Journal of Chromatography, 261 (1983) 153–157
Elsevier Science Publishers B.V., Amsterdam — Printed in the Netherlands

CHROM. 15,736

Note

Improved high-performance liquid chromatographic method for analysis of histidine dipeptides anserine, carnosine and balenine present in fresh meat

P. R. CARNEGIE*, M. Z. ILIC, M. O. ETHERIDGE and M. G. COLLINS
School of Agriculture, La Trobe University, Bundoora, Vic. 3083 (Australia)
(Received January 31st, 1983)

The ratio of the histidine dipeptides which are present in skeletal muscle, has been proposed as a useful aid to the identification of the species or origin of meat used in processed meats[1–3]. These peptides, anserine (β-alanyl-1-methylhistidine), balenine (β-alanyl-3-methylhistidine, ophidine) and carnosine (β-alanylhistidine) can be separated on conventional amino acid analyzers with modified programs[1,2]; however, the elution times are rather long. Nakamura *et al.*[4] described a high-performance liquid chromatographic (HPLC) procedure which achieved a separation in 30 min on a Partisil-10 SCX column. Their procedure has two disadvantages, the use of citrate buffers at 50°C and the need for gradient elution. Partisil-10 SCX is a sulphonated silica column which would rapidly deteriorate in performance due to the effect of citrate on the silica especially at an elevated temperature[5,6]. Secondly, gradient elution requires more elaborate equipment and an equilibration phase.

As there is an increasing need for a rapid method for the monitoring of the species of origin of meat used in meat products, we have developed a simpler and more rapid isocratic system for the separation of the histidine dipeptides present in muscle of animals commonly used for meat.

MATERIALS

Muscle samples were obtained from pigs and lambs raised at La Trobe University. Dr. A. J. Sinclair, Department of Agriculture, Victoria, Australia, provided samples of meat from several species used in the development of his isoenzyme method for species identification[7]; these are detailed in Table I. Samples of meat from rabbits, chickens and cattle were obtained from a local market.

Balenine was a gift from Dr. J. Wolff, National Institutes of Health, Bethesda, MD, U.S.A. Anserine, carnosine and *o*-phthaldialdehyde (OPA) were from Sigma, St. Louis, MO, U.S.A.

The HPLC column was a prepacked Partisil-10 SCX column, 25 cm × 4.6 mm I.D. Lot No. IE 5243 from Whatman, Clifton, NJ, U.S.A.

0021-9673/83/$03.00 © 1983 Elsevier Science Publishers B.V.

TABLE I

CONTENT OF HISTIDINE DIPEPTIDES IN MEAT FROM VARIOUS SPECIES

Ans = Anserine; Car = carnosine; Bal = balenine; standard deviations are given in parentheses. The moisture content of the samples was determined[3] and taken into consideration in the calculations. For buffalo, donkey, horse and goat samples a value of 75% moisture was assumed[9].

Species	*No. of samples*	*Source**	*Dipeptides (µmol/g fresh meat)*				*Ans:Car:Bal ratio*
			Total	*Ans*	*Car*	*Bal*	
Pig	8	a	13.6	0.66	12.2	0.75	1:18.4:1.1
			(5.3)	(0.08)	(4.9)	(0.38)	
Beef	5	b	17.1	2.3	14.7	0.07	1:6.4:0.03
			(3.6)	(0.4)	(3.3)	(0.03)	
Buffalo	2	c	18.2	2.1	15.9	0.2	1:7.5:0.1
Goat	3	d	10.7	8.4	2.3	0	1:0.3:0.0
			(4.2)	(3.1)	(1.2)		
Lamb	44	e	9.9	4.9	4.9	0.1	1:1.0:0.02
			(3.1)	(1.5)	(1.7)	(0.1)	
Sheep	3	f	16.8	8.3	8.4	0.1	1:1.1:0.01
			(3.1)	(2.2)	(1.3)	(0.03)	
Horse	3	g	18.0	0.2	17.8	0	1:89:0.0
			(6.1)	(0.04)	(6.4)		
Donkey	3	h	12.2	0.1	12.1	0	1:121 :0.0
			(6.1)	(0.03)	(6.0)		
Kangaroo	5	i	18.2	15.9	2.3	0	1:0.1:0.0
			(4.6)	(3.8)	(1.9)		
Rabbit	2	j	21.1	18.9	2.2	0	1:0.1:0.0

* Source: (a) four shoulder and four leg samples were obtained from Large White pigs aged 4 to 6 months raised at La Trobe University; (b) samples of topside, rump and shin were obtained from local butchers; (c) two samples of Australian water buffalo meat as used in ref. 7; (d) three samples of goat meat as used in ref. 7; (e) twenty-two samples of shoulder and twenty-two of leg from 6 to 7 month old lambs raised at La Trobe University; (f) three samples of leg from mature sheep as used in ref. 7; (g) two samples of horse rump and one of shoulder as used in ref. 7; (h) three samples of donkey rump as used in ref. 7; (i) three samples from three species of kangaroo as used in ref. 7, and two samples of mince from unidentified species of kangaroo obtained from a pet food shop. There was no significant difference between the species; (j) one wild rabbit obtained from the local market and a sample of mince from a pet food shop.

METHODS

Extraction of dipeptides

Using a Sorvall Omni-mixer at 8000 rpm for 1 min, histidine dipeptides were extracted from 30 g lean muscle samples with 30 ml 0.9% saline and 120 ml 8% 5-sulphosalicylic acid (BDH, Poole, Great Britain). The homogenates were centrifuged at 10,000 *g* at 5°C for 1 h. The supernatant fractions were filtered through a Millipore pre-filter (type AW) and filter (type GS, 0.22 µm diameter pores). Prior to HPLC analysis, the samples were centrifuged at 8000 *g* for 4.5 min in a Beckman Microfuge.

Chromatography

The dipeptides in 5 µl of extract were separated on a Whatman Partisil-10

SCX column with a lithium formate buffer containing 0.2 *M* lithium hydroxide titrated to pH 2.9 with formic acid. The column was operated at 40°C at a flow-rate of 0.7 ml min^{-1} from a Waters pump (Model 6000A). The eluate was mixed with OPA reagent[4] delivered at a rate of 1.2 ml min^{-1} with a Technicon high-pressure micro pump. Since it was found that the OPA reaction is temperature sensitive, the post-column reaction coil was immersed in a 30°C bath. The derivatives of the dipeptides were detected with a Waters Model 420 fluorescence detector. The output from the detector was automatically integrated by a Hewlett-Packard 3390A integrator. The reliable range for determination of the concentration of individual dipeptides was from 0.05 to 7.0 μmol/g of meat. Where higher concentrations were present samples were diluted five-fold or ten-fold with distilled water and rechromatographed.

RESULTS

Typical separations of the histidine dipeptides in muscle from pig, sheep, horse and kangaroo are shown in Fig. 1. The silica column with benzene-sulphonic acid functional groups provided a much faster elution time and a greatly improved separation of the dipeptides from each other and from amino acids compared to that obtained on sulphonated polystyrene columns used in amino acid analyzers. From the latter anserine, carnosine, histidine, balenine eluted close together[3] while from

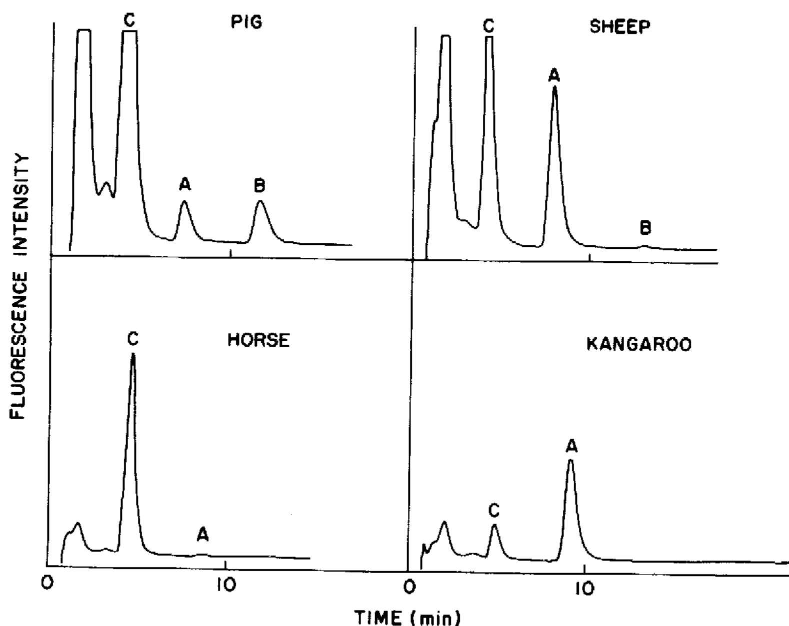

Fig. 1. Typical results of analysis of histidine dipeptides in meat samples from various species. Separation was on a Partisil-10 SCX column with 0.2 *M* lithium formate pH 2.9 at 40°C under isocratic conditions with post-column derivatization with OPA. For the pig and sheep 5 μl of the extract were applied and for the horse and kangaroo samples the extract was diluted ten-fold and five-fold, respectively. The figure was prepared from the output of a Hewlett-Packard integrator. Peaks: A = anserine; C = carnosine; B = balenine.

the HPLC column the order of elution was histidine, carnosine, anserine and balenine with a clear separation between them. Fortunately the numerous amino acids present in meat extracts were eluted prior to the dipeptides. Over 500 samples were applied to the column with no obvious deterioration in its performance other than a slight decrease in the elution times, however after 700 runs resolution of carnosine from amino acids became unsatisfactory. Performance was restored by altering the buffer concentration to 0.1 *M*.

To test the reproducibility of the method four samples of a batch of minced pork were analyzed and the values for the total and individual dipeptides were reproducible to within ±3% and ±5% respectively. The HPLC method was more reliable than the amino acid analyzer method for the determination of carnosine because of the wider analytical range. With fresh meat under normal storage conditions, there is little or no change in the concentration of the dipeptides following slaughter[1,3].

With some species there is a variation in the total concentration of histidine dipeptides in different muscles. For example, in the pig, shoulder has less than leg and the anserine-to-balenine ratio is lower in the former[3]. With lamb no significant difference in the concentration was found between shoulder and leg nor any significant difference with age. Compared to pigs there was more variation in the total histidine dipeptide concentration in lamb muscle from different animals. Further details on the difference in dipeptides between different muscles will be reported elsewhere. As these differences are much less than the differences between meat from different species data from individual species has been pooled and is presented in Table I. The relatively large standard deviations are a reflection on this variation between tissues and between animals of the same species. Statistical analysis of the difference in anserine and carnosine contents between species indicated that these differences were sufficient to obtain a reliable identification of meat from several species Table II. Olsman and Slump[1] came to a similar conclusion. Despite the large differences in ratios of the dipeptides between species it was surprising that the total histidine dipeptide content fell within a relatively narrow range for mature animals. Presumably this is a reflection on their physiological role which remains to be defined. It has been suggested that they function as buffers of lactic acid in muscle[8].

TABLE II

STATISTICAL SIGNIFICANCE OF DIFFERENCES BETWEEN ANSERINE AND CARNOSINE CONTENTS OF MEAT SAMPLES FROM DIFFERENT SPECIES

Data in Table I were analyzed by a BAR 3 statistical package on a VAX 11/780 computer and levels of significance were calculated by the *F* test of Campbell[10]. The first figure in each pair refers to the anserine and the second to the carnosine content. NS = Not significant.

	Pig	*Goat*	*Beef*	*Horse*
Lamb	0.01,0.01	0.05,0.05	0.01,0.01	0.01,0.01
Pig	–,–	0.01,0.01	0.01,NS	0.01,NS
Goat		–,–	0.05,0.05	0.01,0.05
Beef			–,–	0.01,NS

CONCLUSION

The ratios of the histidine dipeptides were found to be quite characteristic in skeletal muscle of animals of the same or related species. For example, if meat from horse, kangaroo or sheep was mislabelled as beef, there would be no difficulty in distinguishing the meats as the ratio of anserine to carnosine and the amount of anserine is so different (Tables I and II). However, it would not be possible to distinguish between meat from buffalo and cattle, from horse and donkey, nor from kangaroo and rabbit. However meat from these species can be distinguished by electrophoresis of isoenzymes[7].

Because the serological and electrophoretic methods for species identification are unsatisfactory with cooked meats the proposed HPLC method could be applied to this problem. Tinbergen and Slump[2] were able to estimate the proportion of chicken meat in luncheon meats and Carnegie *et al.*[3] were able to distinguish between hams of different origins, by measuring the amount and ratio of the histidine dipeptides. Recently we have used the HPLC method to analyse samples of meat pies, luncheon meats, hams and sausages which were suspected to contain meats other than those specified on the label and obtained rapid and clear identification of the source of the meat in the product, these results will be reported elsewhere.

ACKNOWLEDGEMENTS

We thank the Australian Meat Research Committee and the Australian Pig Industry Research Committee for financial support and Dr. A. J. Sinclair for some samples of meat from various species.

REFERENCES

1 W. J. Olsman and P. Slump, *Developments in Meat Science —2*, Applied Science Publishers, London, 1981.
2 B. J. Tinbergen and P. Slump, *Z. Lebensm.-Unters.-Forsch.*, 161 (1976) 7.
3 P. R. Carnegie, K. P. Hee and A. W. Bell, *J. Sci. Food Agr.*, 33 (1982) 795.
4 H. Nakamura, C. L. Zimmerman and J. J. Pisano, *Anal. Biochem.*, 93 (1979) 423.
5 F. M. Rabel, *J. Chromatogr. Sci.*, 18 (1980) 394.
6 P. Griffiths, Whatman Inc., Clifton, NJ, personal communication.
7 W. J. Slattery and A. J. Sinclair, *Aust. Vet. J.*, 60 (1983) 47.
8 E. C. Bate-Smith, *J. Physiol. (London)*, 92 (1938) 336.
9 R. A. Lawrie, *Meat Science*, Pergamon Press, Oxford, 1979.
10 R. C. Campbell, *Statistics for Biologists*, Cambridge University Press, London, 1974.