Quantitative Reversed-Phase HPLC Analysis of Dicumarol in Sweetclover Hay and Silage Samples

Alister D. Muir* and Bernard P. Goplen

Agriculture Canada Research Station, 107 Science Place, Saskatoon, Saskatchewan S7N 0X2, Canada

A quantitative high-performance liquid chromatographic (HPLC) method was developed for determining dicumarol, a fungal-produced anticoagulant, in spoiled sweetclover hay and silage. Samples (1 g) were extracted in 1,2-dichloroethane/phosphate buffer, and the 1,2-dichloroethane phase was purified through a silica column prior to reversed-phase HPLC chromatography using a sodium acetate/methanol (25:75) eluent with UV detection at 303 nm. Recoveries of dicumarol standards subjected to the extraction and purification procedure were 116% and 96% for 20 and 50 ppm solutions, respectively. The SE of replicated analysis of a spoiled sweetclover sample was 5% (n = 8) with 115% recovery of dicumarol added to samples prior to extraction. Analysis time was 6–8 min, and the minimum detectable concentration was 2 ppm. This method uses 10% of the sample and solvent required by previous methods and is adaptable to automation, allowing a significant increase in the sample throughput.

INTRODUCTION

Yellow sweetclover [*Melilotus officinalis* (L.) Lam.] is grown over a wide area in the Canadian prairie as a source of winter feed for cattle. If "high-coumarin" sweetclover becomes moldy during harvesting or storage, then toxic levels of dicumarol [3,3'-methylenebis(4-hydroxycoumarin)] can develop rapidly (Benson et al., 1981; Blakley, 1985). Dicumarol, a potent anticoagulant, is a product of microbial action which interferes with the synthesis of vitamin K dependent coagulation factors and may result in extensive hemorrhaging in cattle and spontaneous abortion in bred cows, a syndrome known as "sweetclover bleeding disease" (Radostits et al., 1980; Blakley, 1985). Dicumarol was first identified as the casual agent for sweetclover bleeding disease in cattle in 1941 (Campbell and Link, 1941; Stahmann, 1941). Caspar et al. (1982) demonstrated that dicumarol levels in excess of 20 ppm were required to generate a toxic response in calves, with levels of 60 ppm causing death. "Low-coumarin" cultivars of sweetclover have been developed (Goplen, 1971, 1981); however, because this characteristic is recessive, outcrossing of the low-coumarin cultivars with high-coumarin wild *Melilotus* is an unavoidable problem (Goplen and Weber, 1966). Goplen et al. (1964) determined that spoiled low-coumarin cultivars contaminated with up to 10% of high-coumarin plants could safely be fed to cattle. Levels of 20 ppm or more have been observed quite frequently in hay and silage samples (Benson et al., 1981); therefore, reliable rapid analytical methods are needed to permit assessment of the hazard of spoiled sweetclover to livestock.

A number of extraction and purification procedures have been proposed for dicumarol, none of which are suitable for rapid routine quantification of a large number of plant samples in a forage quality laboratory. Christensen (1964a,b) developed a paper chromatography method for determination of dicumarol utilizing diazo reagents to detect dicumarol isolated from animal tissue samples by acidic ethylene chloride extraction. Davies and Ashton (1964) extracted dicumarol from spoiled hay into chloroform and quantified dicumarol by paper chromatography and visualization with Brentamine Fast B. Emery and Gear (1970) extracted plant material in aqueous alkali, which was then acidified, and the dicumarol was extracted into chloroform. The chloroform extract was passed through an alumina column and the dicumarol recovered from the dried column material with aqueous alkali. Casper et al. (1981) examined a number of different extraction systems and demonstrated that a 1,2-dichloroethane/phosphate buffer was the most efficient system for extracting dicumarol from sweetclover samples. All of these methods use complicated extraction and cleanup procedures with quantitation by TLC and UV spectroscopy, labor-intensive procedures which are not readily amenable to automation.

HPLC determination of dicumarol in plant samples has not been reported; however, HPLC has been used to determine dicumarol in pharmaceutical products (Vanhaelen-Fastre and Vanhaelen, 1976; Moore and Lau-Cam, 1986) and serum (van den Berg et al., 1977). The methods of Moore and Lau-Cam (1986) and van den Berg (1977) require the use of expensive and hazardous HPLC solvents, making the procedures undesirable for routine use. The methods of Vanhaelen-Fastre and Vanhaelen (1976) have not been tested with plant samples. The method proposed in this paper makes use of the initial extraction procedure developed by Casper et al. (1981), followed by a simplified cleanup procedure using silica gel columns and analysis on reversed-phase HPLC columns. The solvents required for the HPLC analysis described in this paper are less expensive to acquire and to dispose of, and there is a 65% reduction in the volume of 1,2-dichloroethane required compared to the method of Casper et al. (1981).

