A High-Performance Liquid Chromatographic Method for the Determination of Monofluoroacetate

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Abstract

A simple isocratic high-performance liquid chromatographic (HPLC) method for the quantitative analysis of monofluoroacetic acid (MFA), the toxic substance of *Dichapetalum cymosum,* in plant material, rumen contents (gastric contents), and liver samples is described. A suitable HPLC column that gives optimum sensitivity, accuracy, precision, and separation of MFA is identified. A C-610 organic acid analysis column at ambient temperature with 0.02M H_3PO_4 as an eluent and ultraviolet detection at 210 nm is utilized to quantitate MFA. Using this method, the average percentage recovery in plant material, bovine liver, and rumen samples is 94.8%, and a detection limit of 12 µg/L is achievable.

Introduction

The toxic substance of *Dichapetalum cymosum*, a plant known as "gifblaar" or poison leaf, has been identified as CH_2FCOOH, monofluoroacetic acid (MFA) (1). *D. cymosum* is one of the most poisonous plants in southern Africa, causing the sudden death of ruminants in Gauteng, Mpumalanga, North West Province, and Northern Province, as well as in Zimbabwe, Botswana, and Namibia (2). *D. cymosum* is reported as the fourth most economically important plant poisoning syndrome of livestock in South Africa (2). The mortality of cattle following the ingestion of *D. cymosum* leaves, especially during the months of March and August to November, has been reported by various authors (3,4).

Synthetically produced monofluoroacetate (SFA, or compound 1080) is a banned substance in South Africa in terms of the Hazardous Substance Act (Act 15 of 1975). This is mainly due to its extreme toxicity, water solubility, colorlessness, tastelessness, difficult detection, latent period between ingestion and symptoms, and hazards of secondary poisoning. Elsewhere, however, compound 1080 is used extensively as a predacide and rodenticide for the control of animal problems (5). Despite the fact that the substance is banned in South Africa, malicious or incidental poisoning of companion and other animals occurs. Signs of poisoning include sudden death, severe convulsions, and muscular fibrillation (6). The substance is possibly sourced from neighboring states where SFA is not banned or stock obtained legally before SFA was banned.

The lack of a fast, simple, and economical method for the determination of MFA means that cases of poisoning by MFA, either natural or synthetic, go undetected or cannot be confirmed. The importance of determining poisoning by gifblaar in southern Africa cannot be overemphasized, but before any investigation into the concentration of MFA in the plant and its epidemiology can be undertaken, an accurate method for analysis is required. The aim of this work was to find such a method.

Experimental

Chemicals and reagents

The eluent was 0.02M H_3PO_4 (pH 1.5) prepared by diluting HPLC-grade ortho-phosphoric acid with deionized water (Milli-Q, Millipore, Milford, MA). SFA, which was used to establish a calibration curve for monofluoroacetic acid and fortify different matrices, was analytical grade and obtained from Merck (Darmstadt, Germany). Analytical-grade acetic, phosphoric, propionic, and formic acids were supplied by Sigma Chemical Company (St. Louis, MO).

Apparatus and operating conditions

A Waters (Milford, MA) model 600 E liquid chromatograph supported by a model 712 WISP (Waters) auto-sampler injector

was utilized. The HPLC was equipped with a model 490 E programmable multiwavelength ultraviolet (UV) detector (Waters). Column effluents were monitored at a detector wavelength of 210 nm. Quantitation was based on peak area measurements using a 386 personal computer programmed with System Gold version 5 supplied by Beckman Instruments (Fullerton, CA). Analyses were performed isocratically at an optimized flow rate of 0.8 mL/min and at ambient temperature.

Column

The column was a 300 × 7.8-mm-i.d. HPLC C-610H organic acid analysis column (Supelcogel, Supelco, Bellefonte, PA) fitted with a Peek filter holder and a 0.5-µm Peek filter end fitting (Upchurch Scientific, Oak Harbor, WA). The column support was polystyrene divinyl benzene, which is a noninteractive porous solid, and separation occurred by a combination of size exclusion and reversed-phase chromatography.

Sample preparation and extraction procedures

D. cymosum leaves, plant material not containing natural monofluoroacetic acid, bovine rumen contents, and bovine liver tissue samples were used as matrices. Plant material (other than *D. cymosum*) that did not contain MFA, liver samples, and rumen contents were fortified with SFA to determine the extraction accuracy and the percentage recoveries of SFA from different SFA concentrations in each matrix.

