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Note

Determination of uracil, uridine and formic acid in egg products by high-performance liquid chromatography

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Uracil is a potentially useful indicator of deterioration in egg products and uridine appears to be one of its precursors[1]. No method has been reported for determination of uracil or uridine in such products. High-performance liquid chromatography (HPLC) in the reversed-phase mode has become the method of choice for determining nucleosides and their bases in biological matrices[2]. Uracil is so weakly retained on reversed-phase columns, however, that it has been recommended as a void-volume marker for reversed-phase HPLC under a variety of conditions[3]. Thus, reversed-phase analysis of biological materials may result in coelution of uracil with other weakly retained components[4].

The contents of formic, acetic, lactic and succinic acids in eggs have been found to be related to odor and bacterial counts and have been suggested as chemical criteria for egg decomposition[5]. For this reason, AOAC methods have been developed for determination of these acids in eggs using gas chromatography[6]. Different methods are required for the volatile acids and the non-volatile ones, however, and both methods require relatively complex sample preparation. The availability of commercial columns for separation of organic acids by ion exclusion and partition chromatography on a strong cation-exchange resin, a technique often referred to as ion-moderated partition chromatography, prompted evaluation of one such column for simultaneously determining volatile and non-volatile acids in eggs. It was observed that the pH of the mobile phase was such that most nucleotides, nucleosides, and nucleobases were protonated and therefore retained on a guard column, but uracil, thymine and their derivatives were eluted[7]. An application of this HPLC technique for analysis of goats' milk for uridine and uracil was reported during the course of this investigation[8].

This paper describes a selective method for the simultaneous determination of uracil and uridine in egg products by use of a column marketed for HPLC of organic acids. Using the same method with UV absorbance detection at 210 nm allows simultaneous quantitation of uracil and formic acid.

EXPERIMENTAL*

Materials

The reagents used were: uracil (Sigma, St. Louis, MO, U.S.A.), uridine (Sigma), sodium formate (analytical reagent, Mallinckrodt, St. Louis, MO, U.S.A.), sulfuric acid (ULTREX, J. T. Baker, Phillipsburg, NJ, U.S.A.), perchloric acid (Baker Analyzed, J. T. Baker), and acetonitrile (UV, American Burdick & Jackson, Muskegon, MI, U.S.A.). Deionized water was further purified by passing it through a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.).

The eluent consisted of 5% (v/v) acetonitrile in 0.01 *N* sulfuric acid. It was filtered through a 0.45-μm Nylon-66 membrane and degassed by sonication for 20 min.

Samples of liquid whole egg obtained from processing plants and allowed to deteriorate for various times were supplied by the Agricultural Marketing Service, U.S. Department of Agriculture. They were kept at temperatures no higher than -20°C.

Sample preparation

A portion of the frozen egg sample was obtained with the aid of an electric drill. About 0.7 g of egg, 0.7 g of water, 1.12 g of 6% perchloric acid, and enough acetonitrile to make its concentration in the final solution 5% (w/w) were weighed into a stoppered Tefzel ETFE (fluorocarbon) centrifuge tube and the mixture was stirred for 1 min. After standing for at least 15 min, the mixture was shaken for 10 s and centrifuged for 10 min at 12000 *g*. The supernatant was filtered through a 0.45-μm Durapore membrane (Millipore). An aliquot was analyzed on the same day the solution was prepared. In calculating contents of analytes in the egg, the weight of egg in the solution was corrected by subtracting 23.29% of the egg weight[9], the estimated weight of the precipitated solids.

In recovery studies, a portion of one of the standard solutions used to generate calibration curves was substituted for all or part of the added water.

Chromatography

The HPLC system (Waters Chromatography Division, Millipore) included a Model 510 pump, a Model U6K injector, and a Model 481 variable-wavelength UV detector set at 210 or 254 nm. A 300 × 7.8 mm Aminex HPX-87H cation-exchange column protected by a Micro-Guard ion exclusion cartridge (Bio-Rad Labs.) was used. Data were analyzed using Computer Automated Laboratory System (Beckman) software, implemented on a Hewlett-Packard 1000 computer.

