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Journal of Chromatography B, 653 (1994) 47–54

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Capillary electrophoretic profiling of rat hair: a tool for alopecia areata diagnosis

Zdeněk Deyl[a,*], Franco Tagliaro[b], Ivan Mikšík[a]

[a]*Institute of Physiology, Vídeňská 1083, Praha 4, Czech Republic*
[b]*Institute of Forensic Medicine, University of Verona, Verona, Italy*

(First received October 8th, 1993; revised manuscript received November 11th, 1993)

Abstract

Capillary electrophoretic profiling of hair fractions obtained by 0.25 *M* HCl treatment of the tissue with subsequent extraction of the solubilized fraction with chloroform–isopropanol (9:1, v/v) revealed clear differences between hair obtained from alopecia areata affected laboratory rats (both in hair obtained from non-affected areas and hair growing on once hairless patches) and controls. Differences were observed both in the organic-phase extractable material as well as in the aqueous phase after extraction. The separations were carried out in 25 m*M* borate buffer pH 9.2 for the chloroform–isopropanol extractable fraction while profiling of the aqueous phase was done in the same buffer at pH 10. Untreated fused-silica capillaries, 40–45 cm to the detector of I.D. 50 and 75 μm were used at a running voltage of 20 kV. Detection was done either by UV absorbance at a fixed wavelength of 200 nm or by using a diode array detector.

1. Introduction

Alopecia areata is a disease occurring in breeding colonies of laboratory rats with an unknown etiology. It does not exhibit additional clinical symptoms or increased mortality. Still it causes considerable economical losses to the breeders as it brings about doubts about quality and standardization of animals. Besides known hereditary cases of alopecia [1–3], alopecia areata is believed to be connected with stress, nutritional carency, autoimmune processes, application of disinfectants and possibly with other environmental factors. Biological investigation is further complicated by the fact that it is reversible and hair-free patches usually become covered with new hair within 14–21 days. Because of problems caused by this disease in marketing laboratory animals, a method for revealing the susceptibility of animals to this disease is badly needed. Owing to the unknown ethiopathology of this disease it is impossible to look for the disease-causing factor. In this communication we report the differences in hair profiles of the alopecia affected animals as compared with controls.

While human hair has been a subject of intensive investigation for a number of years (for review see Chiarotti [4], Baumgartner *et al.* [5], Harkey and Henderson [6]), particularly in the search for abused drug deposition, there is to our knowledge very limited information on the hair in the rat [7,8]. On the other hand there are several reports on the analysis of drugs in animal

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(93)E0419-Q

hair: most of the work has been focused so far upon the deposition of known drugs in this tissue [9–12]. To our best knowledge there is, however, not any report about the profiles of either human or animal hair.

Selection of a suitable profiling technique is a difficult problem. So far profiling has been limited to the use of chromatographic techniques (TLC, HPLC, GC) (for detailed information see Sweeley and Deyl [13]). However, none of these techniques has been applied to hair fractions. The profiling methods mentioned above have, however, distinct limitations. In TLC some knowledge is needed about the functional groups involved as otherwise it is impossible to detect the separated compounds. Gas chromatography can be applied to volatile compounds only, as derivatization leading to increased volatility of the analyzed solutes may further complicate the analyzed mixture. The last two possibilities are HPLC and capillary zone electrophoresis (CZE). Both these methods offer the possibility of separating compounds within a large polarity and molecular mass range with the possibility to apply non-specific detection at the same time. While HPLC is a well-established profiling technique, capillary electrophoresis (including capillary micellar electrokinetic chromatography) has been used for this purpose in a very limited number of cases (for more information see refs. [14,15]). On the other hand it offers the possibility to obtain fast results and requires a minimum of sample, which may be quite favourable if sections of hair have to be analysed. In addition, since in preliminary experiments HPLC both in the straight and reversed-phase mode did not offer promising results, profiling by capillary electrophoresis was adopted.

2. Experimental

2.1. Animals and material analyzed

Alopetic male rats and controls were obtained from breeding stations in Germany and the Czech Republic. The animals were housed in specific pathogen free (SPF) barrier units, and were fed an Altromin 1324 diet. The animals had a body mass of 140–180 g when used in the experiment. Diseased animals showed the typical symptoms of the disease. All biochemical, mycological, bacteriological and parasitological examinations were negative. Also serological examination did not reveal any difference with controls and both categories of animals did not differ in their body mass.