Sample preparation was identical for each matrix: 1.00 g of plant material, rumen contents, or liver and approximately 20 mL aqueous H_3PO_4 (pH 1) were placed in a preweighed glass bottle. The contents were homogenized with a Heidolph (Kelheim, Germany) Diax 600 ultra turrox at room temperature, and the pH was measured again after homogenization and adjusted to 1 (if necessary) by adding concentrated phosphoric acid to the suspension. The final volume was increased to 50 mL with extraction fluid. The suspension was shaken for 1 min on a Heidolph DSG shaker. Approximately 3 mL was centrifuged in a model GS-15R Beckman centrifuge at a relative centrifugal acceleration of 8500 × *g* for 30 min.

The clear supernatant (50 µL) was injected into the instrument after the sample was filtered through a 0.5-µm syringe filter. Three repetitive injections were performed for each sample to enable the calculation of average peak areas and standard deviation (SD) to be carried out. Percentage relative standard deviation (% RSD) and SD, which were used as a measure of extraction and chromatographic repeatability, were determined.

Determination of retention times of interfering volatile fatty acids

Solutions of fluoroacetic, acetic, formic, and propionic acid were prepared in 0.02M phosphoric acid and chromatographed separately in order to determine the retention times for each acid. Once the individual retention times were established, the acids were injected into the chromatograph as a mixture. The separation of the acids and retention times were evaluated for interferences in the determination of MFA. Fluoroacetic, acetic, formic, and propionic acids were separated when the conditions were applied as described.

Extraction of MFA from *D. cymosum*

Portions of *D. cymosum* leaves (1 g) were macerated to a homogenous suspension with approximately 20 mL of aqueous H_3PO_4, the pH was adjusted to 1, and the volume was increased to 50 mL with water. The suspension was then shaken for 1 min, after which a 3.0-mL pipetted aliquot was centrifuged. The extraction procedure was repeated in order to determine the maximum number of extractions required to remove sufficient MFA from the plant for analysis and to obtain the extraction efficiency of the method.

Calibration and recovery from standard SFA solutions

Seven calibration standards and a blank were prepared by diluting a stock solution of SFA in 0.02M phosphoric acid. Each standard and the blank were injected, and the corresponding peak areas were determined. The concentrations were 25, 50, 100, 200, 400, 800, and 1600 µg/mL SFA respectively. Five injections of each concentration were performed. A calibration curve of SFA concentration versus corresponding peak areas was drawn. The correlation coefficient (r), the slope (b), the error in the slope (S_b), the

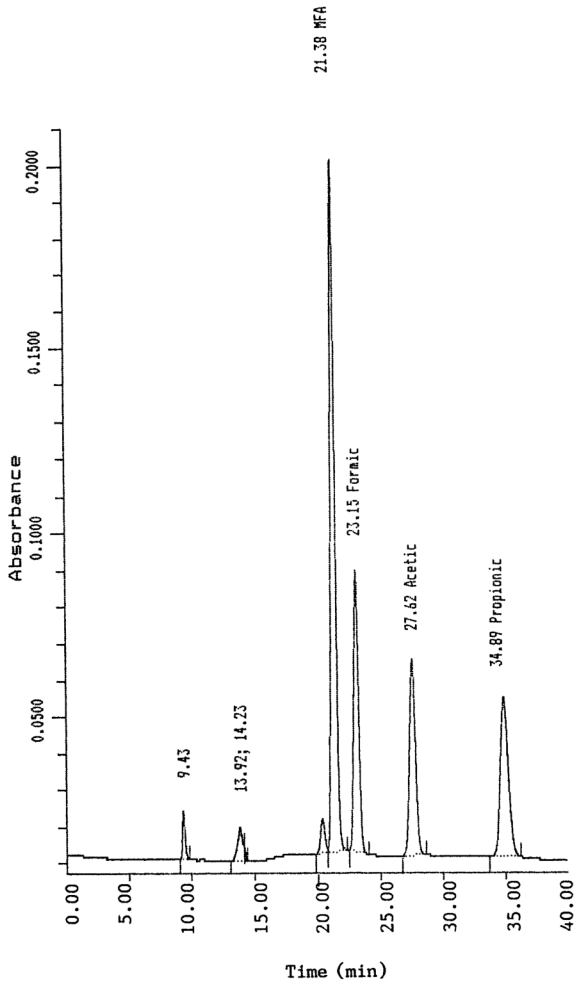

Figure 1. Chromatogram of 50 µL of MFA and formic, acetic, and propionic acids.

intercept (a), the error in the intercept (S_a), the random error in y and x ($S_{y/x}$), and the corresponding confidence limits (p = 0.05) of a and b were determined by linear regression analysis.

