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Contents of biogenic amines, Total Aerobic Counts, and prevalence of nematode larvae and *Listeria monocytogenes* in fish and fish products sold at retail in Vienna

Gehalte an biogenen Aminen, aerobe Gesamtkeimzahl und Vorkommen von Nematodenlarven und Listeria monocytogenes in Fisch und Fischereierzeugnissen in Wien

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Summary

Ready to eat (RTE) fishery products (n = 53) and non-ready to eat (Non-RTE) fish samples (n = 49) were obtained from retail shops in Vienna. The 102 samples were analyzed for content of biogenic amines, total aerobic count and *Listeria monocytogenes* (in 25 g). Raw and marinated saltwater fish was tested for nematodes.

Histamine content >100 mg/kg was observed in one tuna (496 mg/kg) and one trout (280.7 mg/kg) fillet. For histamine and spermine contents, a significant ($P \leq 0.05$) effect of fish species was observed, whereas putrescine was significantly affected by processing mode and storage temperature. No significant correlation was found between amine contents and total aerobic counts. *Listeria monocytogenes* were detected in one RTE sample and four Non-RTE samples. Presumptive nematode larvae were found in 11 Non-RTE and two RTE samples.

Keywords: Fish, market samples, biological hazards, biogenic amines

Zusammenfassung

Verzehrsfertige Fischereierzeugnisse (RTE, n = 53) und nicht-verzehrfertiger Fisch (Non-RTE, n = 49) wurden aus Lebensmittelgeschäften in Wien bezogen. Die 102 Proben wurden auf den Gehalt an biogenen Aminen, aerobe mesophile Keimzahl und *Listeria monocytogenes* (nachweisbar in 25 g) untersucht. Roher und marinierter Seefisch wurde auf Nematodenlarven untersucht.

Histamingehalte >100 mg/kg wurden in je einem Thunfisch- (496 mg/kg) und Forellenfilet (280,7 mg/kg) bestimmt. Histamin- und Spermingehalte wurden signifikant ($P \leq 0.05$) von der Fischspezies bestimmt. Die Putrescingehalte wurden signifikant von der Verarbeitungstechnik und Lagerungstemperatur beeinflusst. Zwischen der aeroben mesophilen Keimzahl und den Amingehalten bestand kein signifikanter Zusammenhang. *Listeria monocytogenes* wurden in einer RTE Probe und in vier Non-RTE Proben gefunden. Präsumtive Nematodenlarven wurden in 11 Non-RTE und zwei RTE Proben gefunden.

Schlüsselwörter: Fisch, Marktproben, biologische Gefahren, biogene Amine

Introduction

From the beginning of history humans are using marine and fresh water animal products for their food (EFSA, 2010). Among them fish represents a valuable source of white meat, providing approximately one-fifth of animal protein consumption and other nutrients in many parts of the world (Esteban, 2012). Fish meat is well suitable for human nutrition, because it contains easily digestible proteins, vitamins, minerals and particularly long chain polyunsaturated fatty acids (n-3) important for cardiac health, brain development and reproduction. It is consumed as fresh fish or as frozen, salted, dried, smoked, canned or as fermented products.

In Austria, the *per capita* consumption of fish amounts to 7.7 kg annually. Most fish and fishery products are imported from Denmark, Poland, France and Germany, i. e. 66,159 tonnes per year which represents 93 % of the total fish consumption (Statistics Austria, 2013).

As regards food safety, fish and fishery products may contain a variety of biological hazards. Among those of bacterial origin, Listeriae are known to be associated with cold-smoked fish fillets (Eklund et al., 1995; Feldhusen et al., 2001; Gram, 2001).

Besides pathogenic bacteria, compounds produced by bacterial metabolism are also classified as biological hazards. For instance, this applies to biogenic amines which are formed by degradation of amino acids (Silla-Santos, 1996). The formation of such amines is largely governed by the abundance of free amino acids as precursors and the presence and activity of endogenous or bacterial enzymes; in particular, the flesh of certain fish species is rich in free histidine, which can be converted to the vasoactive histamine. Quite recently, tolerable values for dietary biogenic amines have been elaborated by EFSA (EFSA, 2011) at EU level, and – more specifically taking into account local dietary habits – for Austria (Rauscher-Gabernig et al., 2009; Paulsen et al., 2012; Rauscher-Gabernig et al., 2012).

Fish-borne parasites are relevant as agents causing infectious disease. Worldwide the number of people at risk, including those in developed countries, is more than half a billion (EFSA, 2010).