Three types of samples were analyzed, *i.e.* hair of the alopetic animals, newly grown hair in the alopetic patches, and hair of control animals. The hair was cut off by scissors next to the skin from the dorsal region and material obtained in this way from 10 animals of each type was pooled.

2.2. Extraction of hair

The hair sample (2.5 g) was first washed with 250 ml of ethanol and phosphate buffer pH 6.0 (0.01 *M*) each on a Büchner funnel; next it was suspended in 125 ml of 0.25 *M* HCl and incubated overnight at 40°C. The extract was filtered and extracted three times with 125 ml of a chloroform–isopropanol (9:1, v/v) mixture. Both the organic extract and the aqueous layer were analysed by capillary electrophoresis.

2.3. Capillary zone electrophoretic profiling

This was carried out with two types of apparatus, *i.e.* ISCO Model 350 manually operated device (ISCO, Lincoln, NE, USA) using a 60 cm (40 cm to the detector) × 50 μm I.D. fused-silica capillary (Polymicro Technologies, Tucson, AZ, USA) equipped with a UV detector set at 200 nm. The other apparatus used at a later stage of the experimental work was an Eureka 2000 (Kontron, Milan, Italy) automated CZE machine equipped with a diode array detector. Differently from the first device here a 50 cm (45 cm to the detector) × 75 μm I.D. fused-silica gel capillary (Polymicro Technologies) was used. The UV detector was also set at 200 nm. The diode array detector scans were recorded three times, *i.e.* at the top of the peaks, and at its positive and negative flanks to get some idea about the homogeneity of the separated peak. The buffer used was 25 m*M* borate pH 9.2 (for

the chloroform–isopropanol extractable fraction) or pH 10.0 (for the aqueous residue) (pH adjusted with 1.0 *M* NaOH when necessary). The samples of the organic phase extractable material were diluted ten times with the running buffer, filtered through a filter (Millex HV 0.45 μm; Waters, Millipore, Milford, MA, USA) and applied to the electrophoretic capillary. Samples of the aqueous phase were taken to dryness *in vacuo* (40°C), and reconstituted in the running buffer in such a way that the applied concentration was ten times less than the concentration of the starting extract.

2.4. Amino acid analysis of the acid extracts

Amino acid analysis of the hair extracts (both organic and aqueous phase) was carried out on a Pico-Tag automated amino acid analyser (Waters, Millipore, Milford, MA, USA) using conversion of the amino acids released by hydrolysis in 6 *M* HCl overnight at 110°C into phenylthiocarbonyl derivatives according to the supplier's instructions.

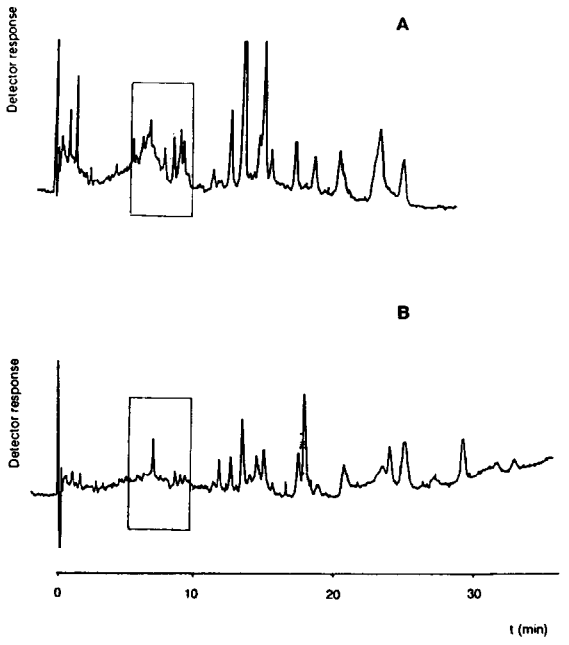

Fig. 1. Capillary electrophoretic profiles of the chloroform–isopropanol extractable fraction after acid treatment. ISCO electrophoresis equipment. (A) Alopecia, (B) controls. For details of separation conditions see Experimental section.

