Food Chemistry 44 (1992) 147–155

Analytical Methods Section

The use of HPLC protein profiles in fish species identification

Sharyn G. Armstrong, David N. Leach

Muscle Foods Research Unit, University of Western Sydney (UWS), Hawkesbury, Richmond, NSW, 2753, Australia

&

S. Grant Wyllie

Centre for Biostructural and Biomolecular Research UWS, Hawkesbury, Richmond, NSW, 2753, Australia

(Received 7 May 1991; revised version received and accepted 24 June 1991)

A method for the rapid identification of fish species by high performance liquid chromatographic (HPLC) analysis of water-soluble sarcoplasmic protein extracts is described. The method relies on the visual comparison of a star-symbol plot constructed from the sample's HPLC profile with those obtained from the mean profile data of morphologically identified species. A library of plots is presented for 15 common edible marine fish species from eastern Australian waters, but any suitable data can be used for star construction. The data presented are applicable to the identification of samples from any season or location, whether they are raw, gamma-irradiated, or dried with infra-red radiation. The procedure involves a single 10 min extraction followed by a 60 min analysis, without the use of an internal standard.

INTRODUCTION

Knowledge of the species of fish destined for human consumption is necessary for both economic and nutritional reasons—including the meeting of quality control requirements and legal regulations, the adherence to fair pricing policies (Sumner & Mealy, 1983; Ashoor & Knox, 1985), and the enabling of species selection for dietary inclusion on the basis of known lipid contents and compositions (Naughton *et al.*, 1983; Brown *et al.*, 1989).

The potential of high performance liquid chromatographic (HPLC) analysis to provide species-specific profiles for raw fish samples has been demonstrated by Ashoor and Knox (1985). It is simple and fast to perform, uses equipment available in most laboratories, and produces reproducible profiles (Osman *et al.*, 1987), thus removing the need for running authentic samples. Some issues, however, remain to be addressed. These include intraspecies variation (within the one sample as well as between samples from different locations and seasons), loss of data through interference from the internal standard, and the utilisation of all collected data in the identification process.

This work addresses these issues and also examines the effects of gamma-irradiation on the characteristic profiles. The use of a statistical package to generate star-symbol plots to enable simple and rapid visual comparisons based on the quantitative data is described.

Food Chemistry 0308–8146/92/$05.00

MATERIALS AND METHODS

Samples

Ten individuals from each of five species (*Acanthopagrus australis* (Black bream), *Zeus faber* (John dory), *Centroberyx affinis* (Redfish/Nannygai), *Genypterus blacodes* (Ling), and *Zenopsis nebulosus* (Mirror dory)) were analysed. These were caught in the spring of 1989 from the coastal waters of New South Wales (33°53'S to 35°21'S). Four of these species were obtained from the same locations in autumn 1990 (Mirror dory not being available). A further 10 Black bream were also taken from both Victorian (37°50'S) and Queensland (25°17'S) waters during spring 1990.

Table 1. Mean HPLC data for each species

Peak number	Retention index	Percentage of total peak area														
		Black bream (n=10)	Redfish (n=10)	Mirror dory (n=10)	John dory (n=10)	Ling (n=10)	Orange roughy (n=5)	Pearl perch (n=3)	Trevally (n=3)	Red bream (n=3)	Mullet (n=3)	Jewfish (n=3)	Whiting (n=3)	Garfish (n=3)	Banana fish (n=3)	Ocean perch (n=3)
1	0·58	—	—	11·3	—	—	—	—	—	—	—	—	—	—	—	—
2	0·67	12·0	12·3	10·8	11·7	13·9	—	12·7	11·9	—	31·4	D31·3	—	—	—	8·8
3	0·73	—	—	—	—	—	13·3	—	—	—	—	—	—	21·9	—	15·6
4	0·81	12·4	13·6	—	—	—	14·2	13·2	27·5	—	—	—	—	—	—	2·1
5	0·89	—	—	—	13·4	—	—	—	—	25·0	—	—	—	—	—	—
6	1·00	16·0	—	D29·9	—	D14·4	—	13·3	—	—	—	D23·1	7·7	D20·0	—	—
7	1·08	—	—	—	13·1	—	—	—	D7·1	—	—	—	—	—	B39·3	6·8
8	1·13	8·9	14·3	—	14·6	—	6·4	—	—	—	21·5	—	24·4	—	—	—
9	1·18	—	D36·1	18·6	—	44·8	—	10·6	—	12·8	—	6·0	—	—	—	—
10	1·23	—	—	—	B20·5	—	D48·0	—	16·1	—	9·7	—	—	31·2	—	B26·9
11	1·29	D7·7	—	—	—	9·2	—	5·4	—	D18·8	—	—	11·9	—	—	—
12	1·49	—	—	—	—	2·5	—	—	—	4·9	3·5	D2·9	—	7·7	12·5	—
13	1·55	—	—	7·8	—	—	—	—	—	—	—	—	3·1	—	—	28·1
14	1·69	—	—	—	—	—	—	—	1·1	—	—	—	—	—	—	—
15	1·74	3·8	—	1·9	—	—	—	—	—	B5·4	D8·8	21·8	—	—	3·6	—
16	1·82	—	4·0	—	D7·8	—	4·0	—	B8·0	—	—	—	—	—	—	—
17	1·86	10·3	—	—	—	—	—	24·2	—	—	—	—	16·8	—	—	—
18	2·17	D10·1	—	—	7·2	—	3·7	3·7	—	—	—	B3·2	—	5·5	—	—
19	2·22	—	6·7	—	—	4·0	1·8	—	—	T12·5	6·1	—	—	—	B9·3	—
20	2·25	—	—	6·3	B4·9	—	2·3	—	—	9·7	—	6·5	6·7	—	—	—
21	2·39	—	8·2	5·1	—	1·1	6·3	—	6·7	—	D13·6	—	2·3	—	D26·0	3·4
22	2·50	B11·0	—	—	—	4·9	—	B3·6	—	3·2	—	—	18·1	B13·8	—	5·5
23	2·69	—	5·0	—	—	D2·2	—	—	12·4	—	5·4	—	—	—	—	—
24	2·72	B7·8[a]	—	—	6·8	—	—	—	—	7·7	—	—	—	—	—	—
25	2·78	—	—	4·9	—	3·0	—	8·2	—	—	—	—	—	—	—	B2·7
26	2·84	—	—	—	—	—	—	—	9·3	—	—	—	—	—	—	—
27	2·93	—	—	3·4	—	—	—	5·3	—	—	—	5·3	9·1	—	—	—
28	3·00	—	—	—	—	—	—	—	—	—	—	—	—	—	9·3	—

