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Note

Liquid chromatography of organic acids in silage extracts using dual detection

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The determination of organic acids produced by the microbial fermentation of silages is one of the principal analytical techniques for the evaluation of their quality. Unlike other methods used so far (fractional distillation, gas–liquid chromatography), high-performance liquid chromatography (HPLC) theoretically offers the advantage of simultaneous determination of volatile and non-volatile organic acids. However, anion-exchange chromatography[1,2], which has been shown to be an effective tool for the analysis of many acids of plant origin, does not assure sufficient separation of lactic and acetic acid in a reasonable analysis time. The ability of other HPLC methods (reversed-phase chromatography[3,4] and ion-exclusion chromatography[4–6]) to resolve all expected organic acid constituents of silage extracts has been not demonstrated until now.

The aim of this study was therefore to find suitable separation and detection conditions for a simple liquid chromatographic determination of silage organic acids, which does not necessitate the use of expensive high-pressure equipment.

EXPERIMENTAL

The set-up for liquid chromatography of organic acids in silage extracts consisted of a MC-300 medium-pressure reciprocating pump (Mikrotechna, Prague, Czechoslovakia) delivering deaerated mobile phase via a pressure gauge (4 MPa) and a six-port sample-application valve (Mikrotechna) to a thermostatted stainless-steel column. The effluent from the column was led first to a RIDK 101 refractometric detector (Laboratory Instruments Works, Prague, Czechoslovakia) and then to a variable-wavelength UV detector UVM-4 (Development Workshops of the Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Chromatograms were recorded using a Model TZ 4200 dual-channel line recorder (Laboratory Instruments Works). One selected recorder output was integrated using a Minigrator integrator (Spectra Physics, Santa Clara, CA, U.S.A.).

A reversed-phase column (25 × 0.4 cm) packed with Separon Si C 18, 10 μm was purchased from Laboratory Instruments Works. Strongly acidic cation-exchange polystyrene resins, Ostion LG KS 0800 (10-μm particles 8% cross-linking) and an experimental batch of 4% cross-linked resin (17-μm particles) were obtained from

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United Metallurgical and Chemical Works (Ustí nad Labem, Czechoslovakia). Ion exchangers were converted into the hydrogen form with 2 *M* HCl and packed into 25 × 0.8 cm columns as 50% (v/v) water slurries using packing pressures of 20 MPa (8% cross-linked resin) or 2.5 MPa for the less cross-linked one.

2% Sodium dihydrogen phosphate adjusted to pH 2.4 with orthophosphoric acid was used as a mobile phase for the reversed-phase method, whereas for the ion-exclusion chromatography 0.35% sulphuric acid was used as the sole eluent. Solutions were degassed *in vacuo* prior to use.

A calibration mixtue of pure organic acids (butyric, isobutyric, propionic, valeric) and their salts (sodium acetate and formate, zinc lactate) in water was used for the external standardization.

Finally cut samples of silages prepared from various plants (alfalfa, maize, beet cuttings, etc.) were extracted overnight with water. Extracts were centrifuged (and diluted if necessary) before chromatography.

RESULTS AND DISCUSSION

Three available column packings have been compared as regards the separation of the three most important organic acid constituents of silages —lactic, acetic and butyric acid. The elution order was identical on all three packings, lactic acid always being eluted first, butyric acid last. However, the reversed-phase column did not yield a complete resolution of lactic and acetic acid, whereas butyric acid, being very strongly retained, was eluted as a broad unsymmetrical peak, characterized by a long retention time (36 min at 0.6 ml/min). The 25 × 0.8 cm column packed with 4% cross-linked cation exchanger gave a good resolution of all three compounds in a reasonable time (t_R for butyric acid was 20 min at 0.6 ml/min), but this resin bed was found to be too compressible for long-term use.

The column packed with Ostion LG 0800 cation-exchange resin did not suffer from either of these disadvantages and was therefore used for further experiments. An examination of temperature effects on the separation of the above organic acids revealed only slight decreases in elution times with increasing column temperature. Although acetic acid was found to be most temperature-sensitive, its complete separation from lactic acid was maintained over the whole temperature range (30–60°C) tested. The gain in column efficiency achieved at elevated temperatures was considered together with the negative impact of high temperatures on detector noise, and 50°C was been chosen as a compromise.

On the basis of our earlier experience with HPLC analysis of organic acids in beverages[2] and samples from the sugar industry[4], we expected UV spectrophotometry to be the most suitable detection method for silage extracts. The optimum wavelength was chosen from the point of view of the lactic acid determination (see Fig. 1).