The operating conditions were: column temperature, ambient; flow-rate, 0.5–0.6 ml/min; and volume injected, 10 μl. The lower flow-rate was preferable because it minimized problems with increasing back pressure.

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* Reference to a company or product name does not imply approval or recommendation by the United States Department of Agriculture.

Quantitation

Quantitation was effected by an external standard method. Five standard solutions in 5% (v/v) acetonitrile were chromatographed in duplicate for each detector setting. The resulting peak areas (for uridine and uracil) or heights (for formic acid) were subjected to linear regression analysis.

RESULTS AND DISCUSSION

Several precipitants reported to be effective for deproteinizing plasma[10] were tried for precipitating the protein and lipid in eggs. Of those tested, perchloric acid was found most suitable because it was reasonably effective and produced minimal interference with peaks in the chromatograms. A more effective precipitant was perchloric acid plus a small amount of acetonitrile, as specified in the section on sample preparation. A chromatogram for a blank prepared using this combination of agents contained no peaks past the solvent front region when detection was at 254 nm; with detection at 210 nm, small blank corrections were required for the formic acid and uracil peaks.

Typical chromatograms obtained using UV detection at 210 nm for an acceptable product and one that had developed spoilage odors after longer storage are compared in Fig. 1. Peaks were tentatively identified by comparing relative retention times with those of standards, and for those of interest supporting evidence was obtained by dual-column chromatography as described previously[7]. For samples with relatively low contents of formic acid, its peak appeared as a shoulder on the much larger uric acid peak; therefore, formic acid concentrations were calculated by comparison of peak heights rather than peak areas, which are more influenced by overlapping peaks[11]. Injection of a solution with an acetonitrile content substantially different from that of the eluent resulted in a small system peak that interfered with the quantitation of uracil. Uridine and succinic acid coeluted under the conditions used, but detection at 254 nm made determination of uridine possible. When the detector was set at 254 nm, the only major peaks after the solvent front region were those for uridine, uric acid, and uracil (Fig. 2).

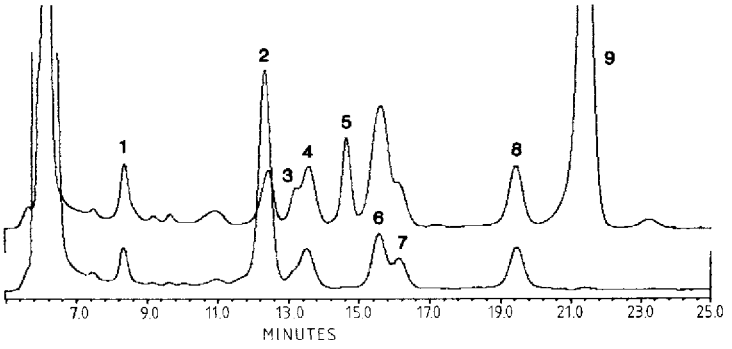

Fig. 1. Chromatograms for liquid whole egg stored for 24 h (lower) and 50 h (upper). Detection, UV absorption at 210 nm; flow-rate, 0.6 ml/min. Retention times (min) are given in parentheses. Peaks: 1 = citric acid? (8.38); 2 = uridine (+ succinic acid?) (12.34); 3 = lactic acid? (13.22); 4 = unknown (13.57); 5 = formic acid (14.64); 6 = uric acid (+ acetic acid?) (15.56); 7 = fumaric acid (16.14); 8 = pyroglutamic acid (19.42); 9 = uracil (21.37).

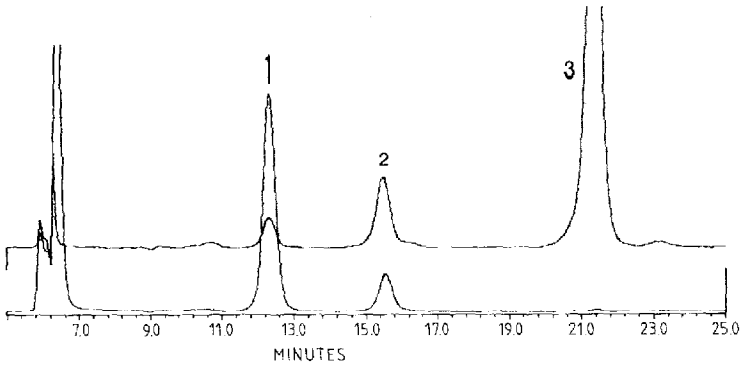

Fig. 2. Chromatograms correspond to those in Fig. 1 except detection was at 254 nm. Peaks: 1 = uridine; 2 = uric acid; 3 = uracil.

