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A novel approach for the determination of freshness and identity of trouts by MALDI-TOF mass spectrometry

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ABSTRACT

Properly handled fish is usually marketed as “fresh fish” until day 10 after fishing. About 40% of the total fishery that is used for direct human consumption is marketed in fresh form stored at temperatures up to +2 °C. Currently, there are no validated methods available for controlling the recommended period of storage. Apart from being a potential source for food fraud, spoiled fish represents a major source of foodborne illnesses and intoxications.

In this study, a rapid MALDI-TOF mass spectrometry based screening method was developed using the vitreous fluid of fish eyes as specimen for the examination of different days of storage. The vitreous fluid was collected from n = 100 freshly fished brown trouts at day 0, 3, 7, 9, and 11 *post mortem* (n = 20 brown trouts each day of examination). The samples were immediately measured by MALDI-TOF mass spectrometry in linear positive mode (mass range *m/z* 2000–20,000 Da). For quality assurance the experiment was repeated with a set of brown trouts (n = 100) originating from the same fish farm and with brown trouts (n = 100) originating from a different fish farm. For specificity testing rainbow trouts (n = 10) were examined accordingly. All obtained mass spectra were processed by means of MALDI Biotyper OC 3.1 and ClinProTools 3.0 software.

The MALDI Biotyper approach showed limited applicability for the identification of the time of storage. However, it was suitable to reliably discriminate between the closely related species brown and rainbow trout. Processing by ClinProTools revealed four crucial mass peaks (*m/z* 2594 Da, *m/z* 4857 Da, *m/z* 4879 Da, *m/z* 4899 Da) which enabled a reliable differentiation between day 0 and 3, 7, 9, 11 (rate of correct identification > 90%) as well as the differentiation between day 3 and 7, 9, 11 (rate of correct identification > 72%). However, this approach showed limited applicability within the end of the tested period of storage when comparing between day 7, 9, or 11.

1. Introduction

Fish is an important part of the human diet. Seafood represents 15% of the total protein that is ingested worldwide (Böhme et al., 2015). In 2014, 146 million tons of fish (87% of total fishery) were used for human consumption. About 40% thereof were marketed as fresh fishery products (FAO, 2016). A German adult consumes 14 kg fish products in average per year. Trout is the most important species of fresh fish in Germany (Fisch-Informationszentrum e. V., 2015). In Europe, fresh fish is defined as fish that has not undergone any treatment except chilling (annex I point 3.5 Regulation (EC) No 853/2004 and German Guidelines for “fish, crustaceans, molluscs and products”). By definition, fresh fish is not allowed to be treated further, i.e. by freezing (Connell, 1995) or coating (Feng, Ng, Mikš-Krajnik, & Yang, 2016; Panchavarnam, Kakatkar, & Venugopal, 2003). Fresh fish is stored at up to +2 °C on melting ice and may be vacuum packed or in a modified atmosphere packaging (MAP) (European Commission, 2004a). Nevertheless, cooling can only offer a short extension in shelf life (Huss, 1988).

According to usual recommendations, “fresh” fish can be sold as “fresh” fish for human consumption up to 10 days after fishing. In general, marine fish takes up to five days until it arrives in the retail stores where it may be stored up to five days until being sold. After this time period the fish is often not discarded but may be frozen and used for human consumption. In every respect, fish is highly subjected to spoilage. The spoilage is associated with the fast

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denaturation of proteins, the oxidation of lipids and potential bacterial degradation processes (Venugopal, 2015).

The consumption of spoiled fish is a health risk for the consumer and is the most frequent source of food poisoning (Huss, Reilly, & Embarek, 2000). The microbial load of fish is very closely related to the aquatic environment and the conservation methods that are applied to the products. These factors influence the growth and survival of bacteria and fungi. If fresh fish is stored under aerobic conditions up to +2 °C, the total number of bacteria can reach 10^8-10^9/g flesh or cm^2 skin when spoilage is apparent (Huss, 1988). Nevertheless, microbial contamination is primarily occurring during the processing stages and prior to packaging (Huss et al., 2000). Psychrotrophic, Gram negative bacteria cause alteration in fresh fish even at lower temperatures (Gram, 1993; Gram & Dalgaard, 2002; Huss, 1988; Eja et al., 2008). In general, cross contamination with pathogenic microorganisms may occur at every stage of production, which may result in serious diseases for humans (Böhme et al., 2010; Ward, 2001). Seafood products are often subjected to heat treatment after packaging or before consumption to reduce the microbial load. However, these treatments do not inactivate preformed toxins and spores of spore-forming bacteria, e.g. *Bacillus* spp. (Böhme et al., 2015).