MATERIALS AND METHODS

Liquid Chromatography. A Waters liquid chromatograph equipped with a M600E gradient system, M700 autosampler, and 991 photodiode array detector (PDA) was used [Millipore (Canada) Ltd., Mississauga, ON, Canada]. The PDA software was used to integrate the signal. Chromatograhy was performed on a Lichrosorb RP-18 (10 μm) column (250 × 4.6 mm) (Phenomenex, Torrance, CA) with an ODS-Hypersil (30 μm) precolumn (20 × 2.1 mm) [Hewlett-Packard (Canada), Mississauga, ON, Canada]. Aliquots (50–100 μL) were applied to the column, which was eluted isocratically with 9 mM aqueous sodium acetate (pH 6.0) (w/v)/methanol (25:75) (35 °C) or 18 mM aqueous sodium acetate (pH 7.50) (w/v)/methanol (24:75) (1 mL/min). The sodium acetate solutions were prepared by diluting a 0.37 M sodium acetate trihydrate solution (50 g in 1 L of HPLC grade

* Author to whom correspondence should be addressed.

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water) with HPLC grade water 1:40 and 1:20, respectively. Detection was by PDA at 303 nm. Spectral information from 250 to 350 nm was also recorded for confirmation of peak identity by comparison with UV spectral scans of authentic dicumarol obtained in a Cary 3 UV–vis spectrophotometer (Varian Canada Inc., Mississauga, ON, Canada).

Thin-Layer Chromatography. Standards and plant samples were chromatographed on Kieselgel 60_{F254} plates with concentration zone (E. Merck, Darmstadt, Germany) developed with 1,2-dichloroethane containing 1% formic acid. Dicumarol was visualized as yellow spots (R_f = 0.70) by spraying with diazotized *p*-nitroaniline [mix fresh; 5 mL of *p*-nitroaniline (0.5 g in 100 mL of 2 N HCl), 0.5 mL of $NaNO_2$ (5 g in 100 mL of H_2O) and 15 mL of aqueous sodium acetate (20 g of trihydrate in 100 mL of H_2O)] (Ziegler and Junek, 1955; Davies and Ashton, 1964).

Materials. Dicumarol was purchased from Sigma Chemical Co. (St. Louis, MO.). All other reagents used were purchased from BDH Inc. (Toronto, ON, Canada). Formic acid, sodium nitrate, HCl, and sodium dihydrogen orthophosphate were of analytical grade. 1,2-Dichloroethane and methanol were of HPLC grade. All other reagents were of laboratory grade.

Plant Material. Samples of yellow sweetclover *M. officinalis* cv. Norgold (low coumarin) and Yukon (high coumarin) and white sweetclover *M. alba* (Desr.) cv. Arctic (high coumarin) were obtained from greenhouse- and field-grown plants. A bulk sample of cv. Yukon was spoiled using the procedure described by Linton et al. (1963). Additional samples were spoiled in the laboratory by placing the fresh sample in a plastic bag and allowing spoilage to occur over a 14-day period. The cv. Norgold material used in this test was obtained from individual plants from a low-coumarin breeder plot. Alfalfa (*Medicago sativa* L.), crested wheatgrass [*Agropyron desertorum* (Fisch. ex. Link) Schult], Kentucky bluegrass (*Poa pratensis* L.), smooth bromegrass (*Bromus inermis* Leyss.), and sainfoin (*Onobrychis vicifolia* Scop.) samples were selected from material submitted for forage quality analysis. Samples of spoiled sweetclover hay submitted to the Saskatchewan Feed Testing Service were also analyzed with the microassay. Samples were freeze-dried or oven-dried and ground to pass a 2-mm screen prior to extraction.

