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Determination of halofuginone in feedstuffs by the combination of capillary isotachophoresis and capillary zone electrophoresis in a column-switching system

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ABSTRACT

A method has been developed for the determination of the coccidiocidic drug halofuginone in feedstuff concentrates which is based on the combination of capillary isotachophoresis and capillary zone electrophoresis in the column-switching mode. The high load capacity of the isotachophoretic step and high sensitivity of the zone electrophoretic step enabled analysis of up to 25 μl of sample solution containing as little as 10^{-8} *M* halofuginone with excellent reproducibility (R.S.D. about 1%). Attention was paid to the possibility of the existence of transient local isotachophoresis in the zone electrophoretic step, and experimental and theoretical methods of revealing zones migrating isotachophoretically in the background electrolyte were shown.

INTRODUCTION

Halofuginone (HFG), DL-*trans*-7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidyl)-2-oxopropyl]-4-[3H]quinazoline (Fig. 1), is included in the category of full broad-spectrum coccidiocides [1] that is added together with minerals, vitamins, amino acids, protectives, stimulants and antioxidants to industrially prepared feedstuffs for poultry. HFG levels in feedstuffs must not exceed the authorized limits, and analytical control is of great importance in order to prevent economic losses due to over- or under-dosage. For the determination of HFG, spectrophotometric methods [2], gas chromatography [3–5] and column liquid chromatography [6] were used, giving results as discussed in the survey [6]. As HFG can easily be protonated on forming cations in aqueous solution, capillary electrophoresis offering high speed, high sensitivity and

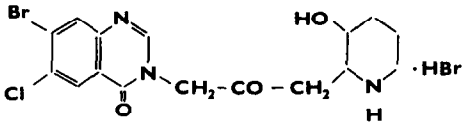

Fig. 1. Structure of halofuginone.

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simple operation may be very useful in its detection [7–10]. Recently, it has been shown that the combination of capillary isotachophoresis (CITP) and capillary zone electrophoresis (CZE) [11,12] offers the advantages of both methods. Isotachophoresis (ITP) offers the ability to inject large amounts of a sample, *e.g.* 30 μl, and thus to analyse trace components below the 10^{-6} *M* level. Column-switching technique offers the ability to cut-off bulk components. Finally, the CZE step offers high resolution and aids the identification of sample components using migration times.

In this paper we show that the combination of CITP and CZE performed using commercial column-switching instrumentation for ITP may be routinely used for analyses of additives in feedstuffs for poultry. Further, we show that the process of switching from ITP to CZE, due to the change of the electrolyte system, is a transient one and may be accompanied by a local existence zones showing self-sharpening boundaries.

EXPERIMENTAL

Instrumentation

A CS isotachophoretical analyzer (ÚRVJT, Spišská Nová Ves, Czechoslovakia) equipped with a column-switching system was used. Pre-separation and analytical capillaries (0.8 and 0.3 mm I.D., respectively) are made of polytetrafluorinated ethylene (PTFE). The distance between the injection port and the conductivity detector is 17 cm. The analytical capillary is equipped with conductivity and UV (254 nm) detectors which are positioned at 17 and 13.5 cm from the starting and bifurcation points, respectively. Solution pH was measured with a Model MS-20 ion activity meter (Laboratorní přístroje, Prague, Czechoslovakia) with glass and silver chloride electrodes.

Absorption spectrum of HFG was measured with a Jasco 875-UV detector.

Chemicals

2-Morpholinoethanesulfonic acid (MES) p.a. and Triton X-100 were from Fluka (Buchs, Switzerland). Other chemicals of analytical grade were from Lachema (Brno, Czechoslovakia). Distilled water was deionized with a mixed-bed ion exchanger Ostion AD and KS (Spolchemie, Ústí n.L., Czechoslovakia). Halofuginone bromohydrate was obtained from Roussel UCLAF (Romainville, France). Vitamin B_1 was a product of Spofa (Prague, Czechoslovakia).