Complex peaks: broad (B); doublet (D); triplet (T).
[a]Peak that may not be resolved from the previous one.

Other fish analysed included *Hoplostethus atlanticus* (Orange roughy) (5), *Glaucosoma scapulare* (Pearl perch), *Argyrosomus hololepidotus* (Jewfish), *Mugil cephalus* (Mullet), *Chrysophrys auratus* (Red Bream/Schnapper), *Hyporhampus australis* (Garfish), *Sillago ciliata* (Whiting), *Paracaesio pedleyi* (Banana fish), *Pseudocaranx dentex* (Trevally), and *Helicolenus percoides* (Ocean perch) (3 of each) (May & Maxwell, 1986). These were caught in eastern Australian coastal waters, and purchased in Sydney fish markets.

Additional Black bream and Redfish fillets were gamma-irradiated in triplicate at doses of 1, 2 and 6 kGy in air. This treatment was performed by ANSTO (Australian Nuclear Science and Technology Organisation) at their Lucas Heights facility (Sydney, NSW). Duplicate fillets of John dory and Redfish were also subjected to infra-red radiation in a far infra-red radiation vacuum dryer (prototype developed by Dr M. Kurumazuka and Showa Manufacturing, Tokyo, Japan) for 15 h at 25°C. This treatment resulted in 55–65% of the initial mass being lost, corresponding to reducing the fillets to 43–56% water.

Sample preparation

Fillets were stored at –22°C prior to thawing and mincing.

Minced white muscle (5 g) was homogenised at high speed for 30 s in Milli Q water (20 ml) using a Waring blender. The supernatant was filtered through a 0·45 μm membrane and refrigerated. Analysis was carried out within 4 h of preparation.

HPLC analysis

Analyses were performed on a Waters HPLC system equipped with two model 501 pumps, a model U6K injector, model 720 programmable system controller, and a model 484 tunable UV detector (Waters Associates, Milford, MA, USA), fitted with a 250mm × 4·4 mm Hi-Pore RP-304 C-4 column (Bio-Rad Laboratories, Richmond, CA, USA).

Data were recorded and integrated using the DAPA computing integrator package (DAPA Scientific Software, Perth, WA, Australia).

The HPLC mobile phase was obtained from 30% (solvent A) and 70% (solvent B) solutions of acetonitrile in 0·1% trifluoroacetic acid. The extract (20 μl) was run using a gradient of 17 to 55% solvent B (37–52% acetonitrile) over 60 min, during which time the absorbance was recorded at 280 nm, 0·10 AUFS. A further 30 min was then required for washing the column with up to 100% solvent B, the return to initial conditions and equilibration.

Star-symbol plots were obtained using the STATGRAPHICS package of statistical software (Statistical Graphics Corporation, Rockville, Maryland, USA).

RESULTS AND DISCUSSION

Direct visual comparison of any two HPLC protein profiles is complicated by changes in peak retention times, widths and resolution. These differences arise due to even slight variations in the chromatographic parameters, such as mobile phase composition, gradient generation and temperature. The long analysis time causes a magnification of these effects in the later peaks. The method described was developed to overcome these problems, and also removed the need for the use of an internal standard.