The regression line for SFA standards was defined as $y = bx + a$, where y is the signal (peak area) of the compound in absorbance units and x is the concentration in micrograms per milliliter. The slope (b) of the line was the response factor for the compound (MFA). Resulting peak areas (signals) were determined, and concentrations were calculated by using the regression line.

Limits of detection and quantitation

The limit of detection (LOD) was calculated from $y_{(LOD)} = a + 3S_{y/x}$ and $y_{(LOD)} = bx_{(LOD)} + a$, where a is the intercept, $S_{y/x}$ is the random error in x and y, and b is the slope. The limit of quantitation (LOQ) was calculated from the formulas $y_{(LOQ)} = a + 10S_{y/x}$ and $y_{(LOQ)} = bx_{(LOQ)} + a$.

Results and Discussion

The chromatogram of a mixture of volatile fatty acids can be seen in Figure 1. Separation was completed within 25 min, and after an additional 5 min reequilibration time, the following injection could be made. Chromatograms of nonfortified and fortified rumen samples are shown in Figures 2A and 2B, respectively, as examples. With the described isocratic system, the four substances of interest (i.e., MFA, formic acid, propionic acid, and acetic acid) were well separated from endogenous peaks (Figure 1).

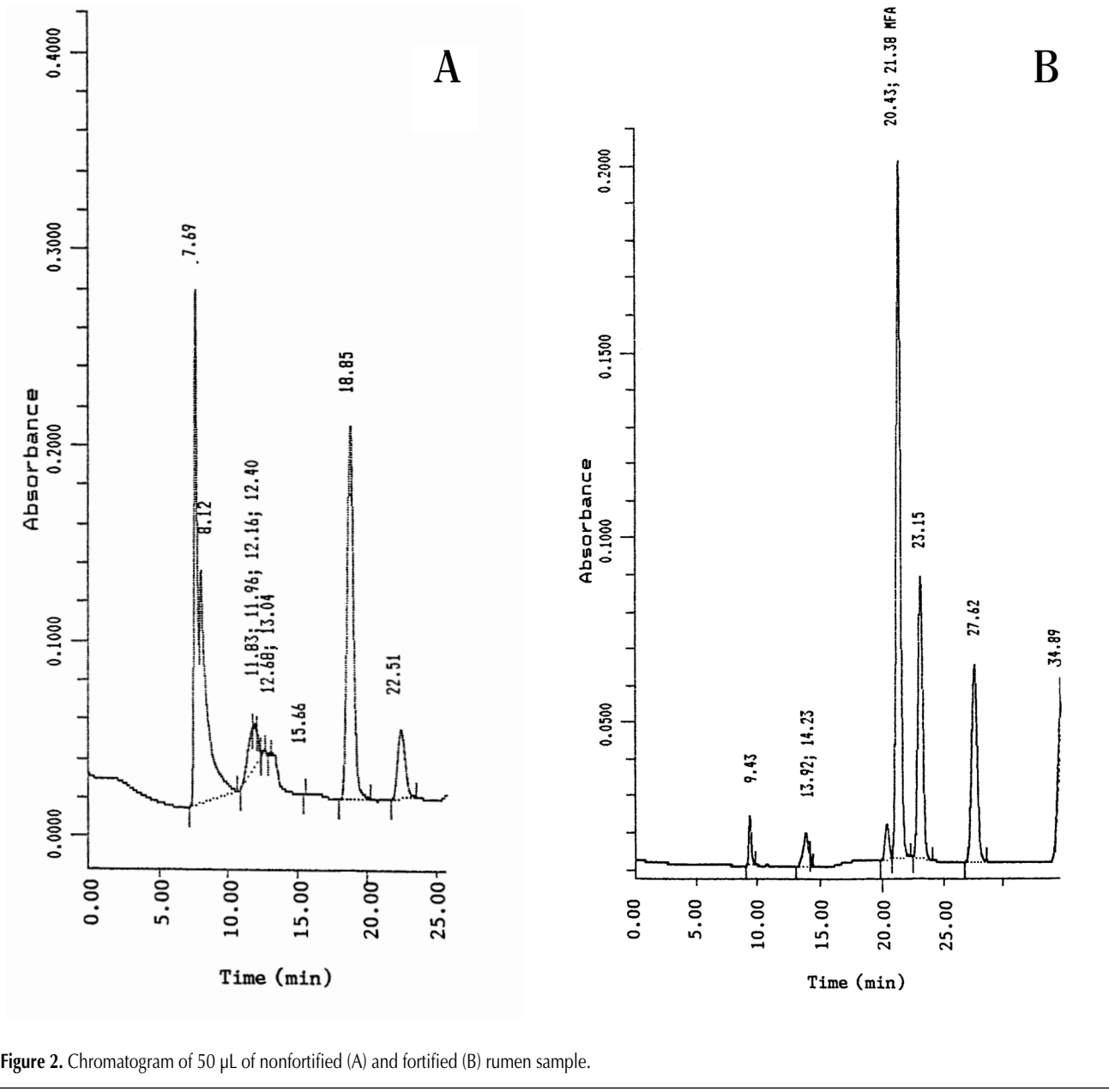

Figure 2. Chromatogram of 50 µL of nonfortified (A) and fortified (B) rumen sample.

The results from the extraction experiments showed that it was only necessary to make one extraction to remove the major portion of MFA (> 98%) from the plant.

In order to obtain equal detection, the initial concentration of MFA in fortified liver samples had to be higher than in matrices other than liver. It is assumed that the complexity of the liver sample was responsible.

