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Determination of fermenting acids in silage by capillary electrophoresis

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Abstract

The analysis of fermenting acids in silage using capillary zone electrophoresis is described. Different separation media including aqueous as well as non-aqueous carrier electrolytes have been tested in combination with direct and indirect UV detection of the analytes. Silage samples obtained from different raw materials and produced by various methods of fermentation have been investigated. UV-transparent carrier electrolytes suitable for direct UV detection with a pH value close to the pK_a of the investigated fermenting acids proved to be most effective for the analysis of silage samples.

Keywords: Carboxylic acids

1. Introduction

For hundreds of years, silage prepared by fermentation of green fodder has been used, in addition to hay as fodder for feeding cattle. Nowadays, the preparation of silage has become as important as the use of hay, especially because of the much lower loss of nutrients during this conservation process. Additionally, the nutrient content of silage may be maximized by optimization of the selection and processing of the raw material as well as the method of fermentation. One of the most important prerequisites for the improvement of the fermentation process is reliable chemical data provided by an appropriate analytical method. The different contents of the fermenting acids, acetic, lactic and butyric acid, is one essential criterion for a proper judgment of silage. Furthermore, these analytical data are also of vital importance for current investigations dealing with possibilities of industrial lactic acid production from liquors obtained from pressed silages.

Usually the quality of silage is evaluated by the so-called Flieg points [1]. This system depends on the content of each acid in relation to the sum of the analyzed fermenting acids, the pH and the dry mass of the sample. The result is expressed in points, derived from a non-linear scale, which are summed up. A high share of lactic acid increases the number of points, whereas high shares of acetic acid and butyric acid decrease the number of points. The higher the number of Flieg points, the better the quality of the silage. A maximum of 100 points can be reached. A method still used to determine the amount of these fermenting acids is based on steam distillation of the sample, followed by titration of the acids. One should be aware that the accuracy of this

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analytical procedure is influenced by the presence of soluble carbohydrates as well as other organic acids, such as propionic or formic acid. Nowadays, most laboratories investigating such fermentation products use gas, liquid or thin-layer chromatographic methods for the determination of the fermenting acids [2–5]. Nevertheless, the sample pretreatment necessary for these analytical techniques still remains time-consuming and tedious. It seems that there has been little progress in the development of efficient analytical methods for silage samples over the last few years.

Currently, capillary zone electrophoresis (CZE) is becoming more and more important as an analytical technique for the separation of low-molecular-mass ionic solutes, e.g., carboxylic acids. It appears to be an interesting technique for the investigation of fermenting acids in silage with promising features such as high separation efficiency, short analysis time and separation selectivity that is completely different from other methods, e.g., gas chromatography (GC) or ion chromatography. CZE separation procedures for a variety of organic acids have already been published [6–13]. Usually, low-molecular-mass carboxylic acids are separated in a co-electroosmotic mode with indirect UV detection. For this purpose, the direction of the electroosmotic flow has to be reversed and directed to the anode by the addition of hydrophobic quaternary ammonium ions to the carrier electrolyte [14,15]. For indirect UV detection, electrolytes like benzoate and phthalate [6,9], hydroxybenzoate and sorbate [11,16], pyromellitate [15], naphthalene–dicarboxylic acid [12], naphthalenesulfonates [11] and *p*-aminobenzoate [13] have been employed. Using an appropriate buffer, direct UV detection is feasible for aromatic carboxylic acids [7] and even for aliphatic carboxylic acids at 185 nm, as published recently by Shirao et al. [9]. Regarding aspects such as the stability of the baseline, direct UV detection seems to be more suitable for the analysis of these solutes.

Recently, even non-aqueous carrier electrolytes have been reported for the separation of small ionic solutes, e.g., carboxylic acids, by CZE with indirect UV detection [17–22]. Non-aqueous buffer systems offer unique selectivity in CZE analysis, so that the information from non-aqueous electrolytes can be regarded as complementary to the information from aqueous electrolytes [23].

