Comparison of odour-active compounds in raw Baltic herring with pH-shift produced protein isolate using gas chromatography olfactometry

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Baltic herring is used in a limited capacity for human consumption in Finland even though it has largest annual catch out of other fish species. There is a substantial amount of interest in improving the rate of consumption for humans instead of most of the catch being used as feed for animals or farmed fish.

The aim of the study was to investigate the difference in important odour-active compounds between the raw Baltic herring and protein isolate produced by alkaline pH-shift process i.e., alkaline solubilisation and isoelectric precipitation. If there are desired changes in the odour of the fish after the pH-shift, the protein isolate produced with the method could have some utility in novel food products by reducing the impact of the odour to consumer perception. The pH-shift was used under alkaline conditions in this study.

There were 28 odour-active compounds in the raw Baltic herring and 21 in the protein isolate that had nasal impact frequency values of 40 % or higher. There were 8 such compounds that had a modified frequency rating of 80 % or higher in the raw Baltic herring and 4 in the protein isolate. These compounds were 2,3-butanedione, 3-methylbutanal, 2,3-pentadione, hexanal, (Z)-4-heptenal, methional, 1-octen-3-ol and (E,Z)-2,6-nonadienal in the raw Baltic herring and in the protein isolate they were 2,3-pentadione, hexanal, (Z)-4-heptenal and 1-octen-3-ol. The pH-shift did have notable effect on the odour-active compounds, additional studies can be done with different pH-shift parameters to see what potential changes would occur in the odour-active compounds. Adding antioxidants during the process could prove to have interesting results because of lipid oxidation on the formation of odour-active compounds.

Keywords: Baltic herring, pH-shift, gas chromatography-olfactometry, odour-active compound

Contents

Abbreviations

EPA	Eicosapentaenoic acid
FID	Flame-ionization detector
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GC-MS/O	Gas chromatography-mass spectrometry/olfactometry
GC-O	Gas chromatography-olfactometry
HHE	4-hydroxy-trans-2-hexenal
HS-SPME-GC-MS	Headspace solid-phase microextraction-gas chromatography-mass spectrometry
MDA	Malondialdehyde
MF	Modified frequency
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
NIF	Nasal Impact Frequency
OID	Olfactory intensity device
PUFA	Polyunsaturated fatty acid
SNIF	Surface of Nasal Impact Frequency

1 Introduction

1.1 Baltic herring usage in Finland

Baltic herring is the most caught fish species in Finland and the annual catch also has the largest economic value. The value of the catch which was an estimated 29 million euros is still far behind the domestic fish farming that generated 70 million euros from growing just rainbow trout and common whitefish. The volume of the catch exceeded 120 million kilos, but its value is still much lower than the 14 million kilos of rainbow trout and common whitefish (Luke, 2017). Most of the Baltic herring caught is not being consumed by the average consumer either. In fact, only 3 % of the annual catch in 2017 was sold to the consumers in the domestic market while most of it ended up as feed for fur animal farms and fisheries (Luke, 2017).

The Baltic herring used to have much larger part in the Finnish diet especially in the 1940s to 1980s and it was especially sought after during the second world-war as the supply of food was not guaranteed. Since the introduction of fisheries and the growth of salmon and rainbow trout farming in Finland and abroad, the popularity of herring as food has plummeted. Salmon and rainbow trout have proven to be more ideal for the consumer and for the retail stores as the availability and quantity of the fish can be guaranteed better with fish farming instead of traditional fishing methods (Luke, 2017). This has had an impact especially on the younger generation who are used to eating salmon instead of herring. It also shows what fish different age groups buy from shops, where the older generations are buying more herring than the younger ones. In Finland Baltic herring was 2.46 times more likely to be consumed by people over the age of 45 than people younger than 45 according to a survey in a study about consumer perceptions (Pihlajamäki et al., 2019). Education level and consumer purchasing power also had an effect, they effected the probability of consumption by 1.54 and 1.33 times at every increase of education level or purchasing power level. The purchasing power levels were separated by qualitative factors to compare Finland with other countries (Pihlajamäki et al., 2019). Consumers in Finland chose Baltic herring for consumption mostly due to its good taste and positive impact on health. On the other hand, the top reasons why consumers chose not to eat Baltic herring included disliking the taste, not used to eating it and because of its poor availability (Pihlajamäki et al., 2019). People who already do eat Baltic herring limit their consumption due to availability issues and potential harm to their health longer term due to potentially consuming too many

dioxins with the fish. These parameters suggest it would be possible to improve the consumption of Baltic herring for human consumption. Ensuring a lower level of dioxins in the fish entering the food market, improving availability in the most cost-efficient manner and better package markings could improve the consumption of Baltic herring especially for people who already eat the fish (Pihlajamäki et al., 2019). Novel food products might help in introducing Baltic herring to consumers who are not used to eating it or do not like how already existing products/dishes taste. Product development can play a part in introducing new products or ways to eat Baltic herring, although the success of new products is far from guaranteed (Pihlajamäki et al., 2019).