The calibration curve of sodium monofluoroacetate was almost perfectly linear over a range of 0 to 1600 µg/mL when using a UV detector at 210 nm as demonstrated by an *r* value close to unity (Table I).

Only one extraction was needed to extract significant amounts (> 98%) of MFA from *D. cymosum*.

Table I. Calibration Data for 50-µL Sample

SFA injected (µg)	[MFA] (µg/mL)	Mean peak area	SD	RSD (%)
0.00	0.0	0.0003	0.0004	133
1.25	25.0	6.02	0.0750	1.25
2.50	50.0	14.6	0.246	1.68
5.00	100	25.7	0.380	1.48
10.00	200	55.6	0.369	0.663
20.00	400	114	0.522	0.456
40.00	800	228	1.13	0.496
80.00	1600	456	1.61	0.353

MFA recoveries from fortified samples

The % RSD for fortified samples of rumen, plant, and liver and percentage recoveries in terms of peak areas are shown in Table II. The precision of the sample preparation method was calculated by analyzing fortified plant, liver, and rumen samples several times on one day to obtain the intraday variation and on different days to obtain day-to-day variation.

Determination of LOD and LOQ

The results of the statistical evaluation obtained from regression are summarized in Table III.

Table II. Recoveries of MFA in Fortified Rumen, Plant, and Liver Samples*

Sample	MFA injected (µg)	Mean peak area	SD of area	Mean MFA recovered (µg)	Mean % recovery	SD of recovery	RSD (%)
Rumen	2.50	11.8	0.0882	2.05	82.2	0.0605	0.746
Rumen	20.0	110	0.298	19.3	96.5	0.260	0.269
Rumen	40.0	222	0.297	39.1	97.7	0.130	0.133
Plant	2.50	11.6	0.0296	2.01	80.4	2.03	2.56
Plant	20.0	105	0.0709	18.4	91.8	0.0619	0.0674
Plant	40.0	221	0.158	38.7	96.8	0.0694	0.0717
Liver	10.0	55.4	0.127	9.72	97.2	0.0227	0.234
Liver	20.0	111	0.0454	19.4	96.8	0.0397	0.0410
Liver	40.0	226	0.263	39.7	99.1	0.0462	0.117

* Samples were fortified with 50 (200 for liver), 400, and 800 µg/mL MFA. A 50-µL sample size was used.