Although a growing number of applications of CZE for the determination of carboxylic acids can be found in the literature, the potential of CZE for the analysis of carboxylic acids in fermentation products, like silage, has not yet been investigated. The main goal of the work presented in this paper was the separation of fermenting acids that are relevant for the evaluation and specification of the quality of silage. Different approaches, including direct and indirect UV detection, as well as a number of carrier electrolytes, have been investigated for the determination of the analytes mentioned above.

2. Experimental

2.1. Instrumentation

The CZE instrument employed was a Quanta 4000 (Waters, Milford, MA, USA) equipped with a negative power supply and a fixed-wavelength detector (mercury lamp) connected to a HP 3359 data acquisition system (Hewlett-Packard, Palo Alto, CA, USA). Separations were carried out using fused-silica capillaries obtained from Polymicro Technologies (Phoenix, AZ, USA), with effective lengths of between 50 and 65 cm, inner diameters of 75 μm and a detection window at a position that was 8 cm from the end. Injection was performed hydrostatically at the cathodic side by elevating the sample at 10 cm for a specified time. Direct UV detection at 185 nm, as well as indirect UV detection at 254 nm, was used.

2.2. Carrier electrolytes

The carrier electrolytes used for the experiments with direct UV detection consisted of boric acid or NaH_2PO_4 of varying concentrations, each containing 0.5 m*M* tetradecyltrimethylammonium bromide (TTAB). The pH value of these solutions was adjusted by the addition of 1 *M* NaOH. Experiments with indirect UV detection were carried out using 5 m*M* sorbic acid containing 0.5 m*M* TTAB. For the preparation of the non-aqueous carrier electrolytes, trimellitic acid was dissolved in 10 ml of 1 *M* NaOH, *n*-butylamine was added and the mixture was diluted to 1000 ml with methanol to give a solution containing 5 m*M* trimellitic acid and 10 m*M* *n*-

butylamine. Analytical-grade reagents and doubly distilled water were used throughout these experiments.

2.3. Carboxylic acids

The following carboxylic acids of the highest purity available were used: malonic acid, lactic acid, formic acid, acetic acid, propionic acid and butyric acid (all obtained from Merck, Darmstadt, Germany).

2.4. Silage samples and sample pretreatment

Silage samples were stored at −18°C. Determination of the dry mass was performed by treating the silage at 105°C until the mass remained constant. Further sample pretreatment was carried out according to a previously published procedure [24]. For extraction, the samples were cut into small pieces using a knife. In a household mixer, a 100-g portion was blended with 1 l of water and homogenized. Afterwards, the sample was transferred to a beaker and left for 5 h, with the pulp being mixed from time to time. The mixture was diluted with water, filtered through a 0.45-μm disposable filter cartridge and injected into the instrument.

Press-liquors were obtained by passing grass as well as corn silage samples through a hand-driven press. Prior to the CZE analysis, the liquid was diluted with water by a factor of 500 and passed through a 0.45-μm disposable filter cartridge.

3. Results and discussion

3.1. Experiments using indirect UV detection

3.1.1. Experiments with aqueous carrier electrolytes

As already mentioned, most papers dealing with the separation of carboxylic acids by CZE describe methods using indirect UV detection. In a series of experiments, we investigated the applicability of 10 m*M* phthalate electrolytes containing 0.5 m*M* TTAB adjusted to pH values between 5.6 and 6.3 with indirect UV detection at 254 nm for the analysis of fermenting acids in silage. Although this buffer system allowed the separation of standard mixtures of fermenting acids, real samples could not be analysed because of interfering matrix effects. Changing to a carrier electrolyte containing 5 m*M* sorbate and 0.5 m*M* TTAB, adjusted to pH 6 by the addition of 1 *M* NaOH, gave much better results. As can be seen in Fig. 1, samples of silage produced from corn yielded acceptable electropherograms with only a few peaks that were caused by the matrix, which allowed quantitation of the fermenting acids present in this sample. Nevertheless, Fig. 1 also shows that this method is not suitable for the analysis