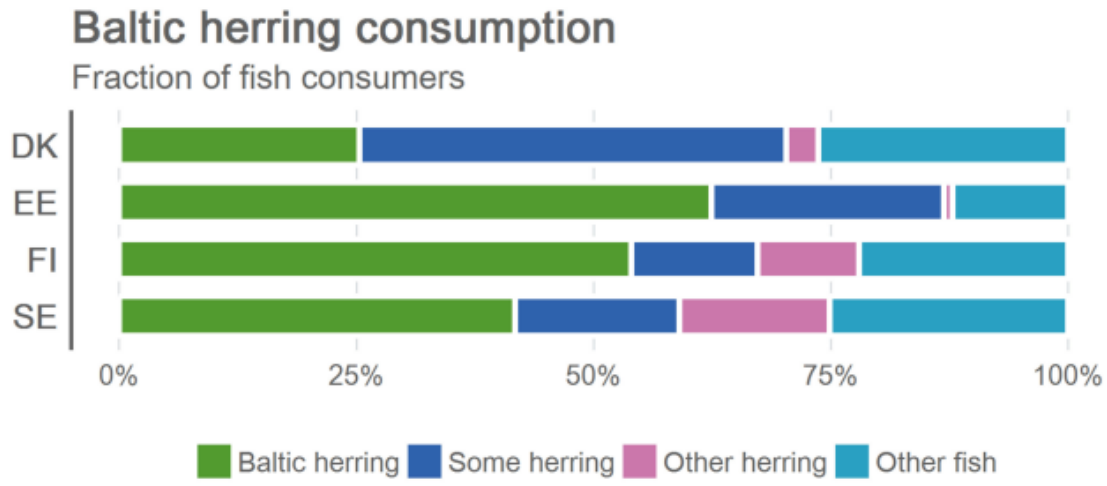

Figure 1. Baltic herring consumption compared to other herring and fish consumption. Baltic herring: people who consume Baltic herring, Some herring: people who do not know the type of herring they eat, Other herring: people who eat other types of herring, but not Baltic herring & Other fish: people who eat fish but do not eat any type of herring (Pihlajamäki et al., 2019).

In **Figure 1.** the people who consume Baltic herring, other herring, or only other fish species are separated into different categories based on the answers of consumers who took part in a survey. The people who did not eat any fish are not included in the figure. The countries included in the figure were Denmark (DK), Estonia (EE), Finland (FI) and Sweden (SE) (Pihlajamäki et al., 2019). The green colour represents the consumers who know that they sometimes consume Baltic herring as a part of their diet, the darker blue represents those that know they consume some herring species but are not sure that if it is Baltic herring or not, the pink colour represents those that know they are eating other herring than Baltic herring and finally the light blue colour that represents the people who eat do not eat Baltic herring. Out of the fish consuming population of the survey, the largest percent of consumers who never eat any kind of herring were in Denmark and Sweden (Pihlajamäki et al., 2019). In Estonia and Denmark there were

more consumers who did not know where the herring they had consumed came from. Having Baltic herring as a part of your fish diet was the most common in Finland and Estonia, and in Estonia it was the most common to eat some kind of herring as part of your diet.

The level of dioxins and PCB compounds were a concern in the Baltic herring regarding health concerns but recently the concentration of these compounds have lowered to more than half of their previous levels and are at a safe level for consuming (Kalakauppiasliitto, 2019). In 2015 a fish processing plant was built in Kasnäs which uses herring as an ingredient to produce fishmeal for the fisheries. This has helped keep the value of herring in Finland instead of it being sold abroad for the same purpose (Kalakauppiasliitto, 2019). Companies in the fish processing business have become interested in utilizing the Baltic herring for food products as there is a lot of potential to increase the number of fish to be caught, which will lead to more value being built in the industry. Baltic herring is a small fish that has a high content of bones, hemeproteins and lipids which makes it difficult to use in some food processes. For consumers the rancidity caused by lipid oxidation in fish and seafood is a major reason why consumers find these types of foods unacceptable (Marmon, 2012).

