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CHARACTERIZATION OF CAPILLARY ELECTROPHEROGRAM PROFILES OF MEAT EXTRACTS BY ENTROPY

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Abstract. Meat samples from a local supermarket were subjected to various sample preparation procedures and later analysed using several capillary electrophoresis separation conditions varying buffer pH and additives. Shannon's entropy function was used for quantitative estimation of the complexity and structural richness of the pherograms of the meat extracts, aiming to find out the optimal sample preparation and analysis protocol for meat species identification by capillary electrophoresis. Modification with ethylene glycol decreases the entropy value of pherograms, offering more characteristic patterns than methods where only the pH value of the buffer is altered. Entropy is minimal for pherograms of capillary gel electrophoresis with linear, non-cross-linked gel, showing suitability of this method for profiling different meat extracts.

Key words: meat analysis, capillary electrophoresis, Shannon's entropy.

INTRODUCTION

Addition of fraudulent substituents into a meat product (for example, mixing cheap, low quality meat with a high quality product) has raised interest in looking for sophisticated analytical techniques to detect such forgery and, in general, to characterize meat samples. Capillary electrophoresis (CE) appears to be a promising technique in this respect because of its high efficiency, which enables to complete separation of meat extract constituents, especially proteins, that eventually determine the quality of the meat product. Also, CE is attractive due to its simplicity of the sample preparation and speed and ease of performing measurements compared with other techniques, for example liquid chromatography [1]. Several investigators have demonstrated that the CE technique gives

highly structured pherograms that are characteristic of the particular meat species [2, 3]. The problem is that a pherogram profile depends significantly on the conditions in which the CE analysis is performed as well as on the conditions of sample preparation. Thus, to the authors' knowledge in this field, it is not yet a commonly accepted separation protocol. During the formulation of an optimal protocol a large number of electropherograms are normally produced. Although the number of experiments was statistically correct, it was still difficult to judge which protocol of sample preparation/analysis could be the most promising for achieving the goal of meat forgery detection set above. Instead of just visual inspection the use of some more formalized parameter is needed. For example, in this work we recorded about 100 electropherograms of extracts of different products – pork and beef from a local supermarket – by varying extraction conditions as well CE analysis conditions. We found Shannon's entropy to be a useful technique for formal characterization of pherogram complexity.

THEORETICAL

As could be expected, different meat sample preparation conditions and different buffer compositions resulted in quite different electropherogram profiles of the extract. It is obvious that the more complex the electropherogram profile, the more characteristic it should be of a particular meat species extract. On the other hand, however, estimation of the "complexity" of a particular electropherogram seems to be a rather subjective procedure, depending much on the judgment of the person performing the analysis. It could be convenient to have a certain formal measure of the "complexity" of electropherograms. In this respect Shannon's entropy seems to be the most convenient measure because it calculates the "smoothness" or "flatness" of a particular function (spectrum, chromatogram). Entropy increases when the graph becomes smoother and vice versa. Thus, electropherograms with many sharp peaks could be expected to have lower entropy values than electropherograms with a few broad peaks.

Let an electropherogram be represented digitally with n points with the i-th point having intensity h_i. Then the entropy can be calculated as follows [4]:

$$S = -\sum_{i=1}^{n} \left(\frac{h_i}{\sum_{i=1}^{n} h_i} \right) \log_2 \left(\frac{h_i}{\sum_{i=1}^{n} h_i} \right). \qquad (1)$$

From this formula it follows that a totally flat electropherogram (all h_i equal) has the maximum entropy value. In this work all electropherograms were measured in 10 000 points, thus $S_{\max} = \log_2(10\,000) = 13.288$.

EXPERIMENTAL

Equipment

An ISCO Model 3850 electropherograph with a UV detector at $\lambda = 214$ nm was used for all analyses. Polymicro fused-silica capillaries (70 cm of total length, 50 cm effective length with i.d. 75 μm) were conditioned every morning by rinsing for 10 min with 0.1 M NaOH, for 5 min with deionized water, and 10 min with buffer. Experiments were performed at room temperature without temperature control.

Chemicals and reagents

Phosphoric acid was obtained from YA-Kemia (Finland), sodium hydroxide from Chemapol (Czechia). Sodium dodecylsulphate (SDS), acrylamide, N,N,N',N'-tetramethylethylene-diamine (TEMED), and ammonium persulphate of electrophoretic grade and 2-mercaptoethanol of reagent grade were obtained from Merck (Germany). Ethylene glycol ($C_2H_6O_2$) (Reachim, Ukraine; analysis grade), sodium chloride (Reachim, Russia; analysis grade), and ethanol (redistilled in the lab) were obtained from the stock of the Institute of Chemistry at Tallinn Technical University.