3. Results

3.1. Profiling of the organic phase extractable material

As shown in Fig. 1 the profiles of the organic phase extractable material obtained from alopecia affected animals and controls differ considerably. Of the set of peaks present in both profiles only those numbered 1, 2 and 3 correspond in both profiles at least as far as their retention is concerned. Two points should be emphasized in this context. First the retention times of individual peaks were unexpectedly well reproducible (C.V. $< 2.5\%$, mostly 1.6; $n = 5$). Second, meticulous care was taken to keep the concentration ratio between alopetic and control samples constant. In other words the amount of the UV absorbing material extractable into the organic phase in alopetic animals is considerably higher than in the control run.

All attempts at resolving further the cluster of peaks in the framed area in Fig. 1 on the ISCO apparatus were unsuccessful (both changes in voltage and in buffer composition, including pH variations, were tested). However a good resolution was obtained with the Kontron electrophoretic equipment. A result typical for control animals is shown in Fig. 2. This profile shows the presence of a single dominant peak (no. 4 in the run) which shows a characteristic two-maxima spectrum (220 and 245 nm). This peak appears to be reasonably pure as the same spectrum was obtained at the positive and the negative flank of the peak. Peak no. 3, with a maximum at 225 nm, is apparently not pure as indicated by the absence of the corresponding maximum in the negative peak flank (the bottom curve in the respective spectrum). There are two other peaks (nos. 6 and 7) exhibiting maxima at 330 and 215 nm respectively. Peak no. 6 is apparently contaminated in its descending part.

In contrast, the electrophoretic profile of the analogous section of the sample from alopetic

