1970). Therefore, the amounts of individual conjugate forms in milks will probably vary depending on the amounts of phenols that are available for detoxification in the animal.

Table VI. Relative Concentrations (Parts per Million) of Free and Conjugated Fatty Acids Found in Concentrated XAD-2 Extracts from Various Species

	sheep			goat			cow		
fatty acid	free[a]	amino acid[b]	glucuronide[c]	free	amino acid	glucuronide	free	amino acid	glucuronide
butanoic	*d*	22.4	*d*	*d*	19.2	*d*	*d*	24.1	*d*
pentanoic	*d*	11.5	*d*	*d*	12.1	*d*	*d*	13.4	*d*
hexanoic	42.4	45.0	46.0	14.5	29.9	35.2	12.1	31.2	14.5
octanoic	115.5	296.6	115.2	129.6	143.8	131.5	20.3	37.1	27.0
nonanoic	3.8	4.0	11.5	13.6	15.0	16.6	0.7	1.3	0.7
decanoic	62.4	65.2	123.5	38.4	141.7	255.3	4.4	7.8	6.8
undecanoic	16.0	20.0	26.0	5.6	15.6	24.3	6.7	7.8	7.5
dodecanoic	9.1	27.0	44.6	19.6	76.8	128.0	2.2	4.3	7.3
tetradecanoic	6.7	13.6	26.5	4.6	17.1	86.4	6.5	6.8	6.5
pentadecanoic	10.7	13.6	47.5	4.9	12.4	33.6	0.8	8.6	5.4
hexadecanoic	20.8	22.0	31.6	12.3	48.1	82.5	8.0	10.5	14.0
octadecanoic	12.4	16.8	16.0	7.8	43.6	75.2	5.0	9.4	6.5

[a] Relative concentration of free fatty acids found in the concentrated extracts of skim milk. Average of duplicate analyses; standard deviation did not exceed ±5% for all determinations. [b] Free and conjugated fatty acids found after mild thermal hydrolysis (pH 1) at 100 °C during 15 min. [c] Free and conjugated fatty acids found after β-D-glucuronidase hydrolysis for 24 h at 38 °C. [d] Not detected.

Nature of Fatty Acid Conjugates. Fatty acids were among the compounds found in the hydrolysates of conjugates from cow's skim milks by Brewington et al. (1973, 1974), but no comment was made about the nature of their precursors. Free fatty acids were also among the most abundant compounds that were found in untreated XAD-2 extracts of the skim milks (Figure 5A; Table V), but upon hydrolysis under mild thermal acidic conditions, notable increases in concentrations of many fatty acids were observed in each sample (Figure 5B; Table V).

Earlier studies have revealed that carboxylic acids may be conjugated to glucuronic acid via 1-*O*-ester linkages (Cooke and Cooke, 1983; Watkins and Klaaser, 1982), and fatty acid ester glucuronides are susceptible to β-D-glucuronidase hydrolysis (Levvy, 1956; Dickinson, 1985). Exposure of XAD-2 isolates from each of the skim milks to β-D-glucuronidase resulted in the incomplete release of only certain of the fatty acids (Table VI). From a comparison of data for concentrations of fatty acids in the free form with those for fatty acids in the glucuronide plus free forms (Table VI), it is apparent that many of the longer chain members occur substantially as glucuronides. Notably, however, neither butyric nor pentanoic acids was found in the free form nor were they released by β-D-glucuronidase.

Since amino acid conjugates of fatty acids have been reported in various animals (Mulder, 1990; Caldwell et al., 1982), studies were initiated to determine whether the fatty acids released by mild thermal acidic conditions were bound as amino acid conjugates. Exposure of XAD-2 isolates from the skim milk samples to *N*-acylase resulted in the release of notable amounts of fatty acids (data not shown). The magnitudes of quantities of fatty acids released by either *N*-acylase or mild thermal acid hydrolysis (100 °C, pH 1, 15 min) were similar, and the relative proportions of individual fatty acids occurring in free and bound forms in each case were also similar (Table VII). This indicated that both treatments resulted in the release of fatty acids from the same precursor pool of amino acid conjugates of fatty acids. Butyric and pentanoic acids were released in especially notable relative amounts by thermal hydrolysis and acylase exposure (Tables VI and VII), indicating that amino acid conjugates of fatty acids were significant precursors of these free fatty acids in milk. Notably, isolates held for 0.5 h in presence of acid (pH 1) without heating did not release fatty acids.