Working procedures

Extraction of halofuginone from feedstuffs. About 10 g of the concentrated mixture of additives are mixed with 5–7 ml of concentrated acetic acid and sonicated for 5 min. Then 50 ml of water are added and the sonication is repeated. Finally, the volume is made up to 100 ml with water. Supernatant obtained after centrifugation or filtration was used as the sample for injection.

ITP or ITP–CZE analysis. The sample was introduced via a sampling valve (24.5 μl) or with a 10-μl Hamilton microsyringe. For ITP measurements, pre-separation and analytical capillaries of the instrument were filled with electrolyte system I and the appropriate current was set (Table I). For the ITP–CZE combination, electrolyte system II and the current given in Table I were used. In this case, current I_1 was

TABLE I

WORKING CONDITIONS

Electrolyte system			Capillary	Current
I	L_1:	10 m*M* KOH + MES[a], pH 6.0, 0.2% Triton X-100	Pre-separation	I_1 = 150 μA
	L_2:	5 m*M* KOH + MES, pH 5.7	Analytical	I_2 = 20 μA
	T:	10 m*M* EACA		
II	L_1:	5 m*M* KOH + MES, pH 5.7, 0.2% Triton X-100	Pre-separation	I_1 = 150 μA
	L_2:	25 m*M* EACA + acetic acid, pH 4.0, 0.2% Triton X-100	Analytical	I_2 = 20 μA
	T:	25 m*M* EACA + acetic acid, pH 4.0, 0.2% Triton X-100		

[a] 2-(N-Morpholino)ethanesulphonic acid.

switched to I_2 exactly 3 s before the bifurcation point. For ITP analysis the timing of column switching is not so critical and a longer period can be chosen.

RESULTS AND DISCUSSION

By orientation experiments, the effective mobility of HFG was found to be $19 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} at pH 4. Hence, electrolyte system I (Table I) was selected where the adjusted pH of the terminating electrolyte was 4.0, ensuring sufficient protonation of HFG and sufficiently low effective mobility of H^+ as a potential terminating electrolyte (TE). To speed up the analysis, ε-aminocaproic acid (EACA) was used as the actual terminator. Analysis of a model mixture of HFG and B_1 carried out by column-switching technique in the ITP + ITP mode is shown in Fig. 2. Obviously,

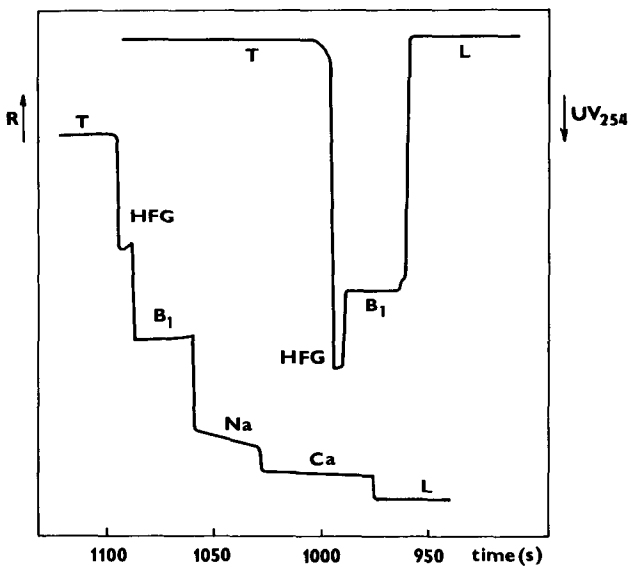

Fig. 2. Isotachophoresis of a model mixture of 2 μl of 2 m*M* HFG and 5 μl of 0.76 m*M* vitamin B_1 in electrolyte system I. R = resistance.