Dicumarol Extraction and Pre-HPLC Purification. Ground samples (2–5 g) were extracted in 25 mL of 1 M sodium dihydrogen orthophosphate (pH 4.2) and 35 mL of 1,2-dichloroethane in a 250-mL round-bottom flask under reflux for 2 h using a heating mantel (Casper et al., 1981). After cooling, the extract was gravity filtered through Whatman IPS (phase separation) paper. The 1,2-dichloroethane fraction was concentrated to dryness under reduced pressure (40 °C) and taken up in 2 mL of 1,2-dichloroethane. The extract was applied to 2 g of silica gel (70–230 mesh) in a glass column (1 cm i.d.), and the dicumarol and coumarin were eluted with 35 mL of 1,2-dichloroethane containing 1% formic acid (prepared by diluting 1 mL of concentrated formic acid to 100 mL with 1,2-dichloroethane). The column eluate was reduced to dryness and redissolved in 1,2-dichloroethane (2 mL) for HPLC analysis.

Dicumarol Microassay. Ground samples (1 g) were placed in 20 × 150 mm thick walled culture tubes, and 10 mL of 1,2-dichloroethane and 5 mL of 1 M sodium dihydrogen orthophosphate (pH 4.2) were added. The samples were extracted uncapped in a miniblock digester for 3 h at 90 °C in a fume hood. The natural draft of a fume hood allows the culture tubes to act as their own air-cooled condensers. After cooling, the samples were gravity filtered through Whatman IPS phase separation paper and the 1,2-dichloroethane phase was reduced to dryness under vacuum at 40 °C. Each sample was redissolved in 5 mL of 1,2-dichloroethane and adsorbed onto 1 g of silica gel (70–230 mesh) in an extraction tube (1.2 cm i.d.) on a vacuum manifold. The dicumarol was eluted with 15 mL of 1,2-dichloroethane containing 1% formic acid. All of the 1,2-dichloroethane passing through the column was combined and reduced to 2 mL for HPLC analysis.

Quantitation. Dicumarol concentrations in spoiled sweetclover extracts were determined by external standards procedures using a calibration curve obtained by duplicate injections of three concentrations of dicumarol (5, 10, and 100 ppm solutions prepared by serial dilution from a 1000 ppm standard).

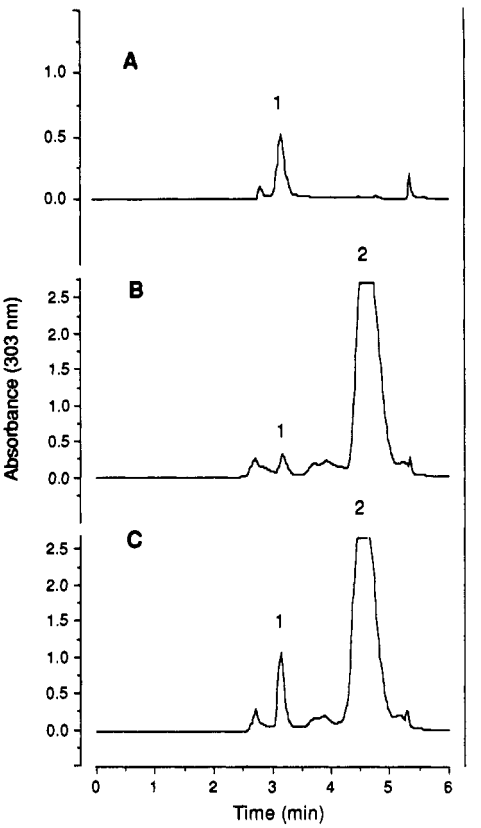

Figure 1. Chromatography of dicumarol and plant samples containing dicumarol: (A) dicumarol, 20 ppm solution; (B) cv. Arctic spoiled to produce dicumarol; (C) sample B spiked with dicumarol (20 ppm) prior to extraction; (1) dicumarol; (2) coumarin. Chromatography: 0.09 M aqueous sodium acetate/methanol (25:75 v/v) (35 °C) (1 mL/min). Detection: 303 nm.