Optimum analytical conditions for the separation of organic acids in silage samples were as follows: column packing and dimensions, Ostion LG KS 0800 (H^+), 10-μm particles, 25 × 0.8 cm; column temperature, 50°C; eluent flow-rate, 0.6 ml/min; mobile phase composition, 0.35% sulphuric acid; detection wavelength, 220 nm. Using these chromatographic conditions, a complete separation of seven organic acids, which may be present in silage samples as natural constituents or as added

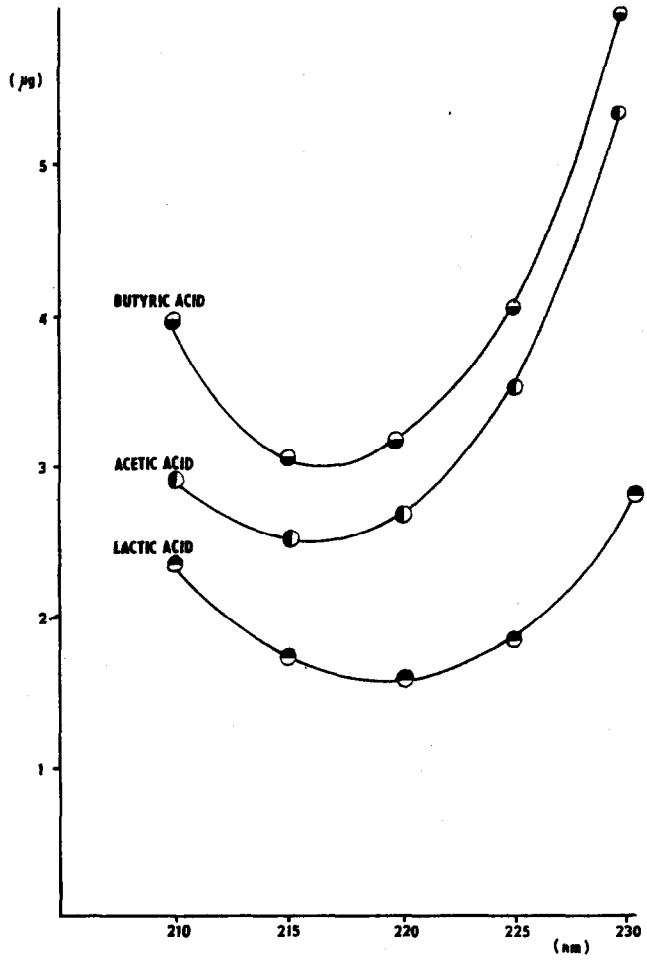

Fig. 1. Spectral optimization of the UV detection for selected organic acids. Dependence of the detection limit (peak height equivalent to three times the noise level) on the wavelength of detection. Chromatographic conditions: 25 × 0.8 cm stainless-steel column packed with Ostion LG KS 0800 (10 μm, H^+); eluent, 0.35% H_2SO_4; column temperature, 50°C; detector, UVM-4.

preservatives, was achieved in 26 min at a column pressure of 2.5 MPa (see Fig. 2). Most common organic acids found in non-fermented plant material (compounds 9–11 in Table I) do not interfere with the determination of acids, whose separation is shown in Fig. 2. The only exception is the co-elution of fumaric and acetic acid. However, simultaneous detection with an UV spectrophotometer and differential refractometer allows one to distinguish these two compounds on the basis of their different RI/UV response ratios (Table I). The values presented in Table I are given relative to lactic acid in order to compensate for the effects of detector construction and peak dispersion, and may be generally employed as a useful identification tool in the analysis of real silage samples.

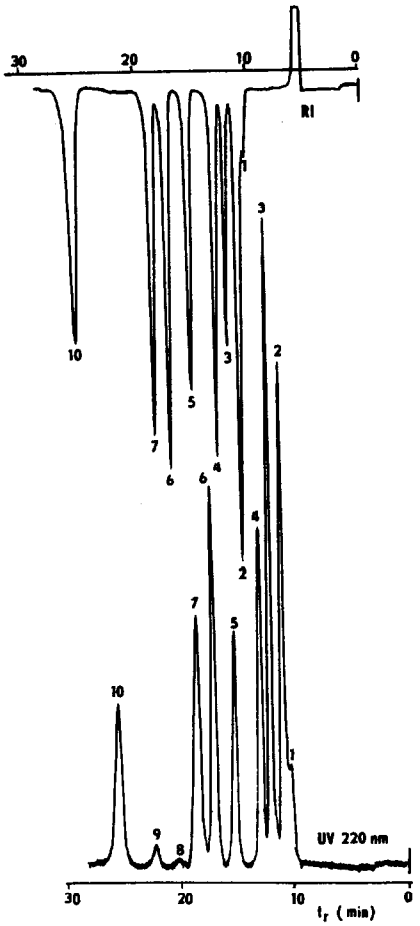

Fig. 2. Separation of a standard mixture of organic acids by ion-exclusion chromatography. Chromatographic conditions: as in Fig. 1, flow-rate 0.625 ml/min. Detection conditions: upper trace, differential refractometer RIDK 101, sensitivity range × 4; lower trace, UV photometer UVM-4 at 220 nm, 0.25 a.u.f.s. Sample: 100 μl of a standard solution containing 98 μg of lactic acid (peak 2), 84 μg of formic acid (3), 92 μg of acetic acid (4), 71 μg of propionic acid (5), 91 μg of isobutyric acid (6), 88 μg of butyric acid (7) and 78 μg of valeric acid (10). Peaks 1, 8 and 9 were not identified.