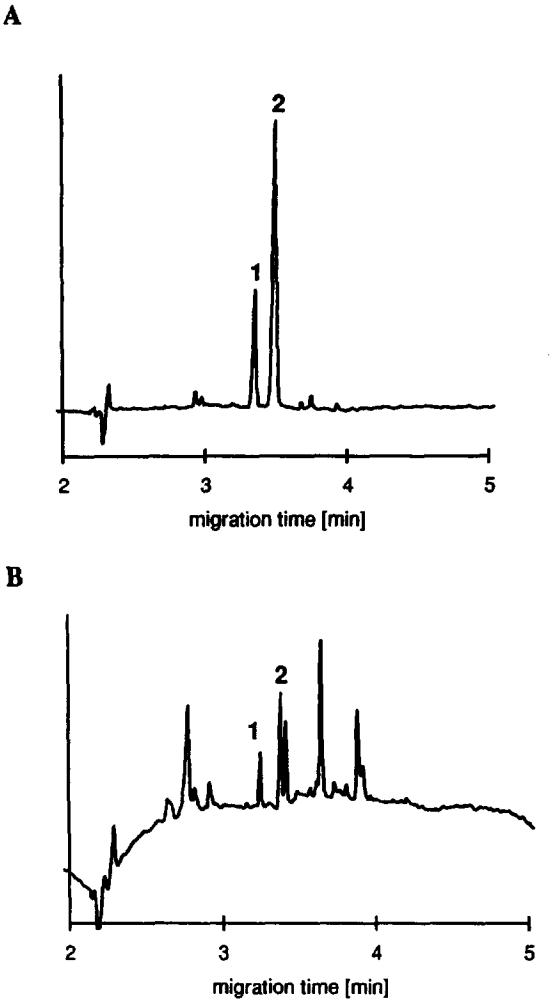

Fig. 1. Electropherograms of a corn silage sample (A) and a grass silage sample (B). Carrier electrolyte: 5 m*M* sorbic acid containing 0.5 m*M* TTAB, pH 6.0. Applied voltage: −25 kV. Injection time: 10 s. Detection: indirect UV at 254 nm. Capillary: 65 cm×75 μm I.D. Indirect UV detection at 254 nm. Peaks: 1=acetic acid; 2=lactic acid.

of grass silages, which represents a matrix with various interfering peaks in the indirect detection mode. In particular, the peak for butyric acid, an important solute in the analysis of silages, could not be clearly assigned. Similar difficulties were encountered in the analysis of press-liquors.

3.1.2. Experiments with non-aqueous carrier electrolytes

In the analysis of complex matrices like silage, cross-validation of peaks using carrier electrolytes with different selectivities seems to be useful. This can be achieved by the use of both aqueous buffer systems and non-aqueous buffers as carrier electrolytes. The non-aqueous buffer employed in these experiments contained 5 m*M* trimellitic acid and 10 m*M* *n*-butylamine dissolved in methanol containing 1% of 1 *M* NaOH. A similar composition containing phthalic acid instead of trimellitic acid, published by Salimi-Moosavi and Cassidy [18] was not suitable for the separation problem investigated. Although the migration order of the selected monocarboxylic acids was identical compared with the aqueous carrier electrolytes employed in this study, this non-aqueous buffer system showed different selectivity for some matrix ingredients. In this way, the reliability of the discrimination between peaks obtained for the selected analytes and peaks originating from matrix substances could be improved. Nevertheless, an exact quantitation, especially for minor components like butyric acid, was not possible. For this reason, no further investigations were performed using this method.