RESULTS AND DISCUSSION

Trials with a number of solvent systems failed either to elute dicumarol from reversed-phase HPLC columns within a reasonable time frame (<30 min) or to separate dicumarol from other components in the extract and would therefore require excessive sample cleanup. Optimum separation was achieved with a sodium acetate/methanol ratio of 25:75 (Figure 1). Reducing the concentration of sodium acetate from 0.18 to 0.09 M reduced the analysis time by approximately 1 min from 7–9 to 6–8 min and reduced the retention time of dicumarol from 3.5 to 3.1 min with a slight improvement in the resolution. Different HPLC columns may require slight adjustments in the ratio, since experiments demonstrated as little as 2% change in the methanol concentration could significantly affect the resolution of dicumarol from other components present in the extracts. Quantitation was obtained using three concentrations of dicumarol. Peak areas were linear over the dicumarol concentration range 5–100 ppm ($y = 1.515 + 178.9x$, $r = 1.000$). Concentrations as low as 2 ppm could be detected; however, the calibration was nonlinear below 5 ppm. Reversed-phase HPLC columns were chosen because they are the most widely used column type for HPLC analysis. Similar separations have been achieved on a Resolvex C-18 (Fisher Scientific, Toronto, ON, Canada) reversed-phase column, indicating that the separations are not utilizing unusual properties of a particular reversed-phase packing. Dicumarol dissolved in the HPLC eluant has two UV maxima, 276 and 303 nm. Detection at 303 nm was used to exploit the characteristic absorption of dicumarol at this wavelength and to minimize any interference from other UV absorbing compounds; how-

Table I. Recovery of Pure Dicumarol and Determination of Dicumarol in Replicated Analysis of a Spoiled Sweetclover Sample Analyzed According to the Microassay Method

sample	dicumarol concn, ppm dry wt ± SE: expected level	observed level	% recovery
dicumarol standard[a]			
50 ppm (n = 3)	50	47.1 (±0.5)	95.9 (±0.7)
20 ppm (n = 4)	20	23.2 (±0.1)	115.9 (±0.5)
plant sample (n = 8)		11.5 (±0.6)	
sample + 20 ppm (n = 8)[b]	31.5	36.2 (±1.1)	114.9 (±2.9)

[a] Dicumarol standards processed through the microassay procedure. [b] Dicumarol added prior to extraction.

ever, satisfactory results can be obtained with detection at 280 nm with only a slight loss in sensitivity.

HPLC analysis of sweetclover samples purified by base–acid–base purification (Casper et al., 1981) indicated that a significant number of UV-absorbing compounds were still present in the extracts, some of which interfered with the detection of dicumarol. Quantitative recovery of added dicumarol was rarely achieved using base–acid–base purification. HPLC analysis of the aqueous acid fraction, which is discarded after 1,2-dichloroethane partition, indicated that in some plant samples dicumarol was still present in this waste fraction. HPLC analysis of crude 1,2-dichloroethane fractions obtained by filtration of the initial extract through phase separation paper indicated that significant improvement in chromatographic resolution was not usually obtained by subsequent base–acid–base extract purification. The only benefit observed from subsequent purification of the crude 1,2-dichloroethane fraction was an increase in column life and a reduction in the frequency with which guard columns had to be replaced due to the removal of the chlorophyll and carotenoid pigments in the purified extracts.

The use of a silica gel column was found to be a more effective and simpler pre-HPLC purification method than the base–acid–base partition technique employed by Capser et al. (1981). Variable dicumarol recoveries were observed in initial experiments when 1,2-dichloroethane was used to elute authentic dicumarol from the silica gel. Recovery of dicumarol was also influenced by the source of silica gel. In our initial experiments, dicumarol could be eluted from silica gel without the addition of 1% formic acid to the eluting solvent; however, when a new stock of silica was purchased, dicumarol was retained on the column. Addition of 1% formic acid to the 1,2-dichloroethane eluent resulted in quantitative recovery of dicumarol from the new silica gel (Table I). Recovery experiments should be conducted with each batch of silica gel used. More than 300 extracts have been prepared according to the microassay and analyzed by HPLC on a single column with no observable deterioration in the chromatography.

Dicumarol is a product of microbial activity (Bellis et al., 1967) and not necessarily uniformly distributed throughout a sample; therefore, initial experiments were conducted using larger samples (2–5 g) using the larger scale method. The larger scale method described utilizes more solvent and more expensive equipment than the microassay method. Subsequently, replicated analysis of a spoiled sweetclover sample (cv. Arctic) was undertaken to determine the variability present in a typical sample (Table I). These results suggest that analysis of a single 1-g sample using the microassay will give an accurate representation of the level of dicumarol in the sample as a whole. This

