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Identification of fish species by reversed-phase high-performance liquid chromatography with photodiode-array detection

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Abstract

A method for the separation of sarcoplasmic fish proteins by RP-HPLC is described. The procedure revealed significant differences useful for reliable identification of fish species. Sixteen of the most common Finnish freshwater fish species were differentiated by species-specific HPLC chromatograms obtained using photodiode-array detection (PAD) at 200–350 nm. The analytical column was a Hi-Pore RP-304 reversed-phase column. The separation was performed by a linear gradient of acetonitrile and water with a small amount of trifluoracetic acid (TFA). Star-symbol plots were constructed from the chromatograms to visualize the data. Clearly different HPLC protein profiles for most fish species were obtained. The chromatograms of salmonoids show similarities, whereas the protein profiles of cyprinids are dissimilar. Minor intraspecific differences were obtained for three types of powan (*Coregonus lavaretus*). © 1998 Elsevier Science B.V.

Keywords: Species identification; Proteins

1. Introduction

There are approximately 60 different natural fish species in Finnish water systems. With occasional visitors and planted fish species the number approaches 100. Many species are regularly used as food and also exported. Chemical identification of fish species is needed when the morphological characteristics of the specimen has been removed by some physical treatment. Morphological identification is not possible, for example, for minced or filleted fish. In these cases the identification of fish species is of great importance for labelling regulations, quality control requirements and establishment of fair pricing policies [1–4].

Both conventional electrophoresis [5–11] and isoelectric focusing [12–24] on different gels have been used for fish and shellfish species identification. Because these methods are time-consuming, include many separation steps and have numerous other disadvantages [25], the use of more sophisticated techniques such as RP-HPLC [2,25–28] and capillary zone electrophoresis (CZE) [29,30] has been reported.

The RP-HPLC technique was first used for protein profile determination with seven marine fish species [2] and later with 31 fish species [25]. Several common edible marine fish species from eastern Australian waters have been studied [27] to find out the intraspecific variation within the sample from the same specimen, as well as between samples of the same species from different locations and seasons. In addition to chromatograms, star-symbol plots have been used for the visualization of HPLC data of

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morphologically identified species [27]. Both CZE and HPLC have been used for monitoring frozen storage changes in sarcoplasmic proteins of cod and pollock fillets [28,29]. Callardo et al. [30] have applied CZE to study aqueous extracts of eight flatfish species. Recently, Esteve-Romero et al. [24] reported an interesting study of both fresh and boiled fish samples by isoelectric focusing of parvalbumins in immobilized pH gradients. To our knowledge, however, there are no data available on HPLC or CZE identification of freshwater fish species living in Finnish or other Scandinavian water systems.

In all the above HPLC studies, the sarcoplasmic proteins have been detected by UV absorbance at constant wavelength, usually 280 nm [2,25,27]. The goal of our study was to obtain reproducible protein profiles suitable for the identification of fish flesh samples representing both individual fish species and also the mixtures of species. For the first time we present the use of photodiode-array detection (PDA) for fish species identification. The major advantages of PDA are the possibility of finding out new information about the differences in amino acid composition of proteins and to check peak purity. The main purpose of our study was to create a collection of HPLC data for 16 most common fish species found in Finnish freshwater systems.

2. Experimental

2.1. Apparatus

HPLC was performed with an instrument consisting of a Waters 510 pump, a Waters 501 pump, a Waters pump control module, a Waters 996 photodiode-array detector, a Waters 717 autosampler and a Waters column oven attached to a temperature control module. The equipment was controlled and the data were handled using Millennium software (Waters, Milford, MA, USA). The analytical column was a 250×4.6 mm I.D. Hi-Pore RP-304 reversed-phase column (Bio-Rad Labs., Richmond, CA, USA), particle and pore sizes 5 μm and 300 Å, respectively. The precolumn was a Waters Delta-Pak C4 300 Å (HPLC precolumn inserts). The samples were homogenized using a Sorvall omni-mixer.

2.2. Reagents and chemicals

Analytical-grade TFA was obtained from Fluka (Buchs, Switzerland), HPLC-grade acetonitrile from Rathburn (Walkerburn, Scotland) and sodium azide (NaN_3) from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was obtained from Sigma (St Louis, MO, USA). The standard solution was prepared by dissolving 8 mg of BSA in 1 ml of ultrapure water.

2.3. Sampling

The samples (except salmon and rainbow trout) were collected from Lake Konnevesi which is the 23rd biggest (187 km^2) lake in Finland. The fish were caught using different kinds of lures and nets and kept frozen at −18°C without being skinned and deboned until sample preparation.