3.2. Experiments using direct UV detection

According to recently published results [25] dealing with the separation of dicarboxylic acids by CZE, borate buffers with different molarity and a pH of 8 can be employed as carrier electrolytes that are compatible with direct UV detection at 185 nm for the analytes of interest in the present study. To reverse the electroosmotic flow and to establish co-electroosmotic separation conditions, 0.5 m*M* TTAB was added to the buffer solution. Starting with a carrier electrolyte of 50 m*M* sodium tetraborate adjusted to pH 8, all analytes could be separated, except for the pair propionic acid–lactic acid. The migration order was acetic acid, propionic acid, lactic acid and butyric acid. Further investigations on the optimization of the separation revealed that the resolution between propionic acid and lactic acid can be improved considerably if the concentration of the carrier electrolyte is increased. In this case, the peak of lactic acid is shifted towards a longer migration time. Using a 200-m*M* borate electrolyte, lactic acid is found exactly in the middle between propionic acid and butyric acid. Specific interactions between boric acid and borate with the analytes (as in the case of diols) seem to be unlikely for the carboxylic acids investigated in this study. Therefore, the changes in separation selectivity can be regarded as a sole effect of the concentration of the carrier electrolyte. This was confirmed by experiments using phosphate buffers of varying concentrations, but with a pH of 8, which lead to results similar to those obtained with borate buffers. These results agree with investigations into the influence of ionic strength on selectivity for inorganic anions [14].

Unfortunately, the high concentration of the carrier electrolyte necessary for a satisfactory separation leads to relatively high currents and increased baseline noise. Therefore, a series of experiments was carried out using buffer systems with a pH value near the pK_a of the carboxylic acids under investigation. In this case, the different degrees of dissociation at a certain pH is the predominant factor for the separation of different carboxylic acids. Best results were obtained with a 50-m*M* NaH_2PO_4 buffer (pH 5.6) containing 0.5 m*M* TTAB and using direct UV detection at 185 nm. Under these conditions, the order of migration was changed compared to that obtained using a borate buffer at pH 8. The migration order was acetic acid, lactic acid, propionic acid and butyric acid. This carrier electrolyte provided a shorter analysis time as well as sufficient resolution between all carboxylic acids of interest. It was used throughout the subsequent analytical work on silage samples.

3.3. Quantitation of fermenting acids in silage samples using direct UV detection

Because of the promising results obtained with the separation conditions mentioned above, a 50-m*M* NaH_2PO_4 buffer (pH 5.6) containing 0.5 m*M* TTAB

combined with direct UV detection at 185 nm was chosen for the analysis of the silage samples. Both the external calibration and the standard addition method were evaluated for quantitation of the fermenting acids. No statistically significant difference could be found between the different procedures. So, further experiments were carried out using an external calibration method for quantitation.

Fig. 2 shows the electropherograms of the same silage samples as already depicted in Fig. 1, but this time obtained using direct UV detection. As can be seen from this figure, the extent of matrix peaks strongly depends on the raw materials of the silage. Nevertheless, with direct UV detection at 185 nm, the content of fermenting acids could be determined without major problems, in all silage samples investigated so far. For a standard mixture, the limit of detection was 4 ppm for lactic acid, 3 ppm for butyric acid and 2 ppm for stronger absorbing compounds like acetic acid.

Ten silage samples made from different raw materials and/or by various methods of fermentation have been investigated. In Table 1, dry masses, fermenting acid contents relative to dry mass and the classification of the investigated silage samples by the Flieg-point system are depicted. High quality silages are defined by high lactic acid contents combined with only low amounts of undesirable fermenting acids like butyric acid.

Corn silages like S 2 and S 5 show high lactic acid contents combined with only low amounts of acetic acid and butyric acid. This is caused by their high portion of easily accessible carbohydrates, which leads to a fast fermentation and a fast decrease in pH, preventing the formation of unwanted fermenting acids.