The free fatty acids present as conjugates in milk, therefore, are bound either as amino acid or glucuronide conjugates because phosphate or sulfate conjugation of the carboxylic acid group would form an unstable anhydride, and this could not occur in aqueous systems. The dose of the fatty acids in vivo has been shown to be determinant of the major conjugation pathway involved. At low doses, amino acid conjugation of fatty acids is almost quantitative, but as the dose increases, glucuronide conjugation becomes more important (Mulder, 1990).

Table VII. Relative Percentages of Fatty Acids in Free and Hydrolyzable Forms Present in XAD-2 Isolates from Cow's Skim Milk

	hydrolysis treatment			
	N-acylase[a]		thermal acidic[b]	
fatty acid	free[c]	bound[d]	free	bound
butanoic	0	100	0	100
pentanoic	0	100	0	100
hexanoic	32	68	39	61
octanoic	34	66	54	45
nonanoic	32	68	53	47
decanoic	37	63	57	43
undecanoic	85	15	85	14
dodecanoic	38	62	52	48
tetradecanoic	87	13	95	5
pentadecanoic	2	98	9	91
hexadecanoic	55	45	75	25
octadecanoic	55	45	53	47

[a] 24 h at 38 °C. [b] 100 °C, pH 1, 15 min. [c] Percent of fatty acids in free form before hydrolysis. [d] Percent of fatty acids in bound form determined by difference after hydrolysis treatment.

A great deal of variation in amounts of individual fatty acids bound in each conjugate form appears to occur among fatty acids within species and between species (Tables VI and VIII). For example, almost all hexanoic acid in sheep's skim milk occurred in the free form, but in goat's skim milk, an equal amount was bound in each of the amino acid and glucuronide conjugate forms (Tables VI and VIII). For cow's skim milk, relatively little hexanoic acid was bound as a glucuronide, but nearly 1.5 times as much was bound as an amino acid conjugate as was present in the free form. The concentrations of fatty acids reported in Table VI under the headings of amino acids and glucuronides include both free and bound fatty acids that were released by the indicated enzyme. The percentages reported in Table VIII are values obtained from calculations which first involved determination of bound and free forms by difference in each instance.

Conjugation of carboxylic acids with amino acids occurs most commonly with glycine (Caldwell, 1982) in a two-stage process. The initial step involves activation of the carboxyl group to a reactive coenzyme A thioester, and this is followed by acyl transfer to an amino acid residue (Killenberg and Webster, 1980; Caldwell, 1982). A fatty

Table VIII. Relative Proportions of Fatty Acids in XAD-2 Isolates from Skim Milks of Various Species Occurring as Free and Conjugated Forms

	free and conjugated forms, %								
	sheep			goat			cow		
fatty acid	free[a]	amino acid[b]	glucuronide[c]	free	amino acid	glucuronide	free	amino acid	glucuronide
butanoic	0	100	0	0	100	0	0	100	0
pentanoic	0	100	0	0	100	0	0	100	0
hexanoic	87	6	7	29	30	41	36	57	7
octanoic	39	61	0	89	10	1	46	39	15
nonanoic	33	2	65	75	8	17	52	46	2
decanoic	49	3	48	11	29	60	43	33	24
undecanoic	53	13	34	16	29	55	78	13	9
dodecanoic	14	28	58	11	31	58	24	22	54
tetradecanoic	20	21	59	5	13	82	95	5	0
pentadecanoic	21	6	73	12	18	70	6	59	35
hexadecanoic	63	4	33	11	30	59	48	16	37
octadecanoic	62	21	17	7	32	61	46	40	14

[a] Percent of fatty acid in free form before treatment. [b] Percent of fatty acids bound to amino acids released by *N*-acylase (24 h at 38 °C) and determined by difference. [c] Percent of fatty acids bound as 1-*O*-glycosidyl esters released by β-D-glucuronidase (24 h at 38 °C) and determined by difference.

acid-activating enzyme that catalyzes the formation of acyl-CoA from a variety of fatty acids has been obtained from beef liver (Mahler et al., 1953). Also, an *N*-acyl transferase specific for certain short fatty acids has been found in bovine liver, and this enzyme catalyzes the conversion of aliphatic thioesters of CoA, including butyric and pentanoic acids to the corresponding acyl derivatives of glycine (Nandi et al., 1979; Schachter et al., 1954).