The following fish species were taken: (1) Vendace (*Coregonus albula*), (2) smelt (*Osmerus eperlanus*), (3a) powan (*Coregonus lavaretus wartmanni*), (3b) powan (*Coregonus lavaretus nilssoni*), (3c) powan (*Coregonus lavaretus pallasi*), (4) trout (*Salmo trutta*), (5) grayling (*Thymallus thymallus*), (6) pike (*Esox lucius*), (7) roach (*Rutilus rutilus*), (8) bleak (*Alburnus alburnus*), (9) bream (*Abramis brama*), (10) perch (*Perca fluviatilis*), (11) ruff (*Acerina cernua*), (12) pike perch (*Stizostedion lucioperca*), (13) burbot (*Lota lota*), (14) minnow (*Phoxinus phoxinus*), (15) salmon (*Salmo salar*) and (16a,b,c) three rainbow trout (*Oncorhynchus mykiss*) of different origin. Species 1–6, 15 and 16a–c are salmonoids and 7–9 cyprinids. The species 10–12 represent perches. Rainbow trout 16a and 16b were obtained from a hatching station. Sample 16c was a rainbow trout grown in the sea.

2.4. Preparation of samples

The frozen fish were thawed and the skin, bones and organs were removed. The fish flesh was cut into small pieces and 15–20 g of them were blended with twice amount of Milli-Q water in the mixer. The blend was filtered through a paper filter (Macherey–Nagel 640 WE, Düren, Germany). Magnesium silicate (Florisil PR, Fluka, Buchs, Switzerland) was added to the filtrate to remove lipophilic substances.

After decanting, the filtrate was filtered again through a 0.45-μm membrane (Schleicher and Schuell ME 25/21 ST, diameter 47 mm). The filtrate was stored in several portions in the freezer at −18°C until analyzed. The analysis procedure was started within 4 h.

2.5. HPLC analysis

The BSA standard and fish protein samples were injected separately because in our preliminary studies we found that the BSA peak interfered with sample peaks in some cases. A 5-μl volume of BSA standard solution and 10 μl or 20 μl volume of the sample solution were used in each injection. The BSA standard was injected first, and exactly 68 s later, the sample was injected from the second vial. The solvent A was prepared by mixing of ultrapure water (MilliQ) and trifluoracetic acid (TFA) (1000:1). The solvent B was acetonitrile–water–TFA (950:50:1).

A linear gradient of 38–70% of the solution B in 90 min was run. The flow-rate was 1.5 ml/min. The column was kept at a constant temperature of 25°C.

The chromatographic data were collected during the first 60 min. The UV spectra were measured from 200 to 350 nm. PDA data were collected at the rate of one spectrum/s with the resolution of 2.4 nm.

2.6. HPLC data handling

The chromatogram plots at 280 nm, 230 nm and at each maximum absorbance wavelength (maxplots) were printed out. The star-symbol plots were obtained based on chromatographic data using Excel5 software. The macro used for symbol generation was based on the relative retention times and peak heights of each chromatogram. The branches of plots represent major peaks of chromatograms. The angles of the branches depend on the relative retention times of the peaks and the lengths of the branches depend on peak areas.

3. Results and discussion

The HPLC separation of sarcoplasmic proteins of fish species is shown in Fig. 1. The retention times relative to BSA-standard and peak areas relative to total sample peak areas are given in Table 1. Fig. 2 illustrates star-symbol plots which visualize the differences between chromatograms obtained at 280 nm. These plots were used for rough preliminary identification. However, the final identification was done by comparing the original chromatographic data of unknown sample and known samples. Generally, species-specific protein profiles were obtained for almost all fish species studied. In some cases, the differences were unexpectedly small.

The relative retention times were found to be reproducible, the standard deviation of three measurements being less than 5% but in most cases even less than 2%. The reproducibility between samples taken from individuals of the same species (perch and roach) was studied in our preliminary investigation. The standard deviation of relative retention times was less than 5%. This makes it possible to compare retention data of library chromatograms, and therefore there is no need to run known samples in each determination as is the case in slab gel electrophoresis.

Among the 16 local freshwater fish species studied are several salmonoids (*Salmonidae*), cyprinids (*Cyprinidae*) and perch fish (*Percidae*). As can be seen in Figs. 1 and 2, there is a quite clear similarity between the protein profiles of salmonoids. The HPLC trace of pike (which is also a salmonoid) resembles remarkably that of other salmonoids although morphologically pike does not resemble any of the other salmonoids. A more comprehensive checking reveals slight but quite interesting characteristic differences between the chromatograms of the salmonoids. There is small intraspecific variation between different types of powans, the most observable difference being the splitting of the peak closest to the BSA peak in the chromatograms of some powan studied. This kind of splitting was observed for *Coregonus lavaretus pallasi* and *Coregonus lavaretus nilssoni* but not for *Coregonus lavaretus wartmanni*. In our separate preliminary experiment, a related phenomenon was found also for rainbow trout (16a–c) of different origin. Conversely, two completely different fish species, vendace (1) and a powan (3a, *Coregonus lavaretus wartmanni*), cannot be unequivocally differentiated by means of their sarcoplasmic protein RP-HPLC profiles.