In the EU, the Rapid Alert System for Food and Feedstuff (RASFF), has, since its establishment in 1979, recorded 494 notifications of parasitic infestion of fish – 366 of which explicitly related to *Anisakis* sp. (2 from Austria) – and among 617 notifications of pathogenic bacteria, 432 related to *Listeria monocytogenes* (52 from Austria). In Austria, cases of fish-derived human Anisakiasis cases have been reported (Auer et al., 2007; Kapral et al., 2009).

It can be argued, that "safe" processing practices would inactivate the parasites, and, thus, eliminate the risk. However, the role of fish borne parasites as allergens should not be neglected. Nowadays, *Anisakis* allergens are included in the standard sets of allergens during allergy investigation (Garcia et al., 2001). These allergens may be present in food as excretory and secretory antigens from living larvae or somatic and cuticular antigens from dead and disintegrating larvae (Audicana and Kennedy, 2008). Among all these, Ani s 4 is a clinically relevant allergen due to its heat-, acid- and pepsin resistant properties and its importance in the anaphylaxis process (Rodriguez-Mahillo et al., 2008; Vidacek et al., 2009; Vidacek et al., 2011).

The main task of this work was to present recent data on biogenic amines contents in fish retailed in Vienna, and to assess if these would exceed tolerable values. In addition, other biological hazards were studied, depending on the type of fish and the mode of processing.

Materials and Methods

Acquisition of samples

A total of 102 samples (one original packing unit or ≥200 g fresh fish fillet) of 23 types of fishes of processed and semi processed fish meat products (i. e. raw chilled, raw frozen, smoked and marinated products) were purchased from major retail stores of Vienna city (Table 1). Particular care was taken that similar types of products originated from different brands (suppliers). All products were transported to the laboratory in chilled storage conditions at 4±3 °C, if refrigerated storage was indicated on the label, otherwise at ambient temperature.

Examination of samples

Examination of samples started within 24 h after arrival at the laboratory, but always before the "use by" or "best before" date. Examination procedure differed according to processing technology (Table 1).

For fish canned in brine or oil, the liquid was drained off, and the flesh homogenized. Then, aliquots were taken for microbiological examination (10 g for TAC; 25 g for *Listeria*) and 10 g for determination of biogenic amines.

Samples were subjected to microbiological analysis, determination of biogenic amines and pH. In brief, total aerobic count was determined by plating out of serial tenfold dilutions (MRD, OXOID CM0733) on plate count

TABLE 1: *Overview on samples and tests conducted.*

Origin of fish	Processing of fish	n =	Ready-to-eat product?	TAC, n =	biogenic amines, n =	Listeria, n =	Nematodes n =	Main species
Freshwater	Raw on ice	5	no	5	4	5	5	Trout (n = 3)
	Raw-deep frozen	5	no	5	4	5	5	Trout (n = 3)
	Smoked	3	yes	3	3	3	3	Trout (n = 3)
	total	13		13	11	13	13	
Saltwater	Raw on ice	9	no	9	9	9	9	Salmon (n = 4)
	Raw-deep frozen	30	no	30	26	30	30	Salmon (n = 15), pollock (n=5), cod (n = 3)
	Smoked	16	yes	16	11	16	16	Salmon (n = 15)
	Marinated	6	yes	6	4	6	6	Herring (n = 4)
	Fully retorted can	28	yes	28	23	28	28	Mackerel (n = 9); sardine (n=6); tuna (n=5); herring (n=5)
	total	89		89	73	89	89	

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agar (Merck 1.05463) and counting colonies after 72 h incubation at 30 °C (ISO, 2003), and the presence of *Listeria monocytogenes* in 25 g aliquots was assessed after enrichment in $^1/_2$ Fraser broth (Oxoid CM0895, SR0166), incubation 72 h at 30 °C, then culturing the enrichment broth on ALOA agar (CM1084, SR0226), incubation 72 h at 30 °C (Fraser and Sperber, 1988). Biogenic amines were determined as follows: 10 g of minced sample are homogenized with 90 g 10 % (w/v) trichloroacetic acid and filtrated. Biogenic amines were derivatised under alkaline conditions with dansylchloride at 70 °C. Dansylated amines were separated using an HPLC method and were detected by UV-VIS absorption (Paulsen et al., 2006).

In addition, 23 types of fish from different fishing zones were analyzed for nematodes by two different techniques, i. e. fluorescence detection (fillets pressed to a thin slice were examined under 350 nm illumination) and peptic digestion method (200 g minced fish are suspended with 6 g pepsin and 8 ml 25 % HCl in 1L water, stirred at 40–45 °C for 20–30 min. and filtered through a 1 mm² diameter mesh. Longitudinal objects retained by the mesh are examined under the microscope) (Brattey, 1988; Karl et al., 2002).