1.2 Baltic herring fat/lipid content related to odorous compounds

The fat content of Baltic herring varies seasonally; from February to June the fat content is about 50 % lower than it is from July to November (Szlinder-Richert et al., 2010). Male herrings also had higher fat content than female herrings. This seasonal variation affects the total number of volatile compounds in the herring. When the fat content is lower the volatile compound content is also lower in the fish (Aro et al., 2002). The overall volatile content increases during storage for fish caught in all seasons when it is stored at 6 °C. Most of the volatile compounds formed in the fish during storage seem to be a result of lipid oxidation, enzymatic formation, or autoxidation (Aro et al., 2003). Straight chain alkanals like hexanal are formed by lipid oxidation from unsaturated fatty acids, 1-penten-3-ol is formed enzymatically from n-3 fatty acids by fish lipoxygenases and hexenals are formed by autoxidation. Autoxidation becomes a more significant formation mechanism of volatile compounds during longer storage (Aro et al., 2003).

The high content of unsaturated fatty acids makes the fish very susceptible to oxidation. The most dominant monounsaturated fatty acid (MUFA) in Baltic Herring was oleic acid and docosahexaenoic acid was the most dominant polyunsaturated fatty acid

(PUFA) (Szlinder-Richert et al., 2010). PUFAs constituted most of the lipids extracted from the muscle of the fish. Percentage wise their amount ranged from 35.9 ± 5.9 to 48.7 ± 2.8 % of lipids in this extract. The concentration of PUFAs varies depending on the month the fish was caught in same as with the fat content but the two are not directly correlated to each other. The variation relates to the feeding period the Baltic herring goes through in October when the PUFA concentration is at its highest and the postspawning period in April when it is at its lowest (Szlinder-Richert et al., 2010). N-3 polyunsaturated fatty acids oxidation is the cause for the formation of potent odorants such as (Z)-3-hexenal, (Z)-4-heptenal, (E,Z)-2,6-nonadienal, (E,E)-2,4-heptadienal and (E,Z)-2,4-heptadienal (Hartvigsen et al., 2000). Not all volatile compounds are odour-active, but an odour-active compound must be a volatile compound so that it will reach the olfactory sensory neurons found in the nose. Odour-active compounds are the main point of interest in this study as they are the compounds that form the overall smell of the Baltic herring.

1.3 Consumers food selection criteria

There are three main principal properties which guide the consumers' selection of food; they are flavour, appearance, and texture. Flavour is divided into the subsets of taste and smell, and it is also defined as the sensation derived from the interplay of signals produced by the sense of smell, taste and other irritating stimuli from food or beverage (Blank, 1996). As discussed in **Chapter 1.1** disliking the taste of the Baltic herring was the major reason why people preferred not to eat it. The compounds that effect the flavour of Baltic herring have not been studied in much detail, thus in this study the Gas chromatography-olfactometry (GC-O) system was utilized to identify compounds that effect the flavour of Baltic herring. With the GC-O odour-active compounds can be detected from the fish, as the name suggest these compounds will mainly affect the odour of the fish, but some also effect taste. Odour is usually used to refer to the smell of food and aroma is referring to the retronasal smell when food is inside the person's mouth (Brattoli et al., 2013). There are three main areas that GC-O studies on food products focus on. The areas include the aroma profile of foods and beverages, changes to odour due to processing techniques and discriminant analysis among a family of foodstuffs (Brattoli et al.,2013). This study focuses on the effect of pH-shift processing on the odour-active compounds found in raw Baltic herring by utilizing the GC-O. The results could provide useful insights for future product development projects.

1.4 Protein isolation using the pH-shift method

The pH-shift protein isolation method was initially invented and subsequently patented in the late 1990s/early 2000s (Marmon, 2012). The method is based on the differences in solubility that muscle proteins have in water at different pH values (Marmon, 2012). The temperature is kept under 10 °C to keep the proteins from denaturizing and to inhibit enzymatic degradation during the process (Marmon, 2012). The pH-shift process first solubilizes muscle proteins at either low or high pH (pH ≤ 3 or ≥ 10.8) after which impurities can be separated with a centrifuge. Next the solubilized proteins are precipitated near the isoelectric point (around pH 5.5) from the remaining solution, and they are collected after another centrifugation from the precipitant that has formed. Since the acid version of the process induces proteolysis (Marmon, 2012) and hemoglobin oxidizes rapidly under acidic pH leading to more oxidation reactions in the fish mass (Richards & Hultin, 2002), the alkaline version was deemed to be better while using Baltic herring as the protein source. More recent studies have found that acidic pH conditions seem to be better in the pH-shift process for inhibiting oxidation than alkaline pH conditions (Abdollahi et al., 2020; Kakko et al., 2022). The protein isolate resulting from the alkaline pH-shift of Baltic herring contained much more free fatty acids, had the highest peroxide values and peak areas of volatile lipid oxidation indicators when compared to the protein isolate extracted with pH-shift under acidic conditions (Kakko et al., 2022). One of the studies pointed out that there are two things that could inhibit or promote lipid oxidation, pro-oxidative activity of heme-proteins increase in acidic conditions and at extremely high pHs such as 3.5 or lower the lipid oxidation can be inhibited (Abdollahi et al., 2020). These effects have a potential influence on oxidation depending on factors such as the raw material quality, heme-protein amounts, and the holding times at different pHs during the pH-shift process (Abdollahi, et al., 2020). Additions of antioxidants can supress oxidation significantly in the protein isolates extracted with the pH-shift.