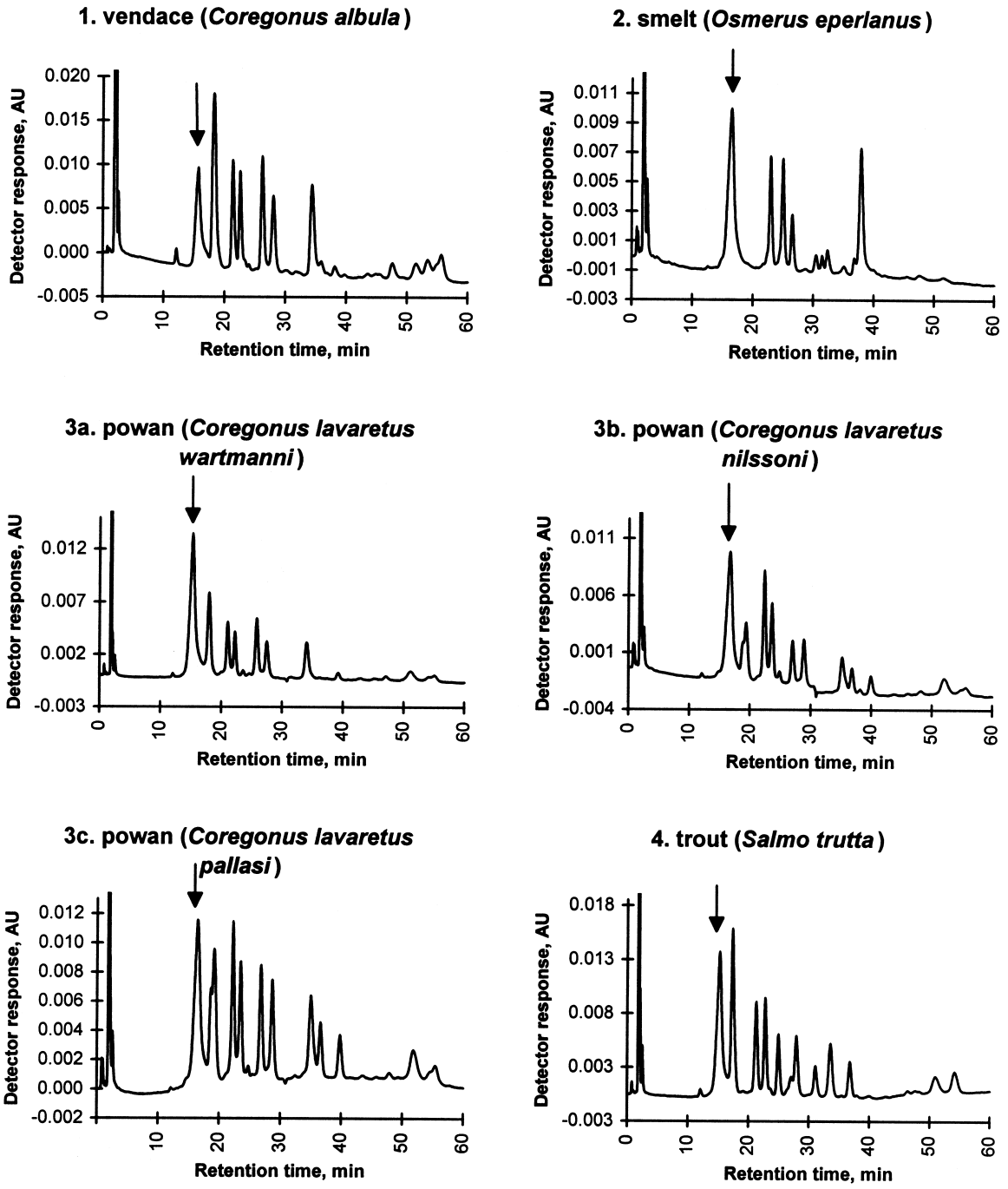

Fig. 1. HPLC chromatograms of fish species at 280 nm with BSA standard. The BSA is marked by an arrow.