The absence of butyric and pentanoic acids in glucuronide conjugates, along with generally low concentrations of hexanoic acid bound in glucuronides, suggests that the glucuronides of fatty acids are formed in the liver or elsewhere in the body rather than in the mammary gland. While some fatty acids occurred as glucuronides (Table VI), the exclusive formation of amino acid conjugates of butyric and pentanoic acids suggests that these are formed in the mammary gland. Butyrate produced during ruminal fermentation is converted to β-hydroxybutyrate in the rumen epithelium (Krehbiel et al., 1992; Stevens and Stettler, 1966), and is transported via the portal vein to the mammary gland where it is again converted to butyric acid for incorporation directly into milk fat or elongated to other fatty acids (Luick and Kameoka, 1966). Thus, butyrate would not be subject to glucuronidation in the liver. These data also indicate that pentanoic acid is transported to the mammary gland in a manner similar to butyric acid. Schachter and Taggart (1954) reported that β-hydroxybutyrate was not conjugated with amino acids, but its susceptibility to glucuronide conjugation has not been established.

Other Compounds Found in Isolates. During GC/MS analysis of XAD-2 isolates after thermal acidic and enzymic hydrolysis, several additional compounds were identified (Table V), and the relative amounts of selected compounds are shown in Table IX. Furaldehyde, benzaldehyde, and furoic acid probably were thermally produced artifacts, and they have been found in heated milk and similar systems earlier (Scanlan et al., 1968; Shibamoto, 1980). Phenylacetic acid, which possesses a very tenacious animal-like aroma (Arctander, 1969), is a metabolic product derived from phenylalanine (Fruton and Simmonds, 1958), and it is commonly conjugated to either glycine or glutamine (Thierfelder and Sherwin, 1914). Acetovanillone possesses a structure which indicates that could be derived from lignin-related precursors (Robinson, 1980). The role of these compounds in milk flavors, however, has not been determined as yet.

Summary. Characteristic species aromas were observed in XAD-2 extracts of cow's, goat's, and sheep's skim milks, and these aromas were greatly enhanced after thermal acidic and enzymic hydrolysis (β-D-glucuronidase, acid phosphatase, and arylsulfatase) of extracts. Alkylphenols and fatty acids which comprised the major portions of the volatiles were considered responsible for characteristic species aromas of the extracts. Quantification of alkylphenols in samples showed that the highest amounts were present in sheep's skim milk. Alkylphenols were found as glucuronide and sulfate acid conjugates in cow's, goat's, and sheep's skim milks, but only sheep's skim milk contained phosphate conjugates. Fatty acids were found in skim milk in the free form and as glucuronic and amino acid conjugates. Fatty acids that were conjugated with amino acids, presumably with glycine, were hydrolyzed by both *N*-acylase and thermal acidic hydrolysis (100 °C, pH 1).

Table IX. Relative Amounts (Parts per Million) of Other Compounds Identified in the XAD-2 Concentrated Isolates from Cow's, Sheep's, and Goat's Skim Milks after Sequential Enzymatic[a] and Thermal Acidic[b] Hydrolysis

compound	cow	sheep	goat
furaldehyde	64	366	913
benzaldehyde	c	102	98
phenylacetic acid	746	1363	359
benzothiazole	105	95	284
acetovanillone	14750	9236	8576
furoic acid	3456	1600	3408

[a] β-D-Glucuronidase, arylsulfatase, and acid phosphatase hydrolysis at 38 °C for 24 h. [b] 100 °C, pH 1, 15 min. [c] Not found.

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