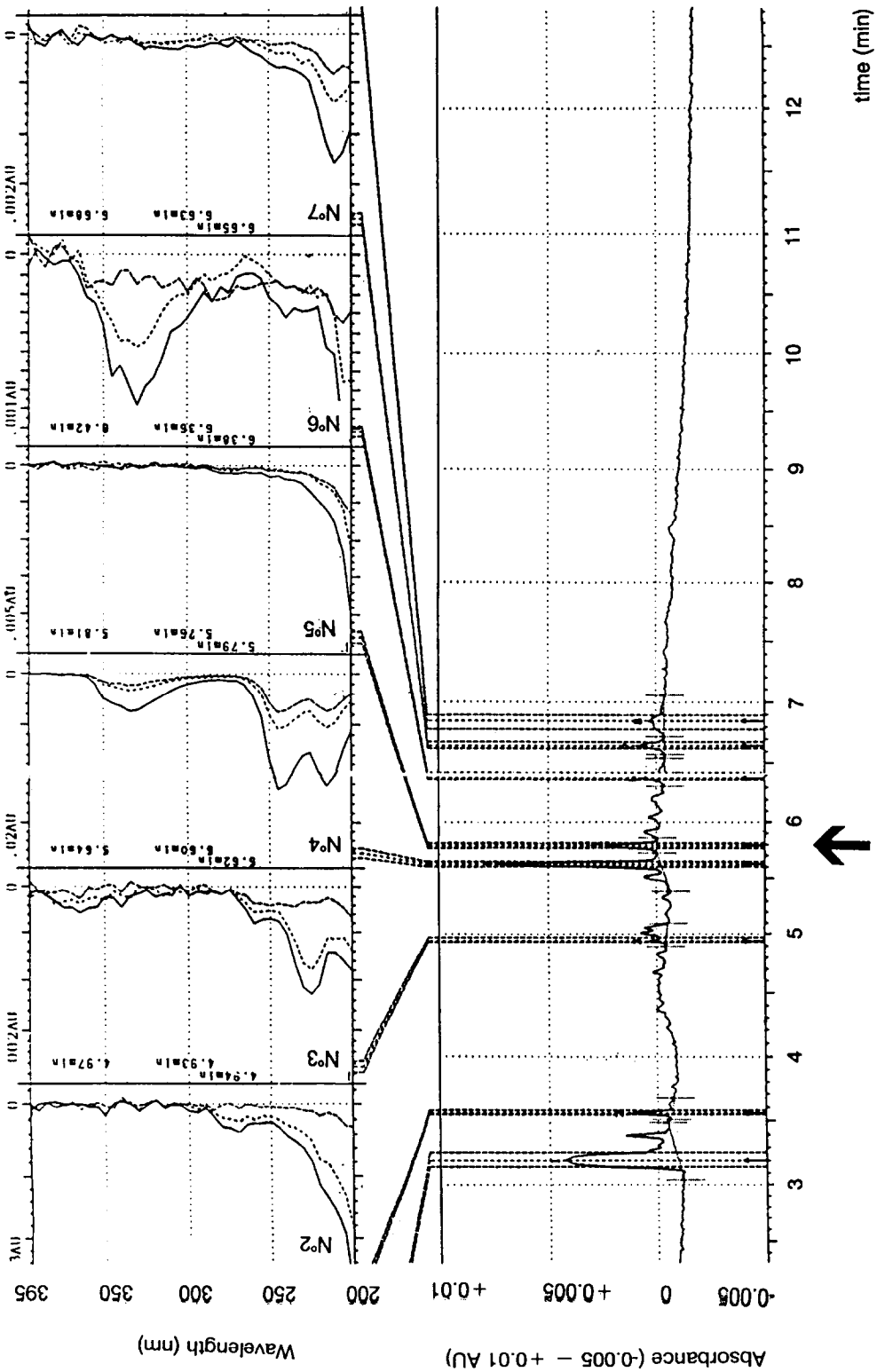

Fig. 2. More detailed fractionation of the framed part of the profile shown in Fig. 1 of control animals. Kontron instrument. For experimental details see Experimental section.