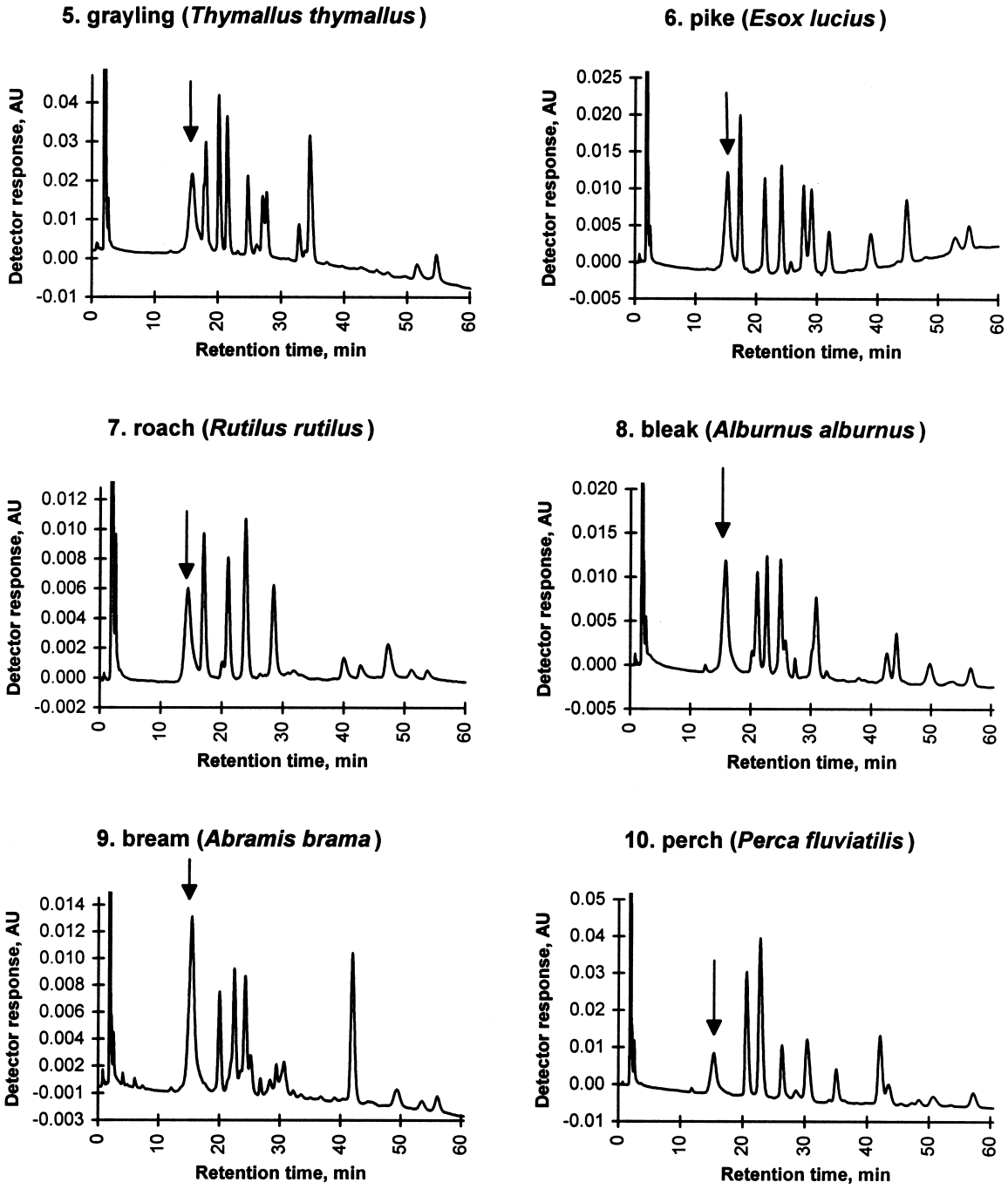

Fig. 1. (*continued*)