Profiles of the 15 species (see Fig. 1) yielded a total of 130 peaks. The majority of them were common to a number of species and consolidation of the data resulted in 28 peaks that were adequately resolved under these chromatographic conditions. Each species profile consisted of up to 10 of these possible peaks, which are indicated in Table 1 along with their percentage areas. Determination of the position of each peak in relation to those from other species was facilitated by the co-injection of 50 combinations of pairs of species extracts. Relative retention indices were calculated with reference to a peak common to almost half (seven) of the species. It was found that the most reproducible indices were obtained by correcting all peaks for an appropriate time value such that this peak (peak 6) was shifted to a constant retention time (which on the present system was 19·0 min) before index calculation. Those fish species not containing this peak required co-injection of their extracts with ones that did, prior to retention index calculation.

To aid in the visual comparison of profiles a series of star-symbol plots was constructed. These consist of a series of radial lines originating from a common centre, each representing one of the 28 possible peaks, with its length in proportion to the corresponding percentage area. In this way unique stars were produced for each species (Fig. 2), from the mean data given in Table 1. Their value lies in the fact that they eliminate complications due to peak broadening and resolution variation, and provide characteristic and easily recognisable shapes, based on complex multivariate data.

The intraspecies variation is also most easily observed by comparison of star-symbol plots. Figure 3 shows ten stars obtained from the individual profiles of the Black bream caught in spring off Sydney, Australia. Also included are stars constructed from the mean data calculated for a similar sample taken 6 months later, as well as ones from Queensland and Victorian waters during the following spring. Although minor differences are displayed, each star can be easily identified as belonging to Black bream, when compared with the species stars in Fig. 2.

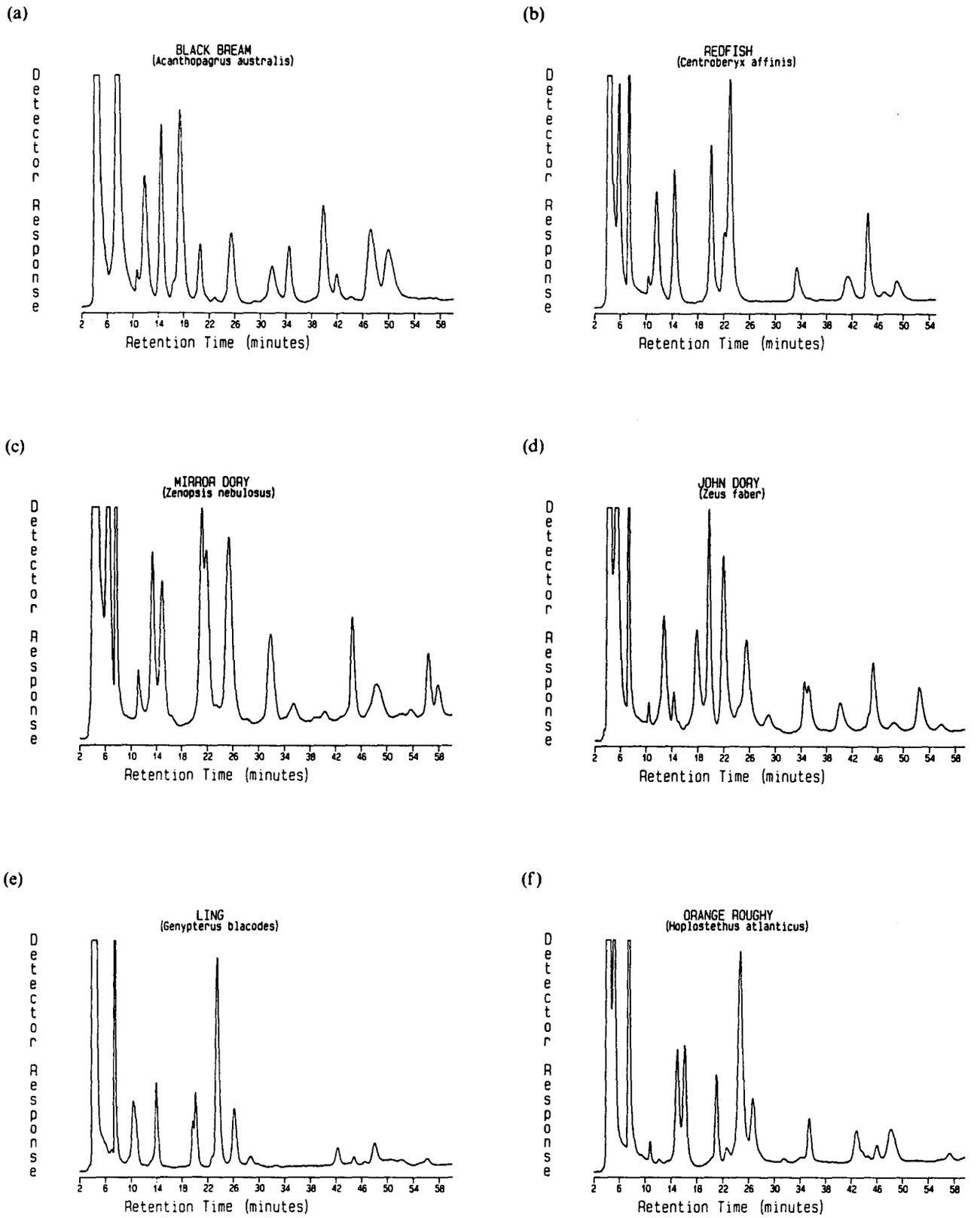

Fig. 1. Chromatograms of authenticated species samples.