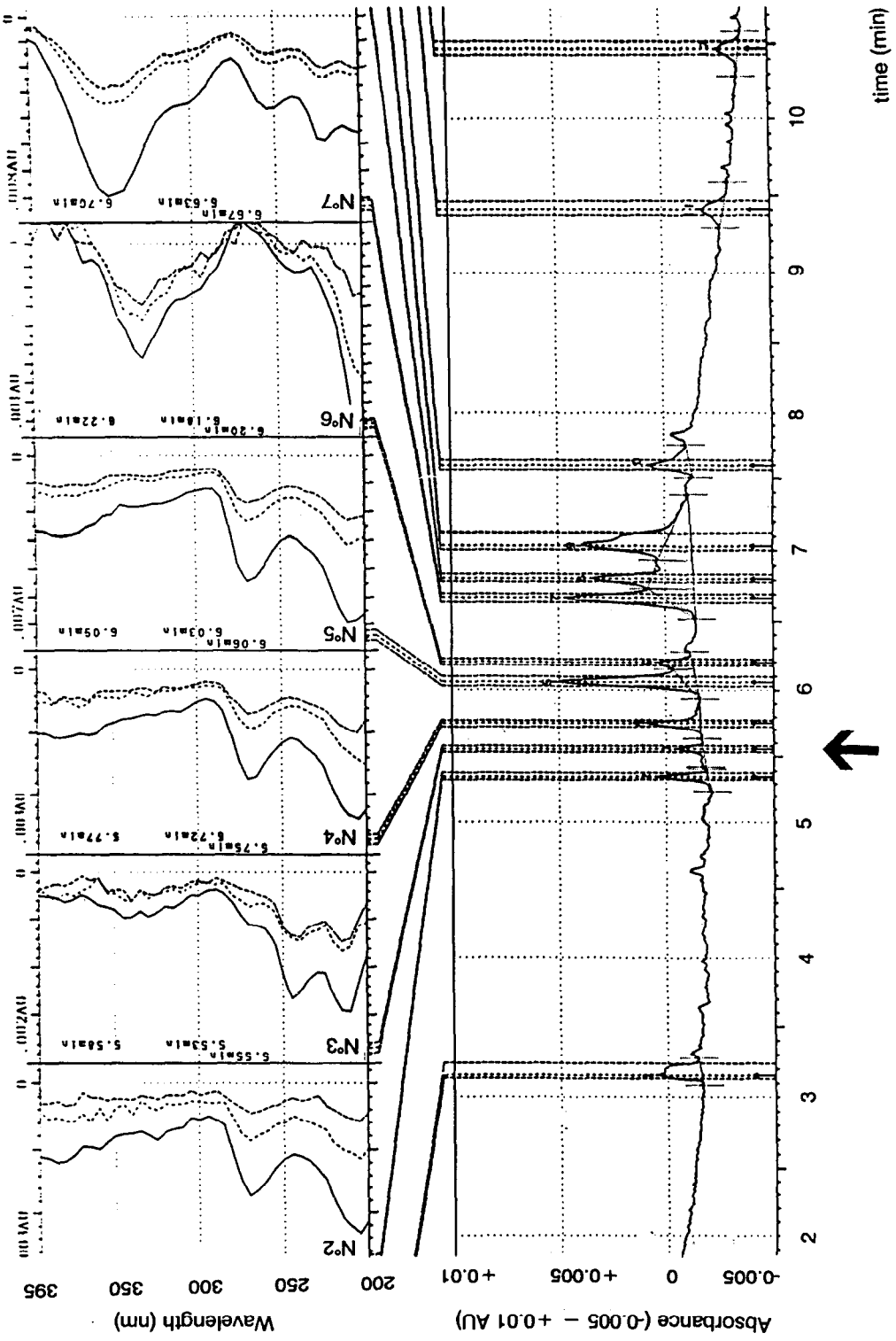

Fig. 3. More detailed fractionation of the framed part of the profile shown in Fig. 1 of alopecia affected animals. Kontron instrument. For experimental details see Experimental section.

animals showed the presence of four dominant peaks designated 5, 6, 7 and 8 (Fig. 3). Of these only the first three could be evaluated, as peak no. 8 is (judged by its spectrum and shape) apparently a complex one constituted of several analytes. Of the whole profile obtained from alopetic animals peak no. 3 is of particular interest. This peak, as judged both by its retention time and spectrum, is identical with peak no. 4 of the control run (compare Fig. 2, retention times 5.55 and 5.62 min respectively). Of the other peaks, peaks no. 6 in both the alopetic and control runs apparently correspond to each other, although peak no. 6 appearing in the control spectrum is apparently contaminated in its negative flank (retention times 6.18 and 6.36 min respectively). There is a clear difference in the retention times of this peak in controls and alopetic animals (compare with the peak exhibiting a double absorbance maximum at 220 and 254 nm, designated no. 4 in controls and no. 3 in alopetic animals).

However the main difference between the two profiles (Figs. 2 and 3) is that except for the peaks just discussed all the other peaks do not have matching counterparts. This means that the composition of this section of the original electropherogram (compare Fig. 1) is different in controls and alopetic animals. In the profile obtained with the control sample there is no peak that exhibits absorbance maxima both at 215 and 270 nm, while there are three such peaks seen in the profile obtained with the sample from alopetic animals (retention times 5.37, 5.75 and 6.06, numbered 2, 4 and 5 in the alopetic animal profile). As a matter of fact peak no. 5 (at 6.06 min) is one of the most prominent peaks in the whole profile. It can be concluded that there are profound differences in the composition of the organic phase extractable material from alopetic animals and control animals. The presence of compounds with absorption maxima at 215 and 270 nm is indicative of alopecia affected hair.

3.2. *Profiling of the aqueous fraction*

After 0.25 *M* HCl treatment *ca.* ten times less UV absorbing material is dissolved in control samples compared with alopetic samples. This difference is preserved in newly grown hair on once alopetic areas (Fig. 4). Three massive peaks are clearly visible in profiles obtained with alopetic samples with retention time between 9 and 11 min. A peak with a retention time of *ca.* 13 min is missing in the acid extracts from hair obtained from non-alopetic regions of the alopecia affected animals. On the other hand in samples prepared by acid extraction of hair from the once alopetic patches there are two distinct peaks at the end of electrophoregram. The profiles are well reproducible with an R.S.D. less than 2% (analyses were done with twenty animals, each analysis/profile was run four times).