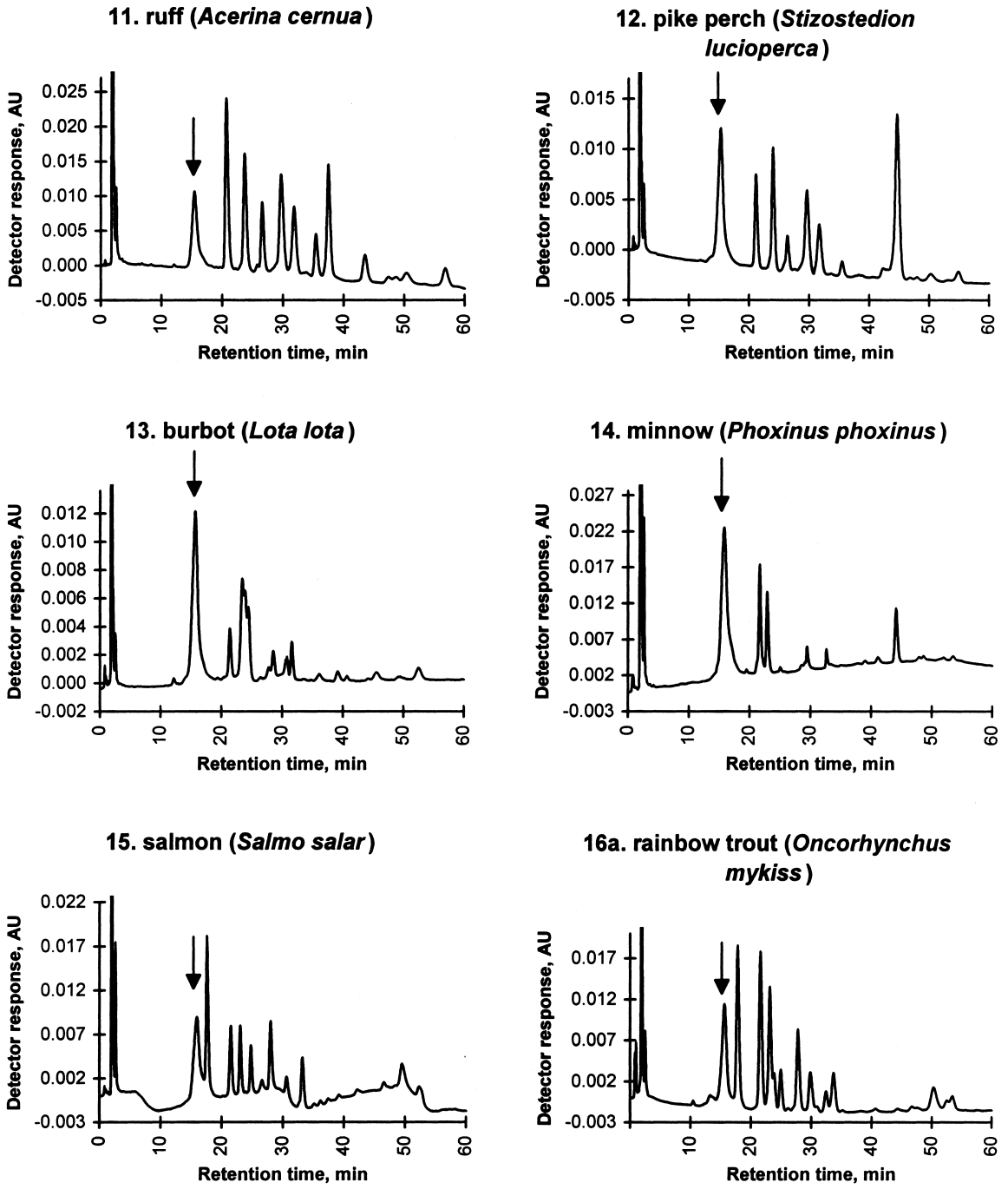

Fig. 1. (*continued*)