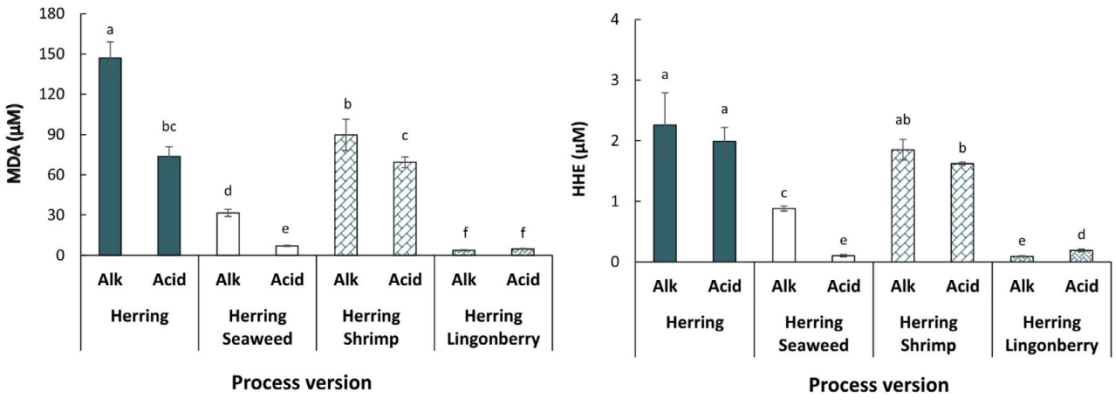

Figure 2. Levels of malondialdehyde (MDA) and 4-hydroxy-trans-2-hexenal (HHE) in protein isolates separating acid and alkaline conditions in the pH-shift (Adbollahi et al., 2020)

Figure 2. shows the differences in MDA and HHE levels between samples with only herring and samples mixed with seaweed, shrimp, or lingonberry press cake under acidic or alkaline pH-shift conditions. Measuring the concentration of MDA and HHE aldehydes is done to assess the extent of secondary lipid oxidation products in the pH-shift process. Adding seaweed or lingonberry to herring had a noticeably positive effect on inhibiting oxidation in the pH-shift, seaweed inhibited oxidation less under alkaline conditions than lingonberry. Flavonols and anthocyanins, particularly proanthocyanidins, that are present in the lingonberry have an antioxidative effect which inhibits lipid oxidation in the process (Adbollahi et al., 2020). The pH-shift process has the additional benefit of removing dioxins and PCBs from the Baltic herring which are harmful at high enough doses. The removal of dioxins and PCBs correlated with the removal of lipids (Marmon, 2012). As the oxidation of long chain unsaturated fatty acids creates volatile compounds such as heptanal and 1-penten-3-ol (Aro et al., 2003), the potential of the process to remove lipids was of particular interest. The lipid and fatty acid composition of the protein isolate are noticeably influenced by the processing conditions of the pH-shift (Kakko et al., 2022). The process steps of the pH-shift are described in **Figure 3.**