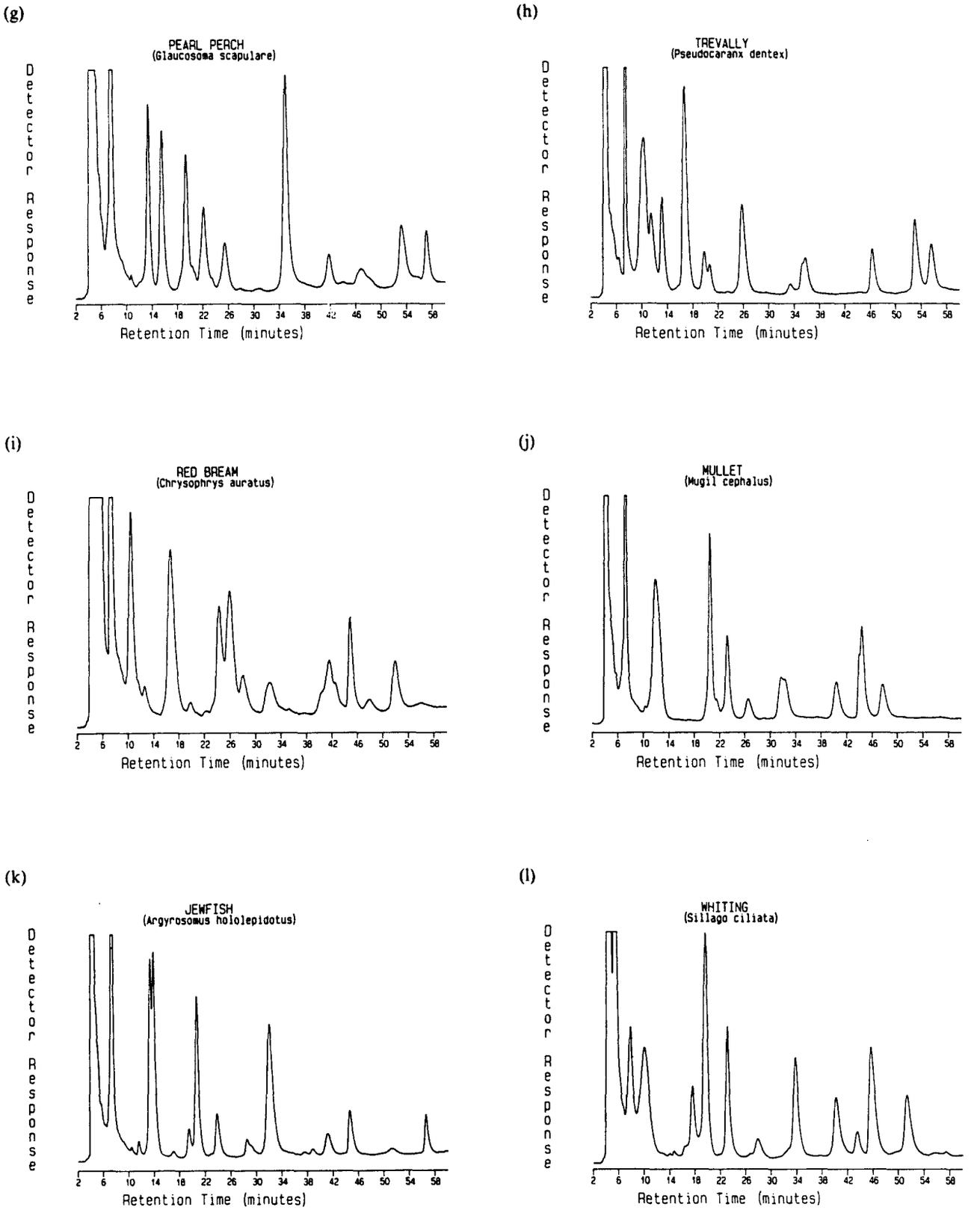

Fig. 1. Chromatograms of authenticated species samples—*continued.*

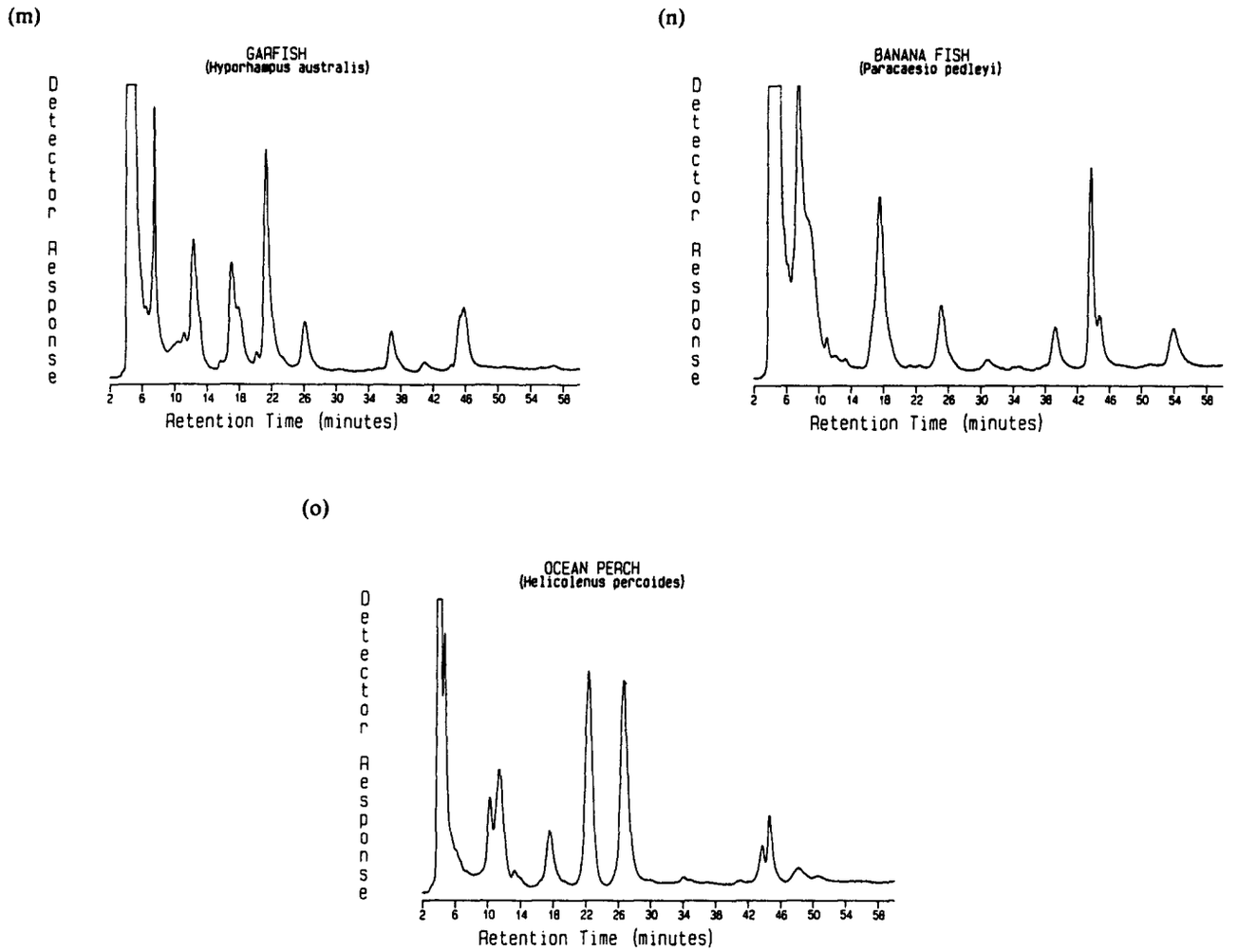

Fig. 1. Chromatograms of authenticated species samples—*continued.*

The low intraspecies variability established enabled the sample size to be reduced from 10 to 3 individuals for subsequently acquired characteristic species profiles.

A procedure for the identification of an unknown sample's species is suggested in Fig. 4. It was validated in the authors' laboratory on 21 unlabelled fillets, representing 12 of the library species. Only one injection was necessary in each case to provide a profile that appeared to match one from the library, and resulted in a matching star. All identifications were correct—matching morphological classifications. The use of such a procedure is necessary when an authentic sample of the species whose profile is a suspected match is not available, or the extra injection not desirable, as co-injection of the two extracts would obviously conclusively prove or disprove the match.

It is envisaged that any laboratory following the experimental procedure given here can directly use the species data provided. The general applicability of the library is justified by its proven lack of variation with sampling changes, and the relative nature of the retention data. Extension to include other species would be possible after only a few co-injections with defined library species, due to the complete coverage of the profile time range by the 28 defined peaks. Their addition would simply involve the determination of which of these possible peaks are present, along with calculation of the mean peak percentage areas.

The authors have successfully constructed 31 easily distinguishable stars from the data of Osman *et al.* (1987), thus demonstrating the general use of this identification procedure. Characteristic stars were obtained using both data sets from a maximum of only 10 peaks. This was adequate to identify even closely related species, including Steelhead and Pink salmon, and Arrowtooth and Witch flounder from Osman's data (*ibid.*); Black and Red bream, and Mirror and John dory from the data presented here (Fig. 2).