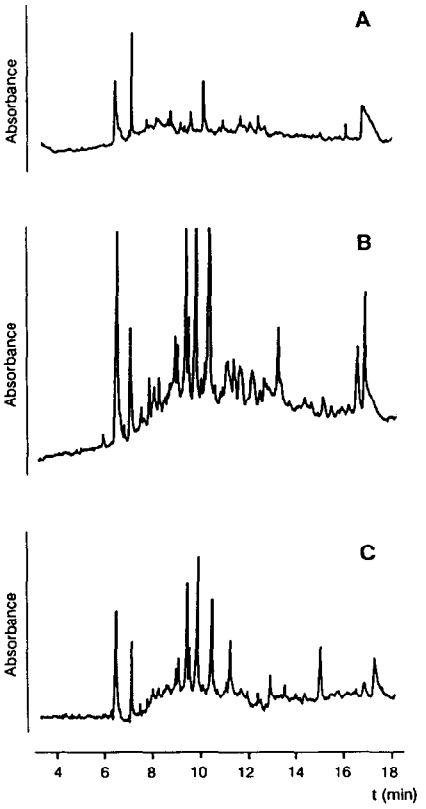

Fig. 4. Capillary electrophoretic profiles of the aqueous fraction after acid treatment. ISCO electrophoresis equipment. UV detection at 200 nm. (A) controls, (B) alopetic patch, (C) non-alopetic region. For details of separation conditions see Experimental section.

4. Discussion

Before starting any considerations about the changes observed in the hair fraction profiles two points have to be stressed: First, the retention times of the peaks in the individual capillary electrophoretic profiles are well reproducible with a C.V. of 2–2.5%. Second, during the whole study all was done to keep the concentrations between the individual types of samples (diseased, healthy) comparable. Consequently peak heights and areas can give some information whether a particular zone in a particular profile is present in a similar proportion as in another profile, or if it is considerably less or more.

Distinct changes were observed in both the organic phase extractable fraction and the aqueous residue obtained after 0.25 *M* HCl treatment of the hair. Running electrophoretic profiles of the organic phase extractable material at pH 9.2 revealed more complex profiles for alopecia affected animals than for control animals. Of particular interest are the three peaks, clean as far as judged by their spectra, showing double UV maxima at 215 and 270 nm. Their presence in the profile is clearly indicative of an alopecia predisposed animal. However, direct measurement without fractionation did not reveal any difference in UV absorbance at these wavelengths between alopecia affected animals and controls; this is apparently due to the complexity of the mixture.

As far as the aqueous phase after acid treatment is concerned, considerably more UV absorbing material was released in both samples obtained from hair growing in the alopetic region as well as from hair from the unaffected areas as compared to controls. Estimation of protein nitrogen showed a 255% increase in samples from non-affected regions of alopetic animals and a 564% increase in samples of hair growing on the once alopetic patches. This indicates a higher susceptibility of the hair core to 0.25 *M* HCl treatment, indicating a lower structural stability of hair from alopecia affected animals. It indicates also that most of the UV absorbing material released by acid treatment of hair is of peptidic nature. Amino acid analysis of the acid extractable fraction revealed slight differences in amino acid composition, e.g. some decrease of the glutamic + aspartic acid (33.53 and 39.80% in alopetic and healthy animals respectively), a drop in cysteine (0.18 *vs.* 0.55%), and an increase of lysine (7.27% in alopetic animals *vs.* 4.98% in controls). Attempts to resolve the peptidic material extractable by 0.25 *M* HCl (either from alopetic or control animals) by polyacrylamide gel electrophoresis were unsuccessful, possibly due to the fact that the molecular mass of the released material is too small for polyacrylamide gel sieving. The occurrence of three distinct UV absorbing peaks in the electrophoretic profile of this fraction between a retention time of 9 and 11 min also appears to be of diagnostic value indicating the susceptibility of the animal to alopecia areata.

From the separational point of view capillary electrophoresis appeared superior to both straight and reversed-phase chromatography as chromatographic profiling did not reveal clear-cut differences between affected animals and controls in preliminary tests. Likewise, alkaline treatment of hair which is also frequently applied for toxicological purposes in hair analysis did not offer easily interpretable results: the fractionation scheme used in this case is analogous to acid treatment: after treating hair with 1.0 *M* NaOH the sample is extracted with a chloroform–isopropanol mixture and the aqueous and organic phases are analysed separately. Electrophoretic profiling of both the organic and aqueous phases did not reveal applicable results. The problem with the aqueous phase derives from the fact that alkaline treatment of hair leads to complete dissolution of the matrix and that possible differences are obscured by the complexity of the resulting hydrolysate. The reason that the organic phase obtained after alkaline treatment did not reveal profiles of diagnostic value remains to be elucidated.

5. Acknowledgement

This work was in part supported by the Czech Ministry of Education, Youth and Sports Grant No. 0711.

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