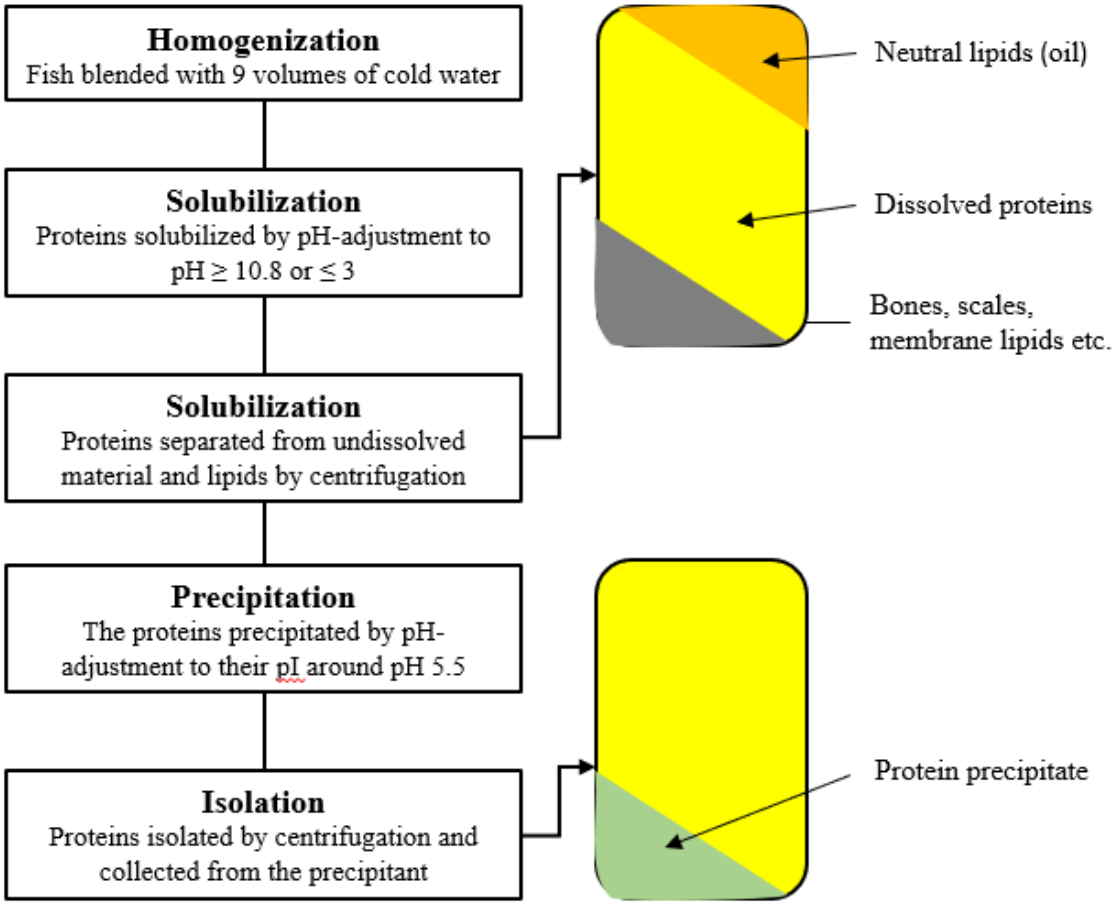

Figure 3. pH-shift process step flowchart (Marmon, 2012).

1.5 Gas chromatography-olfactometry (GC-O)

GC-O refers to the use of human olfaction as a sensitive and selective detector for odour-active compounds that are separated from each other using a gas chromatography (GC) (Vene et al., 2013). A GC-O system utilizes a specifically designed odour port that is connected in parallel to a conventional detector, in this study the parallel detector was a flame-ionization detector (FID). The parallel connection splits the effluent so that it reaches both the sniffing port and detector almost simultaneously. The GC-O can also be set up in a way that it is connected to only one the olfactory port or detector, this ensures that all the volatiles in the sample reaches either connection endpoint (Reineccius & Peterson, 2013). The double connection is achieved by a y-connector that splits some of the flow from the column to the olfactory port instead of it just flowing to the GC detector in use. In **Figure 4.** the GC-O system is shown as a basic schematic with the different components it consists of (Plutowska & Wardencki, 2008). The accuracy of GC-O is thus dependent on the performance of the human assessors, who are sniffing for the odour-active compounds (Delahunty et al., 2006). People associate odours with their own past experiences, from those experiences; they will involuntarily

assess the odour as likable, dislikeable, or indifferent. These responses are individual, and they can vary from person to person (Brattoli et al., 2013). Ideally a gas chromatography-mass spectrometry/olfactometry (GC-MS/O) system is used as it is ideal to get an odour profile and a mass spectrometry (MS) profile at the same time. The identification of odour-active compounds is an easier task with the GC-MS/O system than when using a GC-O and GC-MS system separately even if using the exact same column in both. Because the MS is a machine that creates a vacuum it changes the elution time of the GC column, thus making it more difficult to compare the results of the two systems. Additionally, the FID detector responds to the compounds it detects differently from an MS detector, which results into differences between the peaks from the same compound when comparing the two detectors (Reineccius & Peterson, 2013). Having the olfactometry and MS detectors connected in the same sample run makes it easier to identify odour-active compounds with the help of the data received from both detectors.