With detection at 254 nm, calibration curves were linear in the range 0.25–400 ng for uridine (r = 0.9999) and in the range 0.1–120 ng for uracil (r = 0.9999). With detection at 210 nm, linear response was established in the range 0.2–60 ng for uracil (r = 0.9987) and in the range 13 ng to 2.6 μg for formic acid (r = 0.9987). Approximate detection limits for standard solutions with detection at the specified wavelengths and the corresponding concentrations in egg analyzed by this method (assuming 100% recovery) are listed in Table I. The repeatability of response was tested by repeated injection of the same standard solutions. The coefficients of variation (C.V.) obtained ranged from 1.9 to 4.4% (Table I).

The precision of the method was evaluated by the analysis of solutions individually prepared from the same egg sample. The data presented in Table II for three independent determinations of uridine and uracil in a single portion of the sample indicate that the method is highly reproducible. The reproducibility of results of formic acid determinations also was satisfactory when its content in the egg was relatively high, but was unsatisfactory at a lower concentration, when the formic acid

TABLE I

DETECTION LIMITS AND REPEATABILITY FOR ANALYSES OF STANDARD SOLUTIONS

Repeatability data are for five consecutive injections of the same solution.

	Uridine	*Uracil*		*Formic acid*
	254 nm	*254 nm*	*210 nm*	*210 nm*
*Detection limit**				
ng	0.24	0.10	0.20	13
μg/g egg	0.09	0.04	0.07	4
Calculated concentration				
Mean (μg/ml)	1.092	0.3258	0.5963	24.89
Standard deviation	0.021	0.0084	0.0211	1.09
C.V. (%)	1.9	2.6	3.5	4.4

* Signal-to-noise ratio = 2.

TABLE II

REPRODUCIBILITY DATA FOR ANALYSES OF EGG

Either 5 separate portions of a frozen sample were analyzed, or a single portion was allowed to thaw and stirred before being divided into 3 portions for analysis.

	Uridine *254 nm*	*Uracil*		*Formic acid* *210 nm*	
		254 nm	*210 nm*		
Single portion (n = 3)					
Mean (μg/g egg)	30.11	19.16	20.40	12.49	88.58
Standard deviation	0.10	0.15	0.63	9.53	2.84
C.V. (%)	0.3	0.8	3.1	73.6	3.2
Separate portions (n = 5)					
Mean (μg/g egg)	30.23	18.12	19.52	–	87.06
Standard deviation	2.98	1.34	1.36	–	6.42
C.V. (%)	9.9	7.4	7.0	–	7.4

peak appeared as a shoulder on a much larger peak. The relatively large coefficients of variation resulting from analysis of separate portions of the sample appear to have been due to sample inhomogeneity. The accuracy of the determinations was established by spiking egg samples with standard solutions to approximately double their contents of the compound determined; recovery data are presented in Table III.

TABLE III

RECOVERY FROM SPIKED EGG SAMPLES

	Uridine *254 nm*	*Uracil*		*Formic acid* *210 nm*
		254 nm	*210 nm*	
Concentration before spiking (μg/g egg)	20.2	8.9	6.0	39.9
n	3	3	3	4
Recovery (%)	97.0	95.8	96.5	98.6
Standard deviation	1.9	1.5	2.0	7.5
C.V. (%)	2.0	1.6	2.1	7.6

The results show that chromatography on a column of cation-exchange resin with an acidic eluent and UV detection at 254 nm provided a selective, reproducible, and accurate method for determination of uracil and uridine in egg products. With detection at 210 nm, uracil and formic acid could be determined in eggs, but determination of formic acid at lower concentrations was adversely affected by incomplete resolution under the conditions used.

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