Regarding the investigated grass silages S 3 and S 10, a favourable ratio between lactic acid and the unwanted fermenting acids can be observed. This may be caused by careful selection of the raw material (the carbohydrate content depends on the stage of vegetation) combined with an optimized process of fermentation. On the other hand, S 4, S 7 and S 8 represent silages of poor quality, mainly caused by their high dry mass. Especially in the case of S 7 and S 8, the designation "fermented hay" (50–70% dry mass) fits better than silage. For the correct characterization of the latter two samples, the

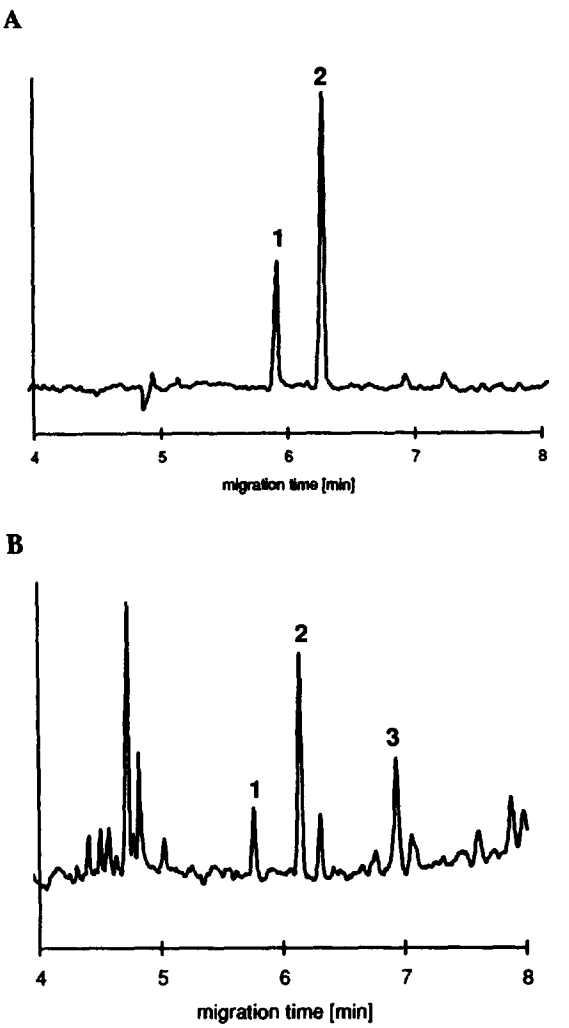

Fig. 2. Electropherograms of a corn silage sample (A) and a grass silage sample (B). Carrier electrolyte: 50 m*M* NaH_2PO_4 containing 0.5 m*M* TTAB, pH 5.6. Applied voltage: −20 kV. Injection time: 20 s. Detection: direct UV at 185 nm. Capillary: 65 cm×75 μm I.D. Peaks: 1=acetic acid; 2=lactic acid; 3=butyric acid.

Flieg-point system is not suitable. The low quality of S 6 and S 9 seems to be caused by a poor fermentation process. As can be seen in the case of S 6, this happens mainly if the dry mass of the silage is too low. Nevertheless, S 1 shows that even silages with a high content of butyric acid may obtain a high number of Flieg-points if they also contain a lot of lactic acid. For the reasons mentioned above, the

Table 1
Dry masses, fermenting acid contents and classification of the investigated silage samples by the Flieg-point system

Sample	Raw material	Dry mass (%)	Fermenting acid content (%)			Flieg-points			
			Acetic	Lactic	Butyric	Acetic	Lactic	Butyric	Σ
S 1	Grass	37	1.0	7.3	2.0	20	28	2	50
S 2	Corn	26	1.9	11.7	n.d.	20	30	50	100
S 3	Grass	30	0.4	10.8	0.4	20	30	20	70
S 4	Grass	51	0.2	2.1	0.7	20	24	0	44
S 5	Corn	30	1.3	8.2	n.d.	20	30	50	100
S 6	Grass	21	1.4	3.8	2.1	18	14	0	32
S 7	Grass	71	0.1	n.d.	0.1	0	0	0	0
S 8	Grass	65	0.1	n.d.	0.4	18	0	0	18
S 9	Grass	34	1.1	1.3	3.7	20	0	0	20
S 10	Grass	41	1.0	7.6	0.4	20	30	15	65

n.d.=not detected.

importance of revised classification systems is increasing (K.B., unpublished results) .

Press-liquors obtained from both corn silages and grass silages were also analysed using the method presented in this paper. Concerning the selected fermenting acids, the composition of the investigated press-liquors was very similar to the pattern detected in the silage extracts described above. The lactic acid content was found to be 3% for an investigated corn silage and 1% for a grass silage.