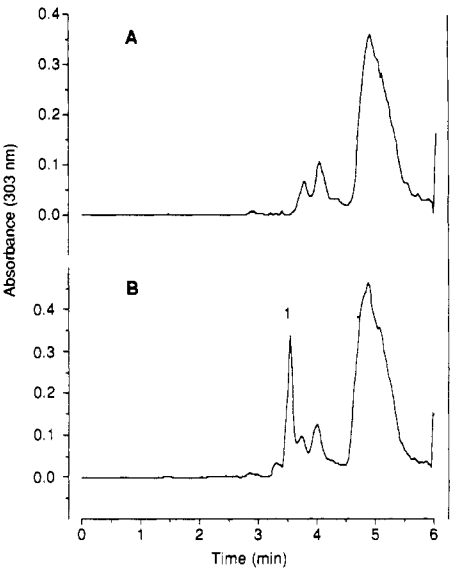

Figure 2. Spoiled sweetclover samples: (A) cv. Norgold; (B) cv. Norgold spiked with dicumarol (20 ppm) prior to extraction; (1) dicumarol. Chromatography: 0.18 M aqueous sodium acetate/methanol (25:75 v/v) (35 °C) (1 mL/min). Detection: 303 nm.

represents a significant reduction in the volume of solvents required compared to the 10-g samples required for previous methods (Casper et al., 1981). All subsequent extracts were prepared according to the microassay method. Dicumarol standards (20 and 50 ppm) were extracted and purified according to the microassay, and the dicumarol recovery was calculated using an external standard calibration. Complete recovery of the dicumarol was achieved (Table I). These results are similar to those obtained by Casper et al. (1981), who reported recoveries of added dicumarol of 104% (n = 3) using a 10 ppm standard processed through the extraction procedure.

In the analysis of feed samples to determine relative hazard, these results suggest simple extraction would provide sufficient indication of hazard. In toxicological studies, addition of a known amount of dicumarol to a duplicate sample would allow a more precise quantification. Sweetclover samples submitted for analysis can contain small amounts of other forage species. To check for the presence of extractable compounds that might cochromatograph with dicumarol, alfalfa, crested wheatgrass, Kentucky bluegrass, smooth bromegrass, and sainfoin samples were analyzed using the standard method. No interfering or cochromatographing compounds were observed in any of these extracts. Unspoiled cv. Arctic and Norgold (a low-coumarin sweetclover) were also analyzed, and no compounds corresponding to dicumarol were observed in either sample. Peaks in plant extracts with elution times corresponding to dicumarol (3.1 min in 0.09 M sodium acetate/methanol) were subject to peak purity analysis and determined to be composed of a single compound. Comparison of the UV spectra of the unknown with that of authentic dicumarol indicated identical absorption spectra.

Samples of cv. Arctic (two), Yukon (one), and Norgold (one) were spoiled under controlled conditions and analyzed for the presence of dicumarol. Significant levels of dicumarol were found in the cv. Arctic (Figure 1) and Yukon samples, but no peaks corresponding to dicumarol were observed in spoiled cv. Norgold samples (Figure 2). To confirm the presence or absence of dicumarol in samples analyzed by HPLC, aliquots of the extracts were subjected to TLC on Kieselgel. Only samples containing dicumarol (by HPLC) exhibited a characteristic yellow spot (R_f =

0.70) when the plates were sprayed with diazotized *p*-nitroaniline. The spot is yellow rathern than red because of the acidic nature of the TLC solvent (Clayton and Larmour, 1935). In samples spiked with authentic dicumarol the spot was correspondingly larger. The use of diazotized *p*-nitroaniline as a TLC spray reagent is based on the observation (Ziegler and Junek, 1955) that the diazo reagent cleaves the dicumarol molecule to yield two molecules of 2,3,4-chromatrione-3-(4-nitrophenylhydrazone), with a red chromophore. Unfortunately, this reagent cannot be used to detect dicumarol directly since 4-hydroxycoumarin (Huebner and Link, 1945), coumarin, and melilotic acid (Clayton and Larmour, 1935) will also form red-colored hydrazones.

The HPLC analysis of four samples of spoiled low-coumarin sweetclover made from individual plants of the cv. Norgold revealed the presence of large peaks corresponding to coumarin in two of the four samples. However, dicumarol was not detected in any of these four samples. This would suggest that coumarin and melilotoside are not the direct precursors of dicumarol but rather *o*-coumaric acid and 4-hydroxycoumarin are the immediate precursors, as suggested by Davies and Ashton (1964) and Bellis et al. (1967).

In conclusion, the method described provides a rapid quantitative assessment of the dicumarol content of spoiled sweetclover hay and silage samples. Using this method, the dicumarol level in 36 samples can be determined per person per day utilizing $^1/_{10}$ of the sample required by previous methods.

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