Species identification was still possible after gamma-irradiation at normal radurisation levels (1 and 2 kGy), as well as the much higher dose (6 kGy). Drying in an infra-red drier under the conditions specified also maintained the characteristic species profiles, and resulting stars (see Fig. 5). It is expected that the method will be

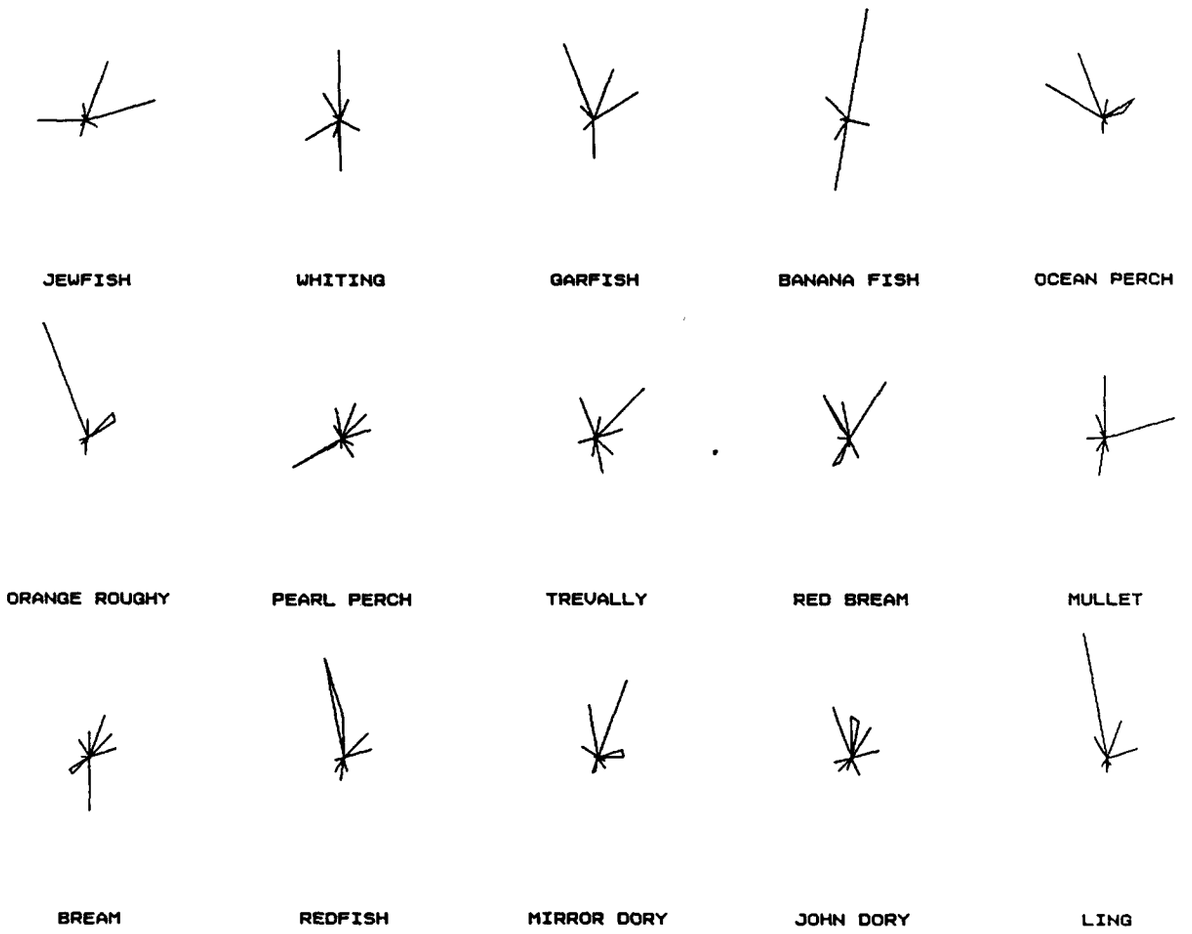

Fig. 2. Species star-symbol plots.

applicable to fish processed by any technique that does not apply heat to or generate it within the sample.

This HPLC method can be used in place of the current electrophoretic techniques for the identification of raw (fresh or gamma-irradiated) or infra-red dried fish samples; however, where samples have been cooked, species identification still requires the use of iso-electric focusing (Rehbein, 1990).

STATISTICAL APPENDIX

In constructing a set of star-symbol plots, STATGRAPHICS scales each peak relative to the range of values for the corresponding peaks in all stars of the data set. This was not desired as it caused distortion of each star in a way that was dependent on the nature of the other observations in the same set. One outcome of this was the loss of the smallest value for each peak over the data set, as it was scaled to be equal to zero. Correction was made by entering two constant value 'dummy' stars with each set of observations with values slightly above the maximum data value, and below the minimum, respectively. An artificial range bounding that of the data was thus imposed. In this way characteristic stars were maintained irrespective of the nature of accompanying stars in the same data set.

ACKNOWLEDGEMENTS

The authors wish to thank Dr K. Hammerton of ANSTO, Lucas Heights, (Sydney, NSW, Australia) for performing the gamma-irradiation of samples. Drying was done by Mr K. Kawaura (Kawaura & Co. Pty Ltd, Crows Nest).