Buffer solutions were prepared by dissolving chemicals of analytical grade in Milli-Q deionized water (water purifying system with 18 M Ω water resistance from Waters).

Procedures

Preparation of the phosphate buffer solutions for CE

Phosphate buffers with different pH values and ionic strength of 0.01 (concentration 0.01 M) were prepared by mixing proper amounts of 1 M H_3PO_4 and 1 M NaOH solutions with water. All buffer solutions were filtered through Millipore glass fibre filters.

Preparation of the linear, non-cross-linked gel for capillary gel electrophoresis (CGE)

The preparation of the gel and the selection of the pH value of the buffer were based on the recommendations given by Widhalm et al. [5]. For the preparation of 100 ml of linear, non-cross-linked liquid polyacrylamide gel of medium chain length and 7.5 g of acrylamide were dissolved in 60 ml of water; 75 mg of TEMED was added, and the solution was degassed with He passing through the solution for 10 min. Polymerization was initiated with 92 mg of ammonium persulphate (dissolved in 40 ml of water). The resulting polyacrylamide gel solution was degassed with He passing through the solution for 20 min.

For the running gel buffer, solid SDS was added to the phosphate buffer ($I = 0.1$) to give a final concentration of 0.5% (w/v). Prepared as described above

5 g of gel was mixed with the SDS buffer, giving a running buffer solution with 10% (w/v) of gel.

Preliminary treatment of meat cuts

The meat for the samples was always obtained from Konsum's, a local supermarket, where the fillet cuts were vacuum packed. Raw meat was minced twice with a mincing machine, 100 g of meat was mixed and blended with 100 ml of ethanol (65%) to precipitate the proteinaceous fraction, according to total protein separation methods described by Suzuki [6]. Ethanol causes changes in non-covalent interactions without any accompanying change in the covalent interactions, i.e. proteins are in unfolded state. The solutions were centrifuged at 800 rpm for 15 min and the supernatant was filtered through Whatman glass fibre filters. The extracts were dried in amounts of 1 ml in exicators kept at 40 °C.

The residue (40 mg from 1 ml of pork or 44 mg from 1 ml of beef extract) makes up about 20% of the average protein amount of the meat muscle.

Preparation of the samples for CE

40 mg of residue from the treatment with ethanol was mixed and blended with 2 ml of water or salt solution (0.6 M NaCl). 0.01% of sodium azide was added as a stabilizer to the sample. The sample dilution was performed with the same phosphate buffer solution that was used in electrophoresis. In the case of modification with ethylene glycol the modificator was added directly to the neutral phosphate buffer (pH 7.2) diluted sample, and the effect of the addition of different amounts (5–20%) of ethylene glycol was studied.

Preparation of the samples for CGE

40 mg of residual from the treatment with ethanol was mixed and blended with 2 ml of water or salt solution (0.6 M NaCl) containing 2% of SDS and 1% of 2-mercaptoethanol. The samples were centrifuged at 800 rpm for 15 min, the supernatant was filtered through Whatman glass fibre filters. To accelerate disulphide bond reduction, the samples were heated at 100 °C for 10 min in water bath and submitted to analysis. The sample dilution was performed with the same buffer solution that was used in the preparation of linear, non-cross-linked gel.

Data processing

Electropherograms were recorded digitally using Keithley ADC/DAC Mini-16 board and processed later with MATLAB 5.3 (Mathworks, Natic, MA, USA). The raw pherograms were subjected to editing using software written in house [7] in MATLAB. It included filtering of spikes and subtraction of the base line. The areas, migration times, etc. for the peaks and also the entropy were calculated using the home written program in MATLAB.

Entropy of electropherograms was calculated according to Eq. 1. Before calculation a small number $\varepsilon = 10^{-7}$ was added to the base line. The change in the calculated entropy value is negligible but this avoids problems with taking logarithms of very small numbers. At least three electropherograms were taken for every experiment condition, and entropy was calculated for every electropherogram. For comparison of entropy values of different experiment conditions the mean value of these three was used.

RESULTS AND DISCUSSION

Examples of electropherograms obtained using different sample preparation/analysis conditions are presented in Figs. 1–3. The main peaks are numbered according the retention time. Coding of the electropherograms corresponds to that in Table 1. It follows from the figures that extraction with salt solution and CGE analysis is superior over the other methods in the sense that the resulting electropherogram has many well-resolved peaks. These results can be quantitatively confirmed with the entropy calculation results presented in Table 1. We made a series of experiments at different pH values using only buffer and calculated the relevant values of entropy. Figure 4 presents these results with a deep minimum at pH 5.0 seen. This pH value was chosen for further processing of meat samples with only buffer used. The entropy value at this pH was also used for comparison with other methods in Table 1.