Statistical processing of results

Biogenic amine contents were related to fish family, temperature and processing technology (independent factors) via ANOVA, with Fisher's LSD to discriminate among means (Statgraphics 3.0; Princeton, NJ, USA). Statistical significance was established at $P \leq 0.05$. Correlation analysis was done for log transformed bacterial numbers against biogenic amine contents.

Results and Discussion

Contents of biogenic amines in fish at retail in Vienna, and comparison with legal limits and tolerable values

Table 2 relates biogenic amines amount in RTE and Non-RTE fish products to legal limits and recommended tolerance levels. None of the RTE samples exceeded legal limits or maximum tolerance levels. Among Non-RTE fish, amine contents above limits or tolerance values were detected in four of 39 samples: trout, 30.9 mg/kg ß-phenylethylamine; Atlantic cod, 622 mg/kg cadaverine; tuna and trout with 496 mg/kg and 280.7 mg/kg histamine, respectively. Considering that histamine levels are hardly affected by culinary preparation of fish (Hagen et al., 2005), a portion could contain roughly 50 mg histamine, a dose which could induce circulatory effects even in non-predisposed humans (Rauscher-Gabernig et al., 2009).

As regards quality assessment, levels of histamine, tyramine, phenylethylamine and tryptamine of exceeding 100, 800, 30 and 25 mg/kg, respectively, are considered unacceptable (Özden and Erkan, 2005). These levels were exceeded by 2, 1, 4 and 0 of the total of 102 samples, respectively. The 4 samples exceeding 100 mg/kg histamine were two samples each of smoked salmon and frozen salmon fillets.

Relation of biogenic amines content to fish species/family and processing technique

Statistically significant relations were observed for processing technology/storage temperature and putrescine content ($P = 0.001$). In detail, higher putrescine contents were associated with raw fish fillets stored on ice. This was not unexpected and reflects incipient spoilage during cold storage of proteinaceous foods.

A statistically significant fish-species specific effect was demonstrated for spermine ($P = 0.04$) and histamine ($P = 0.002$), with higher contents in mackerel, sardine, tuna and salmon. Such fish are known to contain higher levels of free histidine in their flesh, and have been implicated in so-called scombrotoxicosis (Rauscher-Gabernig et al., 2010).

Relation of biogenic amine content to bacterial numbers

Expectedly, total bacterial counts were lower in heat-processed fish than in raw fillets (Table 3). Correlation between bacterial numbers in log cfu/g and individual biogenic amines was generally low and did not exceed $r = 0.4$. This was not unexpected as, in particular, putrescine levels reflect the "history" of storage conditions of perishable foods prior to heat processing, whereas the total aerobic counts

TABLE 2: *Comparison of biogenic amine contents in mg/kg with available legal limits (histamine) or tolerance levels (TL).*

	Legal limit (mg/kg)	Tolerance level (mg/kg)	Contents in the analyzed samples (mg/kg) Min	Max	Median	Samples exceeding limit of detection, %	TL, n =	Type of samples exceeding limits or TL
Ready to eat								
Tryptamine	100–800		0	25.2	0	37.0	0	
ß-phenylethylamine		25[c]	0	16	1.4	16.0	0	
Putrescine		170[b]	2.4	140.9	13.1	0.0	0	
Cadaverine		510[b]	0	62.1	0	25.0	0	
Histamine	200–400[d]	200–400[d]	0	195.3	5.8	8.0	0	
Tyramine		950[a]	0	44.6	1.0	11.0	0	
Spermidine			0	72.2	9.6	4.0	0	
Spermine			0	33.6	4.0	15.0	0	
Non-Ready to eat								
Tryptamine			0	17.3	0	36.0	0	
ß-phenylethylamine		25[c]	0	30.9	2.5	7.0	1	Trout
Putrescine		170[b]	0	93.9	17.8	1.0	0	
Cadaverine		510[b]	0	622.0	4.5	13.0	1	Atlantic Cod
Histamine	100–200[d]	100–200[d]	0	495.9	0.5	20.0	2	Tuna, Trout
Tyramine		950[a]	0	34.7	0.5	15.0	0	
Spermidine			0	29.3	6.8	3.0	0	
Spermine			0	17.0	6.9	8.0	0	

[a]: Rauscher-Gabernig et al., 2009; [b]: Rauscher-Gabernig et al., 2010; [c]: Paulsen et al., 2012; [d]: Rauscher-Gabernig et al., 2012