The STATGRAPHICS (Statistical Graphics Corporation, Rockville, Maryland, USA) package was used to generate star-symbol plots. The advice of Professor D. B. Hibbert of the University of New South Wales is gratefully acknowledged.

The authors are grateful to Associate Professor R. Greig of Ballarat University for his assistance in many aspects of this work.

Samples were supplied by the Sydney Fish Marketing Authority (FMA) and Poulos Bros. Pty Ltd, Sydney. Morphological identifications were made by Mr K. Harada of the Sydney FMA.

One of us (SGA) is in receipt of a UWS, Hawkesbury Graduate Research Scholarship.

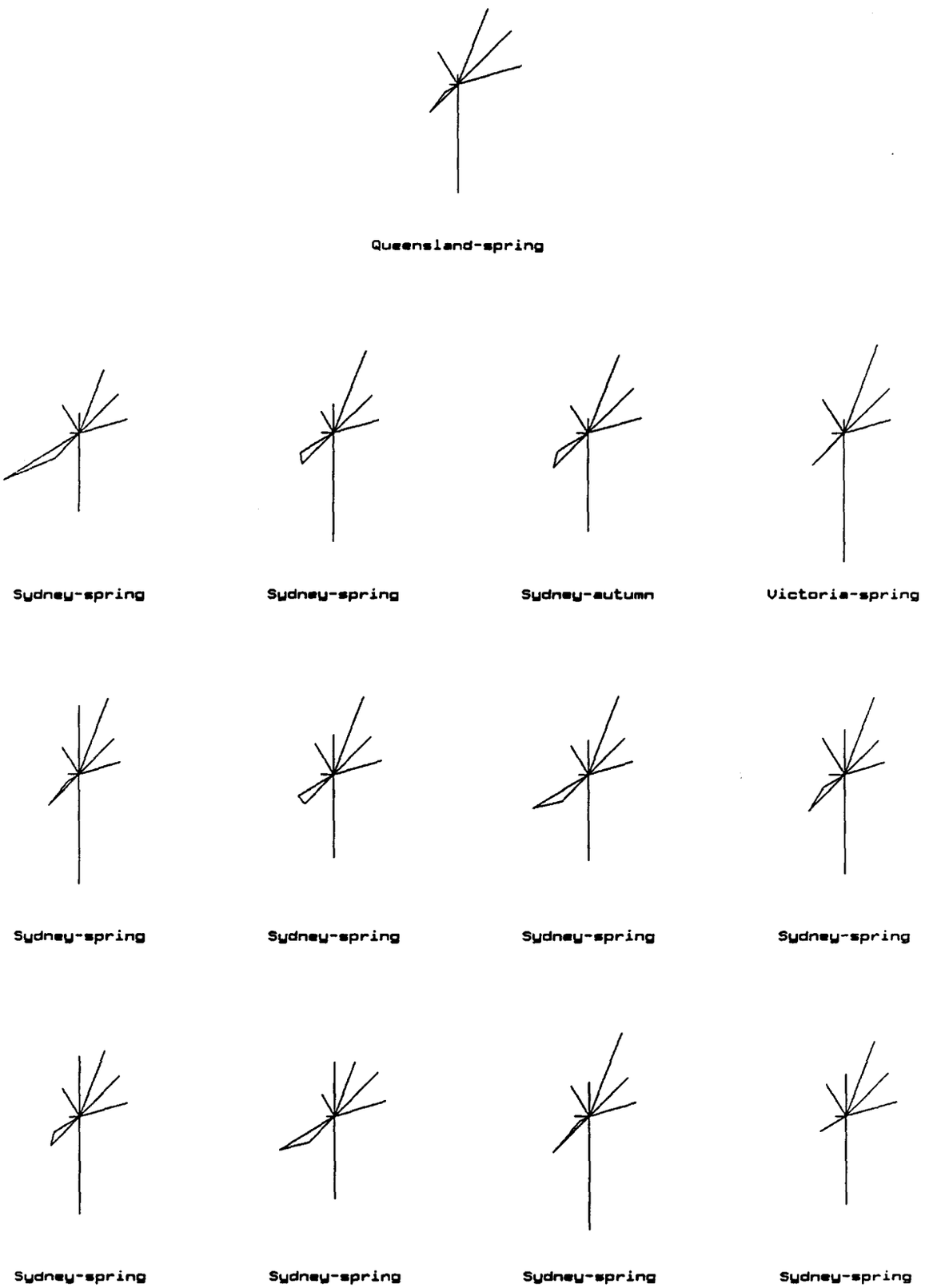

Fig. 3. Black bream (*Acanthopagrus australis*) star-symbols, showing intra- and inter-sample variation.

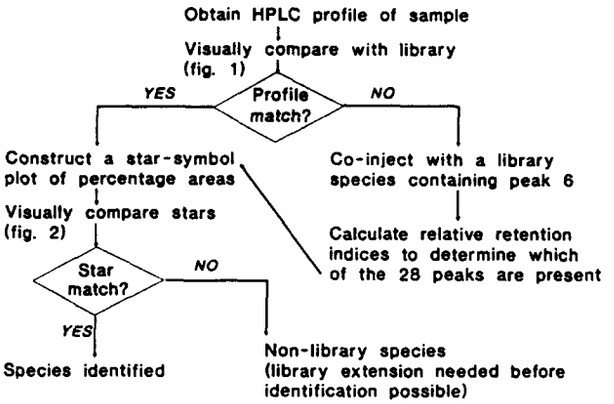

Fig. 4. Species identification procedure.

REFERENCES

Ashoor, S. H. & Knox, M. J. (1985). Identification of fish species by high-performance liquid chromatography. *J. Chromatogr.*, **324**, 199–202.

Brown, A. J., Roberts, D. C. K. & Truswell, A. S. (1989). Fatty acid composition of Australian marine finfish: a review. *Food Aus.*, **41**, 655–66.

May, J. L. & Maxwell, J. G. H. (1986). *Field guide to trawl fish from temperate waters of Australia.* CSIRO Division of Fisheries Research, Hobart, Australia.

Naughton, J. M., O'Dea, K. & Sinclair, A. J. (1983). The fatty acid composition of fish caught in Bass Strait. *Proc. Nutr. Soc.*, **8**, 175.

Osman, M. A., Ashoor, S. H. & Paul, C. M. (1987). Liquid chromatographic identification of common fish species. *J. Assoc. Off. Anal. Chem.*, **70**, 618–25.

Rehbein, H. (1990). Identification of fish species by HPLC of water-soluble proteins. *Informationen für die Fischwirtschaft.*, **37**(1), 25–31.

Sumner, J. L. & Mealy, S. W. (1983). A survey of substitution of fish fillets in Melbourne. *Food Tech. Aus.*, **35**, 332–4.

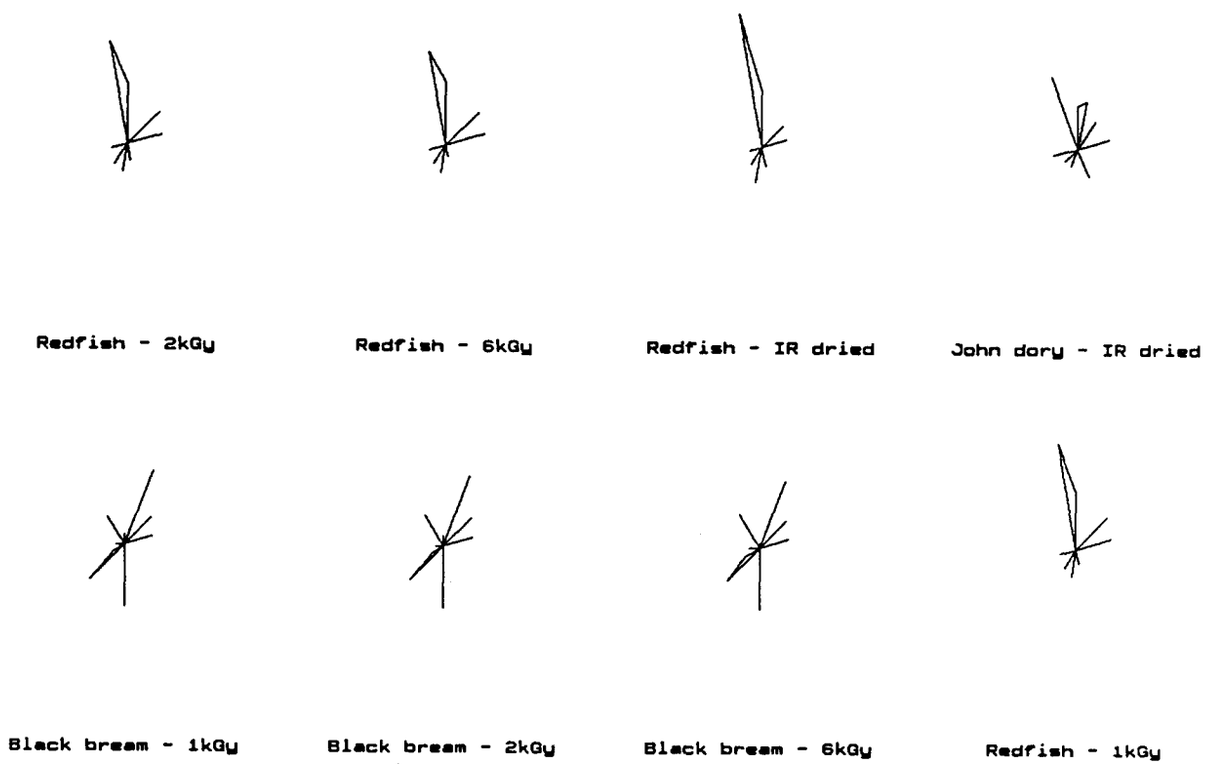

Fig. 5. Star-symbols of gamma-irradiated (n = 3) and infra-red dried (n = 2) samples.