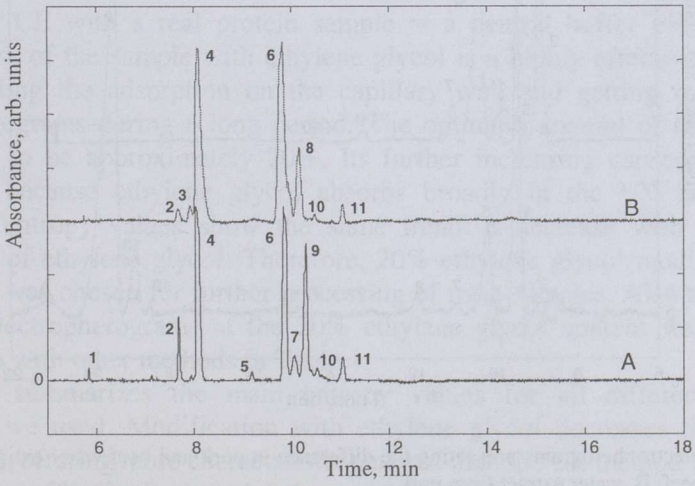

Fig. 1. Electropherograms of profiles from pork and beef samples in CE performed with phosphate buffer. A, extract from beef; B, extract from pork.
Conditions: phosphate buffer (pH = 5.0; $I = 0.01$); detection at 214 nm; applied voltage 20 kV.
Sample: 0.6 M NaCl extract from the precipitated proteins of pork and beef muscle tissue. Diluted up to 10 mg/ml with buffer used in current runs.

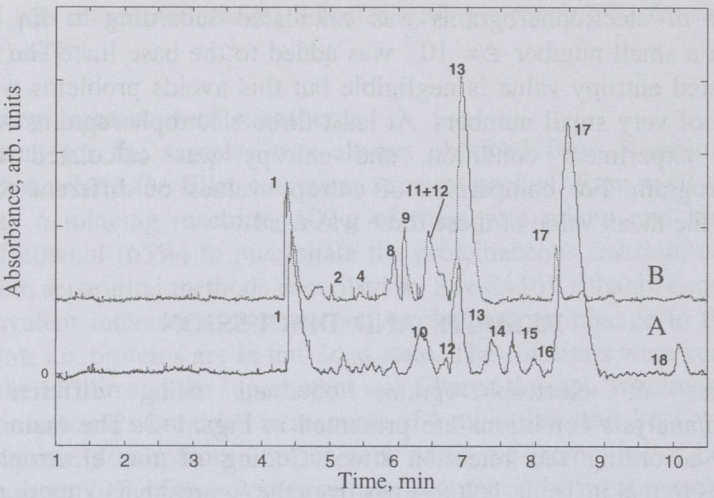

Fig. 2. Comparison of water and salt solution extracts from beef muscle tissue with 20% ethylene glycol as the sample modificator. A, salt solution extract; B, water extract.
Conditions: phosphate buffer (pH = 7.2; $I = 0.01$); detection at 214 nm; applied voltage 20 kV.
Sample: water and 0.6 M NaCl extract from the precipitated proteins of beef muscle. Diluted up to 10 mg/ml with buffer used in current runs.

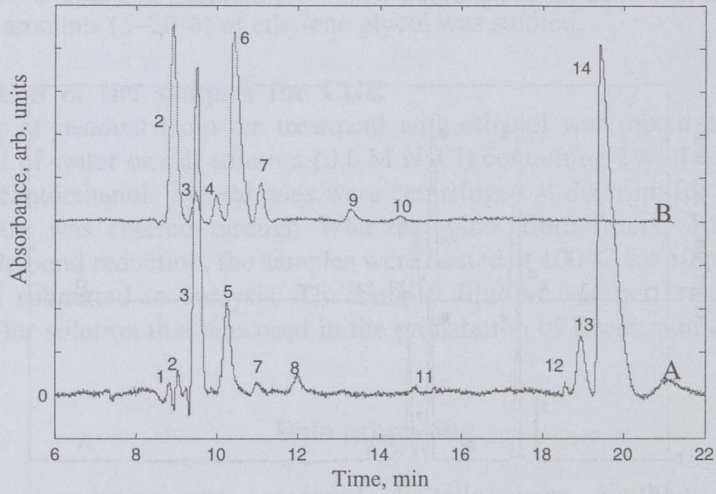

Fig. 3. CGE electropherograms presenting the difference in pork and beef water extracts. A, water extract from beef; B, water extract from pork.
Conditions: buffer solution (pH = 5.5) containing 10% of linear, non-cross-linked gel; detection at 214 nm, applied voltage 20 kV.
Sample: water extracts from the meat muscle tissue with 1% of 2-mercaptoethanol and 2% of SDS. Diluted up to 10 mg/ml with phosphate buffer used in current runs.