A typical chromatogram of a maize silage extract is presented in Fig. 3. In contrast to our previous experience with other types of samples, the UV spectrophotometer was found to give more complicated chromatograms (consisting mostly of 20–30 peaks) than the differential refractometer. As with most other silage samples, the major organic acid peaks in Fig. 3 were tentatively identified as lactic and acetic acid and the identification was confirmed by comparing their RI/UV ratios with those determined for pure acids. No significant differences at a 95% probability level were observed between the quantitative results obtained by RI and UV detectors for these two acids. However, the quantification of minor organic acid peaks was from time to time less satisfactorily. For example, the peak tentatively identified in Fig. 3 as propionic acid on the basis of its t_R has a significantly lower RI/UV response ratio

TABLE I

CHROMATOGRAPHIC DATA FOR SELECTED ORGANIC ACIDS

Chromatographic conditions: as given in Experimental for the Ostion LG KS 0800 column. Detection conditions: RI detector RIDK 101, sensitivity range × 4; UV detector UVM-4, λ = 220 nm, 0.5 a.u.f.s.

No.	*Compound*	*k'*	*Number of plates, n*	*RI/UV response ratio relative to lactic acid*
1	Lactic acid	1.02	6400	1.00
2	Formic acid	1.16	8600	0.422
3	Acetic acid	1.35	8200	1.15
4	Propionic acid	1.75	10,800	1.36
5	Isobutyric acid	2.11	12,100	1.05
6	Butyric acid	2.36	8600	1.45
7	Valeric acid	3.64	8500	1.65
8	Citric acid	0.23	*	0.781
9	Malic acid	0.50	*	1.10
10	Succinic acid	0.82	*	1.75
11	Fumaric acid	1.35	*	0.014

* Not calculated.

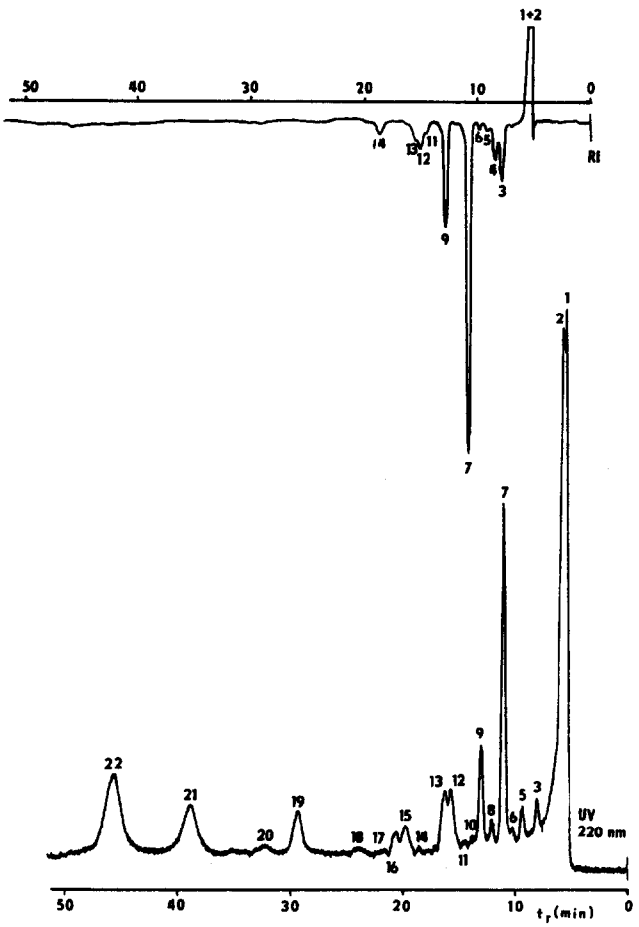

Fig. 3. Separation of a silage extract. Conditions as in Fig. 2. Sample: 100 μl of the centrifuged, water-diluted maize silage extract, corresponding to 2.8 mg of silage. Peaks: 7 = lactic acid; 9 = acetic acid; 12 = propionic acid; 14 = butyric acid; remainder were not identified.

than does the peak of the pure compound, indicating the presence of an UV-absorbing contaminant. This unknown compound was present in all silage samples.

The method presented enables a remarkably long column lifetime despite the very simple sample preparation. More than 200 analyses were made without any significant change in column performance. It is not advisable to use the cation-exchange column for the analysis of silage samples treated with chemical clearing agents, *e.g.*, calcium hydroxide–copper sulphate mixtures, unless the cations present in the supernatant are first removed on another cation-exchange column[7]. Moreover, these extra sample pretreatment steps significantly dilute the sample without affecting the unidentified peaks appearing on chromatograms. Our attention is therefore now being turned to on-line sample pretreatment and/or preconcentration methods, which could improve the analysis of acids present in silages in low amounts.

The described method can easily be accelerated by using more advanced high-pressure equipment, since the column packing is pressure-stable up to 20 MPa.

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