In looking for an alternative method to ensure the trueness of the data obtained in this work, it should be mentioned that, to the best of our knowledge, no standard method dealing with the analysis of fermenting acids in silage exists. For that reason, we compared the data obtained using the CZE method published in this work with results from a more conventional method based on GC. A correlation coefficient of 0.93 was achieved for a set of eighteen data points, showing an acceptable comparability of the two methods.

4. Conclusions

The results obtained in this work indicate that capillary electrophoresis is an attractive technique for the analysis of fermenting acids in silage. Short analysis times in comparison with the well-established techniques of gas or liquid chromatography are one of the main advantages of this analytical method. Additionally, it should be mentioned that the analytical method described in this paper does not depend on the use of chromatographic columns, which, in many cases, deteriorate following use with samples containing problematic matrices like silage. Experiments using both direct UV detection at 185 nm and indirect UV detection at 254 nm lead to the conclusion that direct UV detection is more suitable for the determination of these analytes in complex matrices such as silage. Investigating the content of fermenting acids in silages made from different raw materials allowed us to judge these samples according to the usual standards of assessment.

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References

[1] E. Zimmer, Wirtschaftseigenes Futter, 12 (1966) 299.
[2] V. Mueller, N. Peterhaensel and J. von Lengerken, Agrobiol. Res., 46 (1993) 340.
[3] C. Szathmary, Magy. Allatorv. Lapja, 35 (1980) 522.
[4] P. Daniel, Wirtschaftseigenes Futter, 17 (1971) 234.
[5] P. Daniel, Landwirt. Forsch., 23 (1970) 172.
[6] J. Romano, P. Jandik, W.R. Jones and P.E. Jackson, J. Chromatogr., 546 (1991) 411.

[7] W.C. Brumley and C.M. Brownrigg, J. Chromatogr., 646 (1993) 377.
[8] S.P.D. Lalljie, J. Vindevogel and P. Sandra, J. Chromatogr. A, 652 (1993) 563.
[9] M. Shirao, R. Furuta, S. Suzuki, H. Nakazawa, S. Fujita and T. Maruyama, J. Chromatogr. A, 680 (1994) 247.
[10] O. Devevre, D.P. Putra, B. Botton and J. Garbaye, J. Chromatogr. A, 679 (1994) 349.
[11] S.A. Shamsi and N.D. Danielson, Anal. Chem., 66 (1994) 3757.
[12] E. Dabek-Zlotorzynska and J.F. Dlouhy, J. Chromatogr. A, 685 (1994) 145.
[13] A. Röder and K. Bächmann, J. Chromatogr. A, 689 (1995) 305.
[14] W.R. Jones and P. Jandik, J. Chromatogr., 546 (1991) 445.
[15] M.P. Harrold, M.J. Wojtusik, J. Riviello and P. Henson, J. Chromatogr., 640 (1993) 463.
[16] W.R. Jones, J. Chromatogr., 640 (1993) 387.
[17] R.S. Sahota and M.G. Khaledi, Anal. Chem., 66 (1994) 1141.
[18] H. Salimi-Moosavi and R.M. Cassidy, Anal. Chem., 67 (1995) 1067.
[19] M. Jansson and J. Roeraade, Chromatographia, 40 (1995) 163.
[20] T. Okada, J.Chromatogr. A, 695 (1995) 309.
[21] M. Chiari and E. Kenndler, J. Chromatogr. A, 716 (1995) 303.
[22] H. Salimi-Moosavi and R.M. Cassidy, Anal. Chem., 68 (1996) 293.
[23] S.H. Hansen, J. Tørnelund and I. Bjørnsdottir, Trends Anal. Chem., 15 (1996) 175.
[24] F. Eibensteiner, Ph.D. Thesis, University of Linz, Austria, 1994, p. 49.
[25] W. Buchberger and K. Winna, J. Chromatogr. A, 739 (1996) 389.