Table 1
RP-HPLC analysis of fish sarkoplasmic proteins

Fish sample	Relative retention times[a] and relative peak areas[b]								
(1) Vendace, 280 nm	1.17	1.37	1.45	1.68	1.79	2.21		3.56	
	(0.26)	(0.12)	(0.10)	(0.11)	(0.10)	(0.12)		(0.06)	
(1) Vendace, maxplot	1.16	1.36	1.43	1.66	1.78	2.18	2.47		
	(0.21)	(0.13)	(0.09)	(0.13)	(0.11)	(0.07)	(0.06)		
(2) Smelt, 280 nm	1.39	1.51	1.61	1.96		2.29			
	(0.19)	(0.20)	(0.09)	(0.05)		(0.34)			
(2) Smelt, maxplot	1.39	1.51	1.61	1.90	2.22	2.29			
	(0.18)	(0.19)	(0.08)	(0.15)	(0.11)	(0.20)			
(3a) Powan, 280 nm	1.17	1.36	1.44	1.66	1.77		2.18		
	(0.27)	(0.15)	(0.11)	(0.12)	(0.10)		(0.13)		
(3a) Powan, maxplot	1.17	1.36	1.44	1.66	1.77		2.18		
	(0.23)	(0.18)	(0.10)	(0.13)	(0.11)		(0.07)		
(3b) Powan, 280 nm	1.16[c]	1.34	1.41		1.61	1.72	2.10	2.20	
	(0.12)[c]	(0.20)	(0.14)		(0.08)	(0.09)	(0.11)	(0.05)	
(3b) Powan, maxplot	1.16[c]	1.34	1.41	1.49	1.62	1.73			2.38
	(0.11)[c]	(0.24)	(0.14)	(0.05)	(0.10)	(0.13)			(0.06)
(3c) Powan, 280 nm	1.17[c]	1.35	1.42	1.65	1.76	2.14	2.27	2.47	
	(0.19)[c]	(0.15)	(0.13)	(0.08)	(0.07)	(0.07)	(0.09)	(0.08)	
(3c) Powan, maxplot	1.17[c]	1.35	1.42	1.64	1.75	2.13	2.27	2.44	
	(0.17)[c]	(0.20)	(0.13)	(0.09)	(0.08)	(0.10)	(0.09)	(0.07)	
(4) Trout, 280 nm	1.14	1.39	1.49	1.63	1.82		2.19	2.40	3.53
	(0.24)	(0.13)	(0.12)	(0.08)	(0.09)		(0.09)	(0.05)	(0.05)
(4) Trout, maxplot	1.14	1.39	1.49	1.63	1.82	2.02	2.19		
	(0.21)	(0.12)	(0.11)	(0.08)	(0.11)	(0.10)	(0.05)		
(5) Grayling, 280 nm	1.13	1.26	1.34	1.55	1.69	1.73		2.05	2.16
	(0.12)	(0.17)	(0.15)	(0.09)	(0.06)	(0.07)		(0.06)	(0.18)
(5) Grayling, maxplot	1.13	1.26	1.34	1.54	1.69	1.73	1.99		2.15
	(0.10)	(0.14)	(0.13)	(0.10)	(0.06)	(0.07)	(0.06)		(0.14)
(6) Pike, 280 nm	1.14	1.36	1.49	1.63	1.75	1.86	1.99	2.31	2.92
	(0.20)	(0.05)	(0.12)	(0.07)	(0.06)	(0.11)	(0.09)	(0.06)	(0.11)
(6) Pike, maxplot	1.14	1.37		1.58	1.81	1.90			2.92
	(0.17)	(0.29)		(0.12)	(0.12)	(0.12)			(0.06)
(7) Roach, 280 nm		1.18	1.45	1.65	1.97	3.27			
		(0.15)	(0.13)	(0.18)	(0.12)	(0.06)			
(7) Roach, maxplot	1.10	1.18	1.46	1.65	1.97				
	(0.12)	(0.20)	(0.16)	(0.24)	(0.16)				
(8) Bleak, 280 nm		1.33	1.43	1.58	1.63	1.95	2.69	2.79	3.14
		(0.15)	(0.15)	(0.16)	(0.06)	(0.17)	(0.05)	(0.07)	(0.07)
(8) Bleak, maxplot	1.04	1.33	1.43	1.57	1.73	1.95			
	(0.25)	(0.11)	(0.12)	(0.14)	(0.05)	(0.12)			
(9) Bream, 280 nm		1.30	1.45	1.57	1.63			2.70	
		(0.12)	(0.18)	(0.16)	(0.05)			(0.26)	
(9) Bream, maxplot	1.14	1.29	1.45	1.57	1.62	1.90	1.98	2.70	
	(0.21)	(0.09)	(0.16)	(0.13)	(0.05)	(0.07)	(0.06)	(0.11)	
(10) Perch, 280 nm		1.34	1.48		1.69	1.96	2.24	2.69	
		(0.19)	(0.29)		(0.08)	(0.12)	(0.05)	(0.14)	
(10) Perch, maxplot	1.17	1.34	1.48	1.57	1.69	1.96		2.69	
	(0.07)	(0.15)	(0.25)	(0.16)	(0.08)	(0.12)		(0.08)	
(11) Ruff, 280 nm		1.34		1.53	1.72	1.92	2.05	2.29	2.42
		(0.18)		(0.14)	(0.08)	(0.16)	(0.11)	(0.05)	(0.14)
(11) Ruff, maxplot	1.27	1.34	1.49	1.52	1.72	1.92	2.05		2.42
	(0.05)	(0.13)	(0.13)	(0.18)	(0.07)	(0.13)	(0.10)		(0.10)

(Cont.)

Table 1 (*Continued*)

Fish sample	Relative retention times[a] and relative peak areas[b]								
(12) Pike perch, 280 nm		1.38 (0.11)	1.57 (0.18)	1.72 (0.07)	2.00 (0.09)	2.31 (0.11)	2.75 (0.06)	2.80 (0.06)	2.90 (0.19)
(12) Pike perch, maxplot	1.26 (0.05)	1.38 (0.10)	1.56 (0.14)	1.73 (0.24)	1.93 (0.14)	2.06 (0.08)		2.90	(0.21)
(13) Burbot, 280 nm	1.36 (0.13)		1.49 (0.25)	1.52 (0.14)	1.55 (0.15)	1.81 (0.05)	1.95 (0.05)	2.01 (0.06)	
(13) Burbot, maxplot	1.36 (0.10)	1.39 (0.19)	1.49 (0.13)	1.52 (0.15)	1.55 (0.13)		1.95 (0.07)	2.00 (0.07)	2.57 (0.05)
(14) Minnow, 280 nm		1.36 (0.33)	1.44 (0.23)	1.85 (0.05)	2.05 (0.04)	2.76 (0.19)			
(14) Minnow, maxplot	1.13 (0.28)	1.36 (0.22)	1.44 (0.16)	1.85 (0.11)	2.05 (0.09)	2.76 (0.08)			
(15) Salmon, 280 nm	1.10 (0.28)	1.34 (0.12)	1.44 (0.10)	1.55 (0.07)	1.75 (0.12)	2.07 (0.10)	3.08 (0.07)		
(15) Salmon, maxplot	1.10 (0.25)	1.35 (0.12)	1.44 (0.10)	1.55 (0.07)	1.75 (0.15	2.08 (0.06)	3.09 (0.05)		
(16a) Rainbow trout, 280 nm	1.14 (0.20)	1.38 (0.18)	1.48 (0.14)	1.59 (0.05)	1.77 (0.14)		2.14 (0.08)	3.18 (0.06)	
(16a) Rainbow trout, maxplot	1.14 (0.17)	1.38 (0.19)	1.48 (0.15)	1.59 (0.06)	1.76 (0.16)	1.91 (0.14)			
(16b) Rainbow trout, 280 nm	1.13 (0.22)	1.38 (0.14)	1.47 (0.11)	1.58 (0.08)	1.75 (0.14)		2.12 (0.08)	3.15 (0.07)	
(16b) Rainbow trout, maxplot	1.13 (0.20)	1.37 (0.16)	1.47 (0.13)	1.58 (0.09)	1.75 (0.17)	1.89 (0.09)			
(16c) Rainbow trout, 280 nm	1.13 (0.26)	1.37 (0.12)	1.46 (0.05)	1.54 (0.06)	1.74 (0.16)	2.03 (0.05)	2.10 (0.10)	3.12 (0.07)	
(16c) Rainbow trout, maxplot	1.13 (0.24)	1.37 (0.13)	1.47 (0.05)	1.57 (0.11)	1.74 (0.20)	1.99 (0.06)			