Table III. Statistical Evaluation of the HPLC Determination of MFA

Parameter	Value
r	0.999977
r^2	0.999954
b	0.2859
a	–0.8999
Regression line	$y = 0.2859x - 0.8999$
$S_{y/x}$	1.139
S_b	0.002468
95% Confidence limit of b	0.2859 ± 0.0060
95% Confidence interval of b	$0.2799 < b < 0.2919$
S_a	1.612
95% Confidence limit of a	–0.8999 ± 3.9496
95% Confidence interval of a	$-4.8495 < a < 3.0497$
$x_{(LOD)}$ (µg/L)	12
$x_{(LOQ)}$ (µg/L)	40
R_t (min)	16.6

Further discussion

Existing methods (7–10) for the quantitation of MFA were initially investigated. Using the method of Meyer and Grobbelaar (7) specifically, satisfactory separation could not be achieved, and poor recoveries were experienced. The sensitivity of MFA by UV detection at 254 nm was not sufficient for reliable reporting. Perry (8) attained a sensitive determination of MFA using suppressed ion chromatography; however, this equipment was not available in our laboratory. Plant and rumen extract contain high levels of formic and acetic acid and other volatile fatty acids. These acids were found to interfere with low-level (< 100 µg/mL) determinations of MFA (8). By using the extraction method described in this study, the successful separation of formate and acetate from monofluoroacetic acid was achieved, even at low levels (> 40 µg/mL). Derivatization of MFA was not necessary, and the described method required no organic clean up or organic phase separation and evaporation.

The sample clean up procedure was far less time-consuming than any other method previously described (7–10). The sample handling was minimal; therefore, the laboratory error would be far less than for a sample that requires several extraction and clean up procedures. Due to volatility, monofluoroacetic acid is lost during the evaporation process at high temperature (> 90°C) and is degraded to glycolic acid in solution at high pH (7); therefore, a procedure that requires extraction with organic solvents and then evaporation of these solvents to concentrate monofluoroacetic acid should be avoided. Although SFA was in its salt form (stable form), at a high pH value, it was still possible that it would degrade to glycolic acid. Even when MFA was extracted using a steam bath, severe losses of MFA occurred (7).

Under the optimized conditions adopted, the acids of interest (i.e., acetic, propionic, formic, and fluoroacetic acid) were extracted and separated satisfactorily. These interfering acids were eluted after MFA or SFA. Other unidentified components were separated without coeluting with MFA or causing any interferences.

Another advantage of low-pH extraction was the fact that protein solubility increases as pH decreases, which means that there is no build-up of protein on the analytical column, which in turn will prolong the life of the column. A major reason for the simplicity of this method was that extraction was carried out in an aqueous medium. Most other extraction procedures of MFA were carried out in organic solvents involving additional time-consuming solvent extraction, clean up, evaporation, and derivatization.

Consequently, under the conditions described in this method, large numbers of samples can be analyzed in a relatively short period of time (less than 60 min per sample). This makes the technique useful for epidemiological studies, because it is cost effective in terms of equipment, analysis time, and manpower.

Conclusion

The method described combines simplicity and minimum sample preparation with adequate speed and precision for the simultaneous analysis of MFA in the presence of other volatile fatty acids in various different matrices. This HPLC procedure was well suited to analyze organic acids in plant material, rumen contents, and liver samples based on the following: short preparation and analysis time, a linear calibration curve for the aqueous standards over a broad concentration range, recoveries of over 90% for MFA in all three matrices (only one additional extraction was needed to obtain more than 98% of the MFA present), excellent sensitivity (as manifested by the low LOD and LOQ values), and good, accurate (as given by the recoveries), precise, and specific repeatability.

No direct interferences were found, and the RSD for all recovery determinations was less than 1% (Table II). This was also the case when the concentration of injected SFA exceeded 5.00 µg.

The overriding factor in this study was that the pK_a values of formic, propionic, acetic, and fluoroacetic acid were sufficiently different to allow separation. The stronger acid was the first to be eluted from the column when all other variables were kept constant (i.e., flow rate, eluent composition, column temperature, and acidity of extraction fluid). The eluent pH was of utmost importance for the separation of monofluoroacetic acid from acetic, formic, and propionic acids. Although the acids were all present as ions, separation was achieved due to the influence of the particle size of the resin and its degree of cross-linking.

Investigations into the long-term stability of MFA samples is continuing, as is an investigation of the seasonal influences on the concentration of MFA in *D. cymosum*.

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