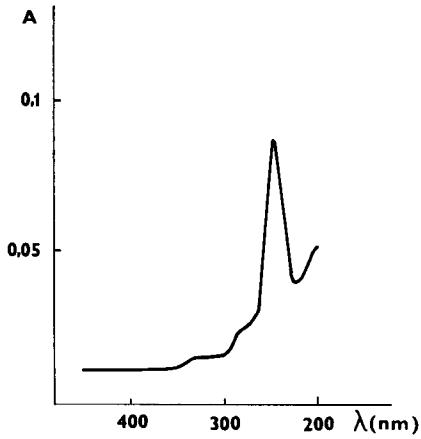

Fig. 3. Absorption spectrum of 0.1 m*M* halofuginone. Optical pathway 10 mm.

good separation of all species was obtained and distinct well developed zones are present in the conductivity detector record. UV detection was carried out at 254 nm which corresponds closely to the absorbance maximum of HFG at 243 nm (see the absorption spectrum in Fig. 3).

Analysis of an extract of the feedstuffs performed in ITP + ITP mode is shown in Fig. 4. Only short zones of HFG and B_1 were obtained, even when full load capacity was utilized. These zones were difficult not only to quantify but also to identify. Even the UV detector gave no unequivocal responses since there were other absorbing compounds in the sample with concentrations and mobilities close to those of HFG.

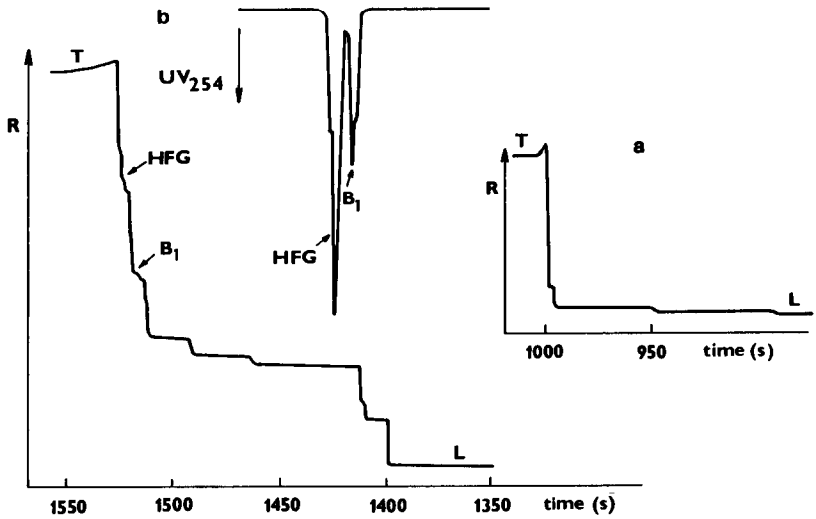

Fig. 4. Isotachophoresis of the extract of feedstuffs supplement. A 10-μl volume of water extract (1.8 g of biofactor supplement per 100 ml of water) was analysed in electrolyte system I. (a) Pre-separation run; (b) analytical run. R = Resistance.

The concentration of HFG in the sample was too low for evaluation from step length in the UV trace, and the presence of other UV absorbing compounds accompanying HFG did not allow use of the spike method for quantification.

The analysis of an extract of the feedstuffs carried out by the combination ITP–CZE is shown in Fig. 5. The first stage was ITP and the electrolyte system was the same as in Fig. 4. The surplus of sodium and calcium was directed towards the auxiliary electrode of the pre-separation capillary, and when the portion of interest appeared close to the bifurcation point the voltage was switched across the analytical capillary. This capillary was filled with TE adjusted to pH 4.0 (electrolyte system II, Table I), which corresponded to the adjusted pH of TE in the ITP mode, and the second stage of analysis was continued in the zone electrophoresis (ZE) mode.