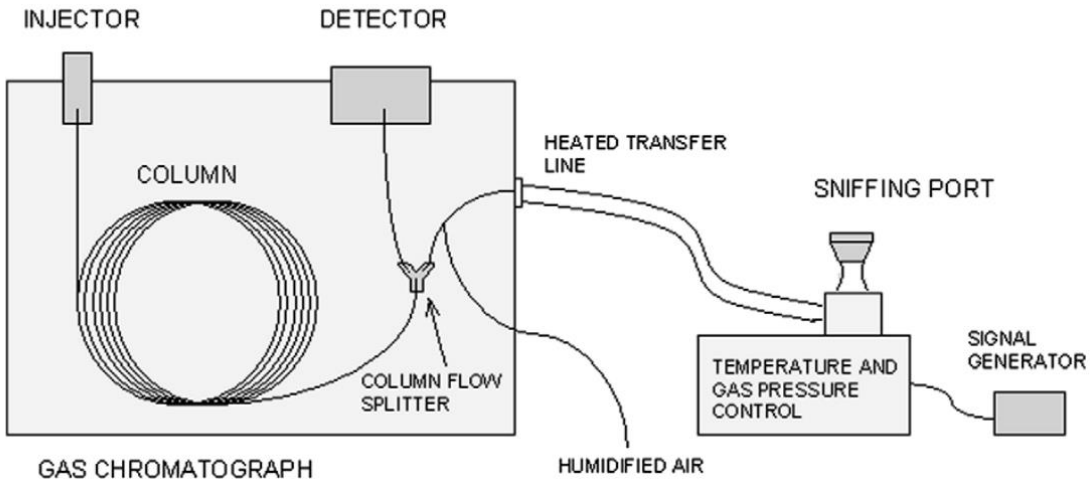

Figure 4. Basic schematic of a gas chromatograph connected to an olfactometric port and a detector (Plutowska & Wardencki, 2008).

Odours are complex mix of different volatile compounds, and these compounds also have different concentrations that influence the overall odour. Food products have been a point of interest in many studies regarding the pleasantness of their odour because the odour most likely influences the overall appeal of the food product in question. Odour threshold is the minimum concentration at which 50 % of the sensory panel can detect the presence of an odour without the need to characterize it (Brattoli et al., 2013). Some compounds that have an intense odour can cross this threshold even in small concentrations, the panel's ability to recognize odours of course influences what

compounds cross the 50 % threshold. Each one of these odour-active compounds that have an odour detection threshold high enough for a human assessor to detect the odour can be analysed further (Vene et al., 2013). Once the assessor detects an odour, they can for example try to describe the smell, estimate the duration of odour activity, and rate its intensity as additional information.

Most olfactometric ports are similar in their design; the eluate travels through the chromatography column reaching the sniffing port that is a conical port molded to fit the shape of a nose (Brattoli et al., 2013). Humidified air is flown to the conical port through a separate line to prevent the assessors' mucous membranes in the nose from drying during long analysis sessions. The panellists should have as comfortable sitting positions as possible and other things such as presence of hot chromatography components need to be considered as potential sources of discomfort. A button/switch that sends an electric signal is connected to the system for the panelist to send a signal when they detect an odour (Brattoli et al., 2013). This signal is utilized in analyzing the sample with voice recording systems to collect the panellist's description of the detected odour and other potential ratings.

Several factors determine the quality of the data that is collected by the CG-O method. The extraction technique of volatile compounds from the samples determines the composition of the extract which affects the eluate that will be perceived (Brattoli et al., 2013). The settings and overall set-up of the GC instrument control the quality of the output such as the overall elution time and peak separation. Non-polar columns enable elution of odour-active compounds at lower temperatures, but very polar compounds eluate in wide peaks. Polar columns enable greater selectivity depending on the composition of the sample (Brattoli et al., 2013). The odour character of some compounds depends a lot on their concentration. These compounds are affected by poor chromatography or poor chromatographic separation. As an example, 3-methyl indole has a faecal odour at high concentrations, but the odour becomes pleasant, sweet, and warm at very low concentrations (Leland et al., 2001). However, compounds with such characteristics are rare and as such they are not something that necessarily require pre-planning in case one of them shows up in the sample and GC-O. Some compounds on the other hand have their odour detectable at very low concentrations and they can be part of complex matrixes (Brattoli et al., 2013). This can make it very hard to identify odour compounds even with the use of a GC-O/MS as some compounds co-elute with each other.

The human detectors most likely have differences in their olfactory ability which will affect the gathered data. Odour thresholds can vary significantly between individuals, and some are unable to detect families of similar smelling compounds (Brattoli et al., 2013). The olfactory response of an individual is known to vary, even during a single day. Things such as speed of breathing, current health (flu etc.) and mood can affect the person's olfactory system. When choosing potential assessors, they can be evaluated for their olfactory sensitivity, motivation, concentration, and ability to recognize odour qualities (Brattoli et al., 2013). Multiple people are chosen to analyse the GC-O sample to minimize the potential data error in the results due to specific anosmia for individual panellists (Reineccius & Peterson, 2013). The panellists should not smoke nor eat or drink strongly flavoured foods or drinks for at least 1 hour before doing a GC-O analysis. Strong deodorants, perfumes and aftershaves should not be used on the day of the GC-O analysis (Brattoli et al., 2013). The instrument should be in a dedicated laboratory with temperature and pressure control. At most the time an assessor spends sniffing should be around 25 to 30 minutes to keep the assessor performance stable (Brattoli et al., 2013). Because the panellists have these individual differences and other factors influencing their performance which may affect their GC-O analysis, the GC-O can be criticized of being a subjective method that yields inconsistent results (Reineccius & Peterson, 2013). Even with well-trained and experienced panellists this variation between individual panellist results cannot be entirely removed.

1.5.1 Frequency Detection Methods

The frequency detection methods usually include a panellist group of 6 to 12 people who analyse the same sample from which odour compounds at a given retention times are ranked as a percentage by how many of the panellists detected the odour. The Nasal Impact Frequency (NIF) value is set at zero or one based on the panellist's ability to detect a given odour at a particular retention time (Brattoli et al., 2013). Then the number of panellists that have detected the odour will be divided by the number of total panel members to get the NIF-% value for the odour, this can be also referred to as a detection frequency value. The NIF value thus corresponds to the olfactometric signal's peak height. The NIF value can be the same for two compounds that have different intensity ratings or odour thresholds by the panel since it only considers the detection of the compound. This means that compounds that have a weak intensity and that are barely over the detection threshold are treated the same as compounds that have a strong intensity and are clearly above the detection threshold in some cases (Vene et al., 2013).

The Surface of Nasal Impact Frequency (SNIF) value describes the peak area by multiplying the frequency percentage by duration. This makes it possible to construct an aromagram to visualize how the panellists were able to detect different odours during the GC-O analysis (Brattoli et al., 2013).

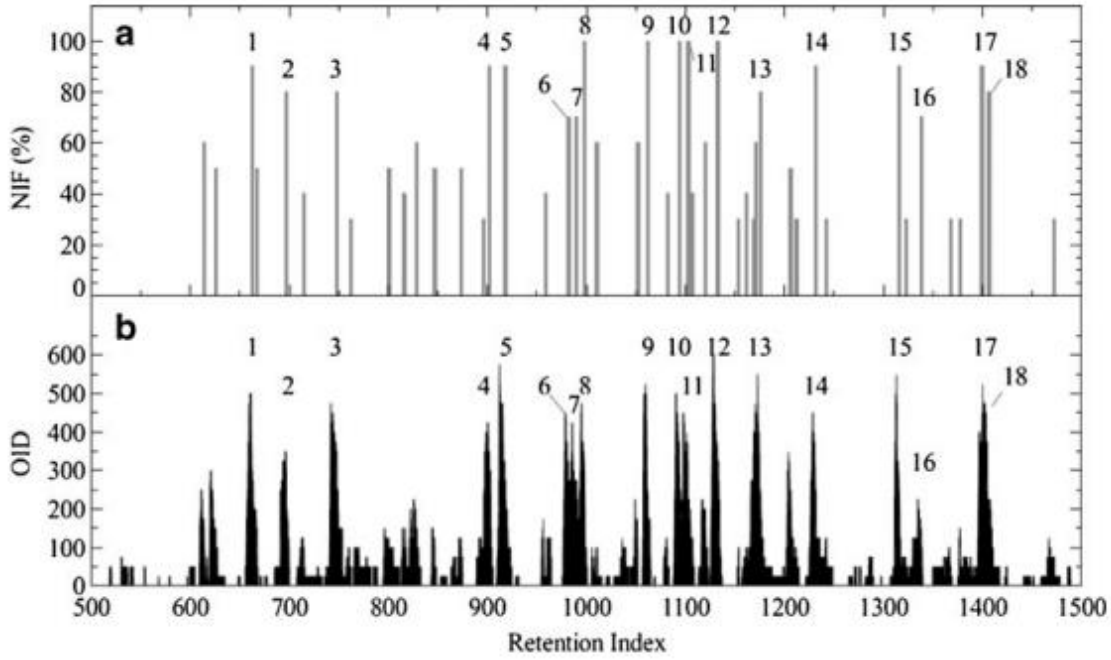

Figure 5. Two different frequency detection methods compared with each other, NIF (**a**) and SNIF (**b**) (Vene et al., 2013).

The two frequency detection methods utilizing either the NIF or SNIF techniques are compared in **Figure 5.**, the NIF-% graph was built manually with the use of a spreadsheet and the olfactory intensity device (OID) value was calculated with the SNIFF program in Chemstation (Vene et al., 2013). The OID value was calculated by multiplying frequency (%) with duration (s). The frequency (%) is received from the signal the panellists give when they detect an odour, with a button or a switch of some kind. When all panellists give this signal at the same time it shows up as the maximum value possible on the SNIF aromagram. The numbered peaks in **Figure 5.** are for odour-active compounds that exceed the NIF value of 60 % for the sample in question (Vene et al., 2013). The two results are comparable but with two more noticeable differences being between peaks 16 and 18. Peak number 16 does not really visualize a compound with a 70 % NIF value in the SNIF aromagram, especially when comparing to peaks number 6 and 7. Peaks 17 and 18 eluted so close with each other that the SNIF method has difficulties in separating the two compounds into separate peaks. This results from the way the signal from the button press is recorded as if two compounds eluate with each other the panellists are required to press the button twice with very fast reactions.

Sometimes they keep holding the button down during these situations giving one longer signal and then in the recording they will specify that they smelled two different odours. Otherwise at times the signals do not overlap which results into the aromagram peaks not showing up at the correct level measured on the signal axel. Both issues can be fixed when making a NIF-% chart manually. The voice recordings of panellists can be used to separate compounds that eluate close to each other that are not separated on the SNIF aromagram. The signals that do not overlap well can also be accounted for with the help of recordings and comparing retention/signal timing between panellists for the odour in question.

The main advantage of frequency-based methods is their simplicity, which means they do not require highly qualified evaluators for the analysis. If only odour detection is required during the analysis, the panellists do not require training before the GC-O session. However, training is still beneficial because it improves the sensitivity of the method by reducing signal to noise level of panellists (Van Ruth & O'Connor, 2001). The methods can be repeated, and the results will reflect the differences in sensitivity between the evaluators. The detection frequency will very likely relate to the intensity of the odours perceived by the panellists, but the peak height will not correlate with the real concentration of the odour compound (Brattoli et al., 2013). Odour-active compounds that have different concentrations in the sample will produce peaks of equal intensity in the aromagram if the compounds are detected by all panellists. The aromagram data can be used to focus on specific areas of interest such as an area in the aromagram where certain off-flavour odours have been detected by the panellists. One can then focus on identifying the compound or compounds of interest that are causing the off-flavour (Reineccius & Peterson, 2013).

1.5.2 Posterior Intensity Methods

Panellists are instructed to assign an appropriate value from a previously defined and trained intensity scale to each detected odour-active compound. The panel's ratings are recorded, and the average result is treated as the overall intensity rating. This value can be used in addition with the detection frequency percentage to calculate a modified frequency, which can be used to determine the most important odour-active compounds in the sample (Brattoli et al., 2013). The formula for calculating the modified frequency percentage (MF (%)) is:

$$\mathrm{MF}(\%) = \sqrt{\mathrm{F}(\%) \times \mathrm{I}(\%)}$$

F (%) is the detection frequency of an odour compound expressed as a percentage that is also used in aromagrams. I (%) is the average intensity of the odour expressed as a percentage of the maximum intensity (Brattoli et al., 2013). The intensity scale that is used is decided in each study by the researchers on what they think is an appropriate scale, and so it can vary depending on the study in question. A larger scale is better as it helps with discriminating between odour-active compounds but a larger is also more difficult for panellists to master. When the MF (%) is higher than 50 percentage for a specific compound it can be regarded as an important odour compound in the sample (Brattoli et al., 2013). Considering both the intensity and detection frequency of the odour gives a better representation for the odour-active compounds that are not just detected by many panellists but are also rated higher based on their intensity.

1.5.3 GC-O Methods used in studying odour-active/volatile compounds in different fish species

Detection frequency methods are commonly used in studies utilizing GC-O to detect and identify odour-active compounds. The methods help in determining the compounds that are the most important in the overall aroma profile of each fish. A study on the key odour-active compounds in steamed meat of *Coilia ectenes