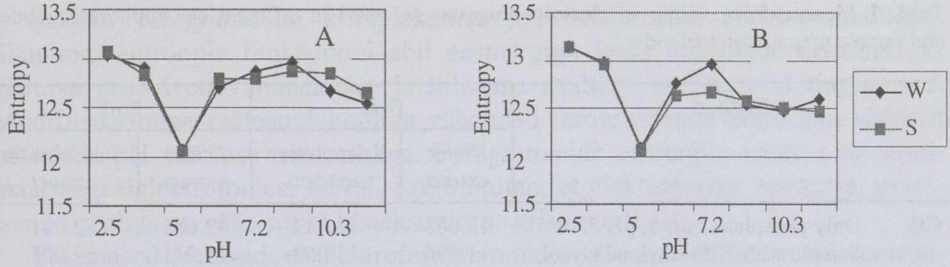

Fig. 4. Mean entropy of electropherograms of meat extracts (W – with water, S – with salt solution) at different pH values. All electropherograms have the same length and the pre-processing with the same parameters. A, extracts of beef; B, extracts of pork.

In the case only buffer was used the shapes of the peaks indicate a protein adsorption to the capillary wall. The second through fourth runs of the sample under the same conditions showed the migration times and peak areas changing from run to run. The fifth continuous run of the same sample under the same conditions sometimes caused a clog in the capillary. According to Gordon et al. [8], the problems of wall adsorption of protein components are easily solved by modification of the sample with ethylene glycol, a hydroscopic liquid, miscible in water. Besides preventing the proteins from adsorbing to the capillary wall, ethylene glycol is believed to reduce protein–protein interactions. According to the literature the exact mechanism is not completely understood and probably it involves several interactions between proteins, ethylene glycol, capillary wall, and buffer. From the electropherograms it was concluded that in performing CE with a real protein sample at a neutral buffer pH value, the modification of the sample with ethylene glycol is a highly effective technique for preventing the adsorption on the capillary wall and getting reproducible electropherograms during a long period. The optimum amount of the modifier was found to be approximately 20%. Its further increasing caused a reduced sensitivity because ethylene glycol absorbs broadly in the UV region. The calculated entropy values show the same trend: a decrease with increasing percentage of ethylene glycol. Therefore, 20% ethylene glycol modification of the sample was chosen for further processing of meat samples. Also the entropy value of electropherograms at the 20% ethylene glycol content was used for comparison with other methods in Table 1.

Table 1 summarizes the main entropy values for all different analysis conditions we used. Modification with ethylene glycol decreases the entropy value in CE, offering more characteristic patterns than the CE method where only the pH value of buffer is altered. It is seen from Table 1 that entropy is minimal for CGE, which once again indicates that CGE with linear, non-cross-linked gel is a very suitable method for profiling different meat extracts.

Table 1. Mean entropy values of electropherograms recorded in different electrophoresis modes and sample preparation methods

Method		Beef		Pork	
		Water extract	Salt solution extract	Water extract	Salt solution extract
CE	Only phosphate buffer, pH 5.0	12.063	12.042	12.085	12.071
	Sample with 20% ethylene glycol, buffer pH 7.2	11.496	11.069	11.241	11.001
CGE	Buffer pH 5.5, 10% liquid polyacrylamide gel added	11.021	11.013	11.075	11.034

Our results show that characterization of the electropherograms by their entropy fits well with human understanding of their complexity and structural richness. Nevertheless, this approach is completely formal not paying much attention to the chemical basis of the separation obtained.

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LIHAPROOVIDE KAPILLAARELEKTROFEROGRAMMIDE ISELOOMUSTUS NENDE ENTROOPIA ALUSEL

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Erinevate protseduuridega ettevalmistatud lihaproove analüüsiti kapillaarelektroforeesi meetodiga varieerides puhvri pH väärtust ja modifitseerivaid lisandeid.

Saadud ferogrammide komplekssust ja struktuurilist rikkust analüüsiti Shannoni entroopia funktsiooni abil eesmärgiga leida lihaliikide eristamiseks võetava proovi ettevalmistamise ja hilisema analüüsi optimaalsed tingimused. Modifitseerimine etüleenglükooliga vähendab ferogrammide entroopia väärtust võrreldes pH väärtuse muutmisega. Ferogrammide entroopia väärtus on minimaalne geelelektroforeesi korral. See kinnitab, et elektroforees lineaarse, mitteseotud geeliga on väga sobiv lihaekstraktide eristamiseks.

Tulemused näitavad, et elektroferogrammide iseloomustamine nende entroopia põhjal langeb hästi kokku levinud ettekujutusega nende komplekssusest ja struktuurilisest mitmekesisusest. Siiski on ferogrammi entroopia arvutamine täiesti formaalne lähenemine eksperimendi tulemustele ning ei pööra tähelepanu saadud segu komponentide lahutamise keemilisele küljele.