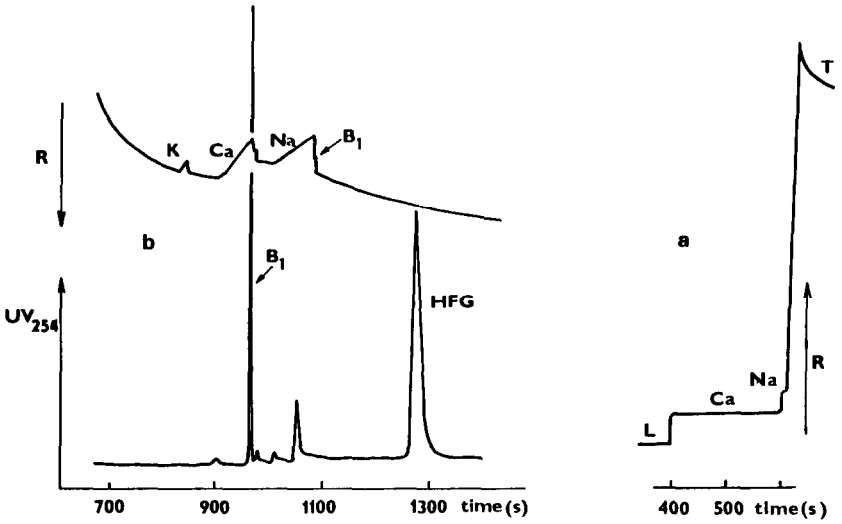

Fig. 5. ITP–ZE of the extract of feedstuffs supplement. A 24.6-μl volume of ten times diluted extract of 1.8 g of biofactor supplement per 100 ml water was analysed in electrolyte system II. (a) Pre-separation run; (b) analytical run. R = Resistance.

A critical point was found to be the timing of the column switching. Three seconds before the sample portion of interest reaches the bifurcation point was found to be optimum. When the time was shorter, a part of the analyte could be lost by migration towards the auxiliary electrode. When a larger portion of the isotachophoretic zones migrates to the analytical capillary, they remain in isotachophoretic migration for a time, and a shorter path is available for ZE separation. Obviously, a universal conductivity detector situated before the bifurcation point is of key importance here.

For the analysis of HFG by the ITP–CZE combination in the electrolyte system II the calibration was evaluated in ZE mode with UV_{254} detection. Linear dependence was found when peak area was plotted *versus* amount of HFG in the range 7.5–480 μg/l (10^{-8}–10^{-6} *M*), and the equation of the calibration line was $y\,(mm^2) = -1.5 + 51.75\,x$ (pmol) with correlation coefficient $r = 0.9999$. Linearity of the plot confirms the

reliability of the procedure. The amount of electricity necessary for the zone to pass the detector was found to be very reproducible, differing by less than 1% for individual runs, which is in good agreement with similar observations [11]. The same holds for the crude extracts of feedstuffs. The results of analyses of HFG in various kinds of feedstuff supplements are given in Table II.

TABLE II

AMOUNTS OF HALOFUGINONE EXTRACTED FROM FEEDSTUFFS AND ANALYSED USING THE ITP–ZE TECHNIQUE

A 24.6-μl volume of the 10–50 times diluted extract was injected and analysed in electrolyte system II.

Sample	Halofuginone content (mg/kg)	Relative standard deviation (n = 3) (%)
Ia	104.9	0.33
Ib	97.5	0.95
Ic	102.0	1.16
II	19.4	2.56

Finally, we discuss certain phenomena that may occur with the ITP–ZE combination and which may be exemplified here on zones of B_1 and HFG.

In general, three types of combination of electrolytes can be used depending on the background electrolyte applied in the second stage. Either leading electrolyte (LE) or TE or some other electrolyte (XE) can serve as background electrolyte (BGE): TE–LE–LE, TE–LE–TE, TE–LE–XE. The combination TE–LE–TE is the simplest as it needs no manipulation with electrolytes during analysis. From the point of electromigration, in all the above cases there exists a possibility of the existence of a transient, local ITP migration during the second stage (ZE) of the analysis [13,14]. Let us examine our cases, namely, the TE–LE–TE scheme. When some compounds are present in the sample having mobility higher than that of the co-ion of the BGE used in the ZE stage of the analysis, they may influence the ZE migration by forming transient local regions. Compounds migrating at the rear boundaries of these high-mobility zones migrate isotachophoretically and show very sharp peaks. Here, this is the case with vitamin B_1 (see Fig. 5), which is present in the extract of feedstuffs. In such a case a very high number of theoretical plates can be adjudged to be in ITP zones migrating apparently in a ZE mode. To give an example we can calculate the maximum plate number (N_{max}) using the relation [15]:

$$N = 0.82\,\bar{u}\,\frac{l}{r}\sqrt[3]{\frac{\lambda}{D^2\,\delta\,\chi\,\bar{u}}}$$

where $\bar{u}$ is the effective mobility, l is the length of the capillary, r is the radius of the capillary, λ is the thermal conductivity of BGE, D is the diffusion coefficient of the substance, δ is the temperature coefficient of the mobility and χ is the specific

conductivity of BGE. This relation includes diffusion and Joule heat as the only sources of zone broadening and does not include any other dispersion processes, *e.g.* injection of the sample. Therefore, using this equation we can calculate the theoretical limit of separation efficiency which is not accessible in practice. For HFG the experimentally found [10] N (11 700) is lower than N_{max} (126 000) confirming ZE migration, while for vitamin B_1 N (264 000) is higher than N_{max} (259 000) and therefore B_1 cannot migrate in the ZE mode.

As can be seen in Fig. 5, conductivity detection reveals the range of local ITP zones. The zone of vitamin B_1 in this system can be found by analysing a higher amount of this compound. The slower migration of HFG (compared to vitamin B_1, the effective mobility of which was found $42.4 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} at pH 4.0) is not influenced, which can also be recognized from the diffuse shape of the zone.

CONCLUSION

The combination of CITP–CZE in column-switching mode is a very promising method for trace analysis of substances in the presence of other micro- and macrocomponents. Even with a commercial apparatus designed for column-switching ITP and equipped with an analytical capillary I.D. about ten times wider than those commonly used in CZE nowadays high sensitivity (10^{-8} M) and reproducibility (R.S.D. 1%) of analyses can be easily reached. The pre-condition for accurate results is the positioning of the detector in the isotachophoretic step that enables exact sampling for the ZE step and having a universal detector in addition to the UV detector in the ZE step to reveal transient isotachophoretic zones in ZE and to achieve high sensitivity of detection.

REFERENCES

1 *Stenorol*, Division Agro-Vétérinaire, Roussel-Uclaf, Paris.
2 A. C. Bratton and E. K. Marshall, Jr., *Biol. Chem.*, 128 (1939) 537.
3 N. T. Crosby and E. Q. Laws, *Analyst*, 89 (1964) 319.
4 J. Pellizarni, *J. Chromatogr.*, 98 (1974) 323.
5 W. Korol, S. Matyka and T. Harenza, *Med. Weter.*, 39 (1983) 303.
6 K. Frgalová, J. Valová and A. Hera, *Biol. Chem. Vet.*, 24 (1988) 407.
7 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis. Theory, Instrumentation and Applications*, Elsevier, Amsterdam, 1976.
8 Z. Deyl (Editor), *Electrophoresis. A Survey of Techniques and Applications. Part A: Techniques*, Elsevier, Amsterdam, 1979.
9 P. Boček, M. Deml, P. Gebauer and V. Dolník, in B. J. Radola (Editor), *Analytical Isotachophoresis*, VCH Verlagsgesellschaft, Weinheim, 1988.
10 F. Foret and P. Boček, in A. Chrambach (Editor), *Advances in Electrophoresis*, Vol. 3, VCH Verlagsgesellschaft, Weinheim, 1989, pp. 272–347.
11 D. Kaniansky and J. Marák, *J. Chromatogr.*, 498 (1990) 191.
12 F. Foret, V. Šustáček and P. Boček, *J. Microcolumn Sep.*, 2 (1990) 299.
13 J. L. Beckers and F. M. Everaerts, *J. Chromatogr.*, 508 (1990) 3.
14 J. L. Beckers and F. M. Everaerts, *J. Chromatogr.*, 508 (1990) 19.
15 F. Foret, M. Deml and P. Boček, *J. Chromatogr.*, 452 (1982) 601.