Serious health problems related to toxins can be caused by spoiled fish (Gram & Huss, 1996; Huss et al., 2000; Iwamoto, Ayers, Mahon, & Swerdlow, 2010). Scombroid poisoning is one of the most important food poisonings. Fish of the *Scombridae* family such as mackerel or tuna are naturally rich in the amino acid histidine. If the fish is not properly stored, bacterial spoilage is associated with decarboxylation of histidine to histamine (e.g. trimethylamine, TMA) causing severe allergy-like symptoms in humans (Böhme et al., 2015; Flick, Oria, & Douglas, 2001; Lehane & Olley, 2000). However, the TMA content varies considerably between different species. It provides no information about early autolytic changes or the degree of freshness (Connell, 1995). Furthermore, the TMA analysis cannot be used for the evaluation of freshwater fish, because they do not contain sufficient amounts of TMAO.

In order to prevent food fraud and health risks for consumers resulting from spoiled fish, it is necessary that authorities control the freshness of fish. The currently available methods to examine the quality and the freshness of fish are listed in Table 1. The *post-mortem* changes can be evaluated by sensory, physical, chemical and microbiological methods in order to assess the general quality and to determine the degree of spoilage or the health risk. These methods are not always applicable to all fish species of marine and freshwater origin (European Commission, 2004b; Heude, Lemasson, Elbayed, & Piotto, 2014; Huss, 1988). Most of them are time consuming and expensive and not very practicable for routine usage. The freshness is mainly analyzed by sensory means using the "Quality Index Method (QIM) evaluation scheme" (Bernardi, Marsico, & Queiroz de Freitas, 2013). Official controls of fishery products are based on the Regulation (EC) No 854/2004 Annex III. For certain fish species maximum values for total volatile basic nitrogen (TVBN) (European Commission, 2005b) and maximum values of histamine exist. Microbiological criteria for fish products are defined in regulation (EC) No 2073/2005 (European Commission, 2005a).

Identification of fish species by measuring muscle and/or liver material with MALDI-TOF MS was performed for processed seafood products and for whole fish such as Gadidae and Pleuronectiformes or freshwater fish such as *Alosa agne*, *Coregonus macrophthalmus* and *Rutilus rutilus*. It is a fast and reliable method for species identification (Mazzeo et al., 2008; Mazzeo & Siciliano, 2016; Siciliano, d'Esposito, & Mazzeo, 2016; Volta, Riccardi, Lauceri, & Tonolla, 2012). However, currently no control method is available to determine the exact day of death for fresh fish. This favours fraudulent activities and misleading information regarding the duration of storage which is illegal according to Article 16 Regulation (EC) No 178/2002 in combination with Article 7 paragraph 1 point a Regulation (EU) No 1169/2011.

In this study, we report on a MALDI-TOF MS based method for the determination of the freshness and identity of two trout species (rainbow and brown trout). In order to reduce external influences (e.g. microbial contamination and spoilage processes) we have chosen the vitreous body of the eye as sample material.

2. Materials and methods

2.1. Chemicals and reagents

Acetone, α-cyano-4-hydroxycinnamic acid (CHCA), and aqua dest. were purchased from Fluka (Fluka, Dagebüll, Germany). Trifluoroacetic acid (TFA) and ethanol (p.a.) were obtained from Merck (Merck, Hamburg, Germany). The matrix for MALDI-TOF MS measurements was freshly prepared every day with the following

Table 1
Methods for the control of fish quality, freshness and degree of spoilage.

Parameter	Examination by	References
Freshness and degree of spoilage	Appearance	Huss (1988), Bernardi et al. (2013), Federal Institute for Risk Assessment (2008), Dutta et al. (2016), Gil et al. (2008)
	Texture	
	Flavour	
	Odour	
	Nucleotides (*K*-value)	Heude et al. (2014), Ehira (1976), Jones and Murray (1964), Burt (1977), Cheng et al. (2016)
Freshness	pH	Huss (1988), Abbas, Mohamed, Jamilah, and Ebrahimian (2008)
	Total volatile bases (TVB)	Connell (1995), Huss (1988), Cheng et al. (2016)
	Peroxide value (PV)	Connell (1995)
	Thiobarbituric acid value (TBA)	
	Water-Binding Capacity (WBC)	Hamm (1972)
	Trimethylamine (TMA)	Heude et al. (2014), Connell (1995)
Quality	Mineral or ash content	Bligh and Dyer (1959), Huss (1988)
	Protein content	
	Water content	
	Fat content	
Quality and degree of spoilage	Texture of tissue	Johnson et al. (1980), Dunajski (1980)
Degree of spoilage	Electric potential of skin and tissue	Huss (1988)
	Standard plate count	Huss and Eskildsen (1974)
	Spoilage bacteria	Böhme et al. (2015)
Degree of spoilage and health risk	Pathogenic bacteria	Böhme et al. (2010)