[a] Relative to BSA standard peak, averages of three measurements.
[b] Relative to total sample peak area (in parenthesis).
[c] Includes two partly overlapping peaks.

Unlike salmonoids, cyprinids have major differences in their chromatograms. For example an intense peak at the retention time of about 42 min is characteristic of the chromatogram of bream (9). The chromatograms of perch fish (perch, ruff and pike perch) are quite different. The peak eluted at about 45 min is the most characteristic one in the chromatogram of pike perch.

Generally the resolution of peaks in the chromatograms is good. In each chromatogram there are four to nine resolved peaks having an area of more than 5% of the total sample peak area (Table 1). The highest number of overlapped peaks was observed for burbot (13). Additional information of the resolution can be obtained comparing the chromatograms of perch (10) and roach (7) (Fig. 1) and their mixture (Fig. 3). This comparison shows that all UV-absorbing sarcoplasmic proteins of perch and roach were resolved.

In our study, a photodiode-array detector (PAD) was used for the detection of sarcoplasmic proteins. The PAD is a powerful tool giving more information than traditional detectors operating at constant wavelength. Chromatograms can be recorded at any wavelength within the detection wavelength region. We have presented results at 280 nm where sarcoplasmic proteins have a broad and weak absorption maximum. In addition, we obtained ‘maxplot’ chromatograms, which represent detector responses at the wavelength of maximum absorbance within the wavelength range of 200–300 nm. Using ‘maxplot’ technique, we observed one to three additional peaks

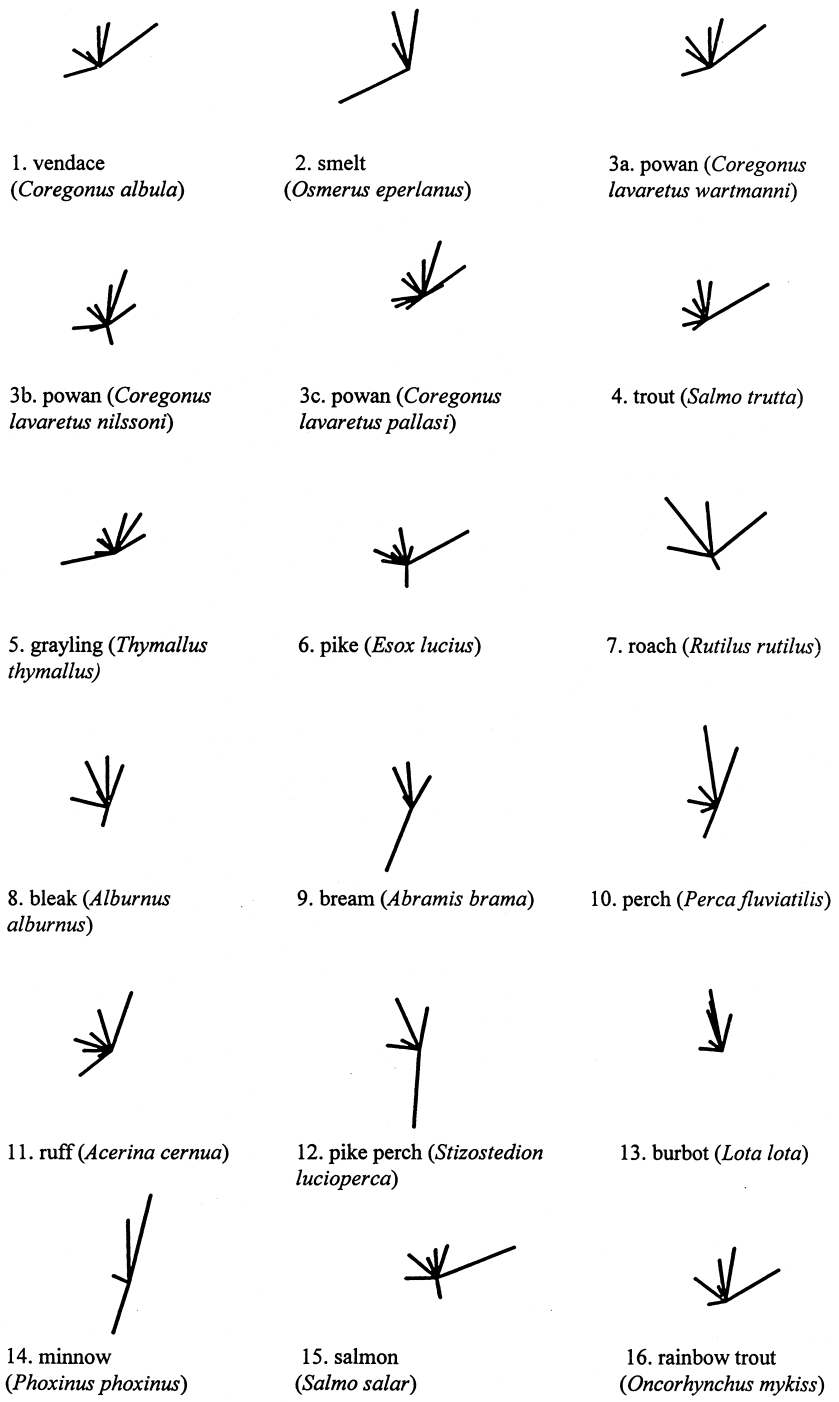

Fig. 2. Star-symbol plots of fish species based on chromatographic data at 280 nm. For details see Section 2.

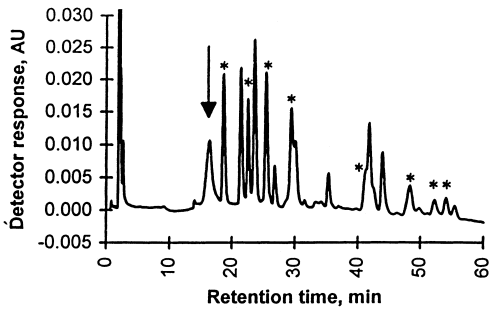

Fig. 3. HPLC chromatogram of sarcoplasmic proteins in the mixture of roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) flesh at 280 nm. Peaks of roach are marked by an asterisk (*). The BSA is marked by an arrow.

which had no absorption at 280 nm. This made it possible to obtain further information when differentiating closely related fish species. An example of the foregoing phenomenon is the comparison of the normal (at 280 nm) and 'maxplot' chromatograms of grayling (Figs. 1 and 4).

4. Conclusions

The present HPLC method provides new information about the differences between the chromatographic behaviour of sarcoplasmic proteins of several freshwater fish species. The procedure has many advantages over conventional electrophoresis, which requires that sufficient reference fish samples are available for each analysis of unknown fish species. The main disadvantage, however, is the rapid spoiling of the samples before HPLC experiment due to the degradation or aggregation of UV-absorbing sarcoplasmic proteins. In addition, the present HPLC procedure is suitable only for analysis of fresh (untreated) fish. The alteration of the absolute retention times is also a problem due to the continuous column contamination. Although there is some variation of absolute retention times, the relative retention times were found reproducible. The use of a suitable internal or external reference standard (BSA) and known standard fish samples derived after taxonomic verification of identity make it possible to identify unknown fish species reliably. In addition, solvent cleaning with 2-propanol is recommended to maintain good column performance. The identification is a routine operation if the chromatograms of the corresponding reference species are available in the chromatogram library. For identification experiments, the application of either HPLC or CZE (or both of them) is recommended. Additional experiments will be carried out at our laboratory to compare HPLC and CZE with freshwater fish samples and to study further the intraspecific differences of fish species such as various different powan.

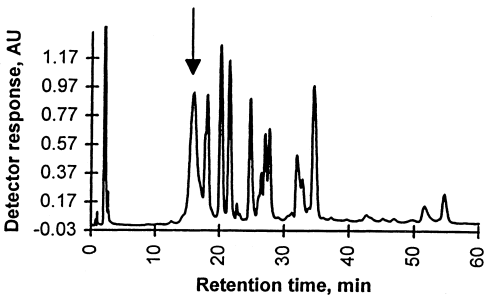

Fig. 4. Maxplot chromatogram of the grayling (*Thymallus thymallus*) presenting absorption at a wavelength of maximum absorption at any given time.

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