TABLE 3: *Relation of biogenic amine contents to total aerobic counts (TAC).*

Sample type	TAC (log cfu/g) mean ± std. dev.	Biogenic amines in mg/ kg (wet weight), median with maximum in brackets							
		tryptamine	ß-phenylethylamine	putrescine	cadaverine	histamine	tyramine	spermidine	spermine
Freshwater									
Raw on ice	3.8±1,7	<1 (1.8)	4.3 (11.8)	13.7 (41.6)	<1 (6.8)	5.8 (20.3)	2.7 (3.4)	10 (23.1)	7.8 (16.2)
Raw deep-frozen	4.7±0.9	<1 (<1)	3.3 (30.9)	17.1 (34.9)	7.4 (215.5)	4.9 (280.7)	<1 (23)	6.2 (8.3)	6.5 (10.5)
Smoked	2.8±1.5	<1 (<1)	4.1 (11)	25.3 (93.9)	3.5 (4.7)	66.3 (166.6)	2.6 (34.7)	10.3 (17.6)	14.1 (17)
Saltwater									
Raw on ice	4.1±1.3	<1 (10.1)	1.8 (15.5)	26 (51.2)	1.2 (41.5)	6.3 (496)	1.3 (12.8)	10.4 (27.8)	5.3 (14.1)
Raw deep-frozen	4.2±1.5	<1 (17.3)	2.7 (21.8)	17.4 (78.7)	5.8 (622)	<1 (55.5)	<1 (26.3)	6.7 (29.3)	6.2 (16.3)
Smoked	2.7±1.2	<1 (14.5)	3.8 (12.9)	30.1 (93.9)	<1 (62.1)	1.6 (80.4)	2.3 (44.6)	9.6 (46.2)	10.3 (33.6)
Marinated	2.2±0.4	<1 (25.3)	4.5 (9.9)	27 (140.9)	8.6 (41.6)	20 (95.9)	<1 (23.2)	3.8 (44.1)	<1 (19.3)
Canned	<2	<1 (5.4)	<1 (6.8)	7.8 (46.8)	<1 (39)	35.1 (195.4)	<1 (23.4)	11.3 (72.2)	<1 (17.6)

include not only this "history" but also reductions in bacterial numbers due to heat treatment (Bauer and Paulsen, 2002); likewise, the ability of bacteria to form of biogenic amines is not always species- or family specific, but rather a strain-dependent characteristic (Pircher et al., 2007).

Detection of presumptive nematodes

In 13 of 102 samples (12.7 %) mainly those from wild salmon *(Onchorhynchus)* and herring samples nematodes were detected. Eleven of these infected samples were Non-RTE, so the nematodes would most likely be inactivated during culinary preparation (Karl, 2008). Still, *Anisakis* antigens can be a source of hypersensitive immune response (Audicana et al., 2002). Among these allergens only Ani s 4 is heat-, acid- and pepsin sensitive (Rodriguez-Mahillo et al., 2008).

Detection of *Listeria* sp.

In four (salmonids and trout fillets) of 49 Non-RTE and in one of 53 RTE samples (smoked salmon), *Listeria monocytogenes* were detected in 25 g aliquots. The presence of this pathogens in aquatic environments (Dijkstra, 1982), and colonisation of processing plants requires different control options to reduce the prevalence in RTE products (Huss et al., 2000). In 2012, *Listeria monocytogenes* could be recovered from 3 to 6 % of fishery products in Austria, which was similar to meat, but higher than in a variety of other foods examined (AGES, 2013). In Austria, 26 confirmed listeriosis cases were reported for 2011 (ECDC, 2013) and 36 for 2012 (AGES, 2013). In fish samples examined in Germany in the period 1998–2000, *Listeria monocytogenes* was detected in 3.5 to 100 % of samples, depending on fish species and type of fish processing (Feldhusen et al., 2001). The authors concluded that despite the abundance of the pathogens, fish was rarely implicated in clinical human listeriosis, maybe due the low concentration of Listeria in most of the samples. Although the fraction of cases attributable to fishery products is unclear, it is obvious that such foods present some reservoir for human foodborne infections.

Conclusions: Significance for public health

In 53 RTE samples, frequency of pathogenic agents was low, i. e. *Listeria monocytogenes* and presumptive nematode larvae were detected in one sample each. A higher frequency of *Listeria monocytogenes* in Non-RTE foods (4/49) will not necessarily result in higher consumer exposure, as the latter depends also on culinary processing. For nematode larvae, the allergenic potential of already inactivated larvae (recovered from 12 of 49 samples) should be considered, as well as the fact that histamine contents >200 mg/kg could be detected and will not be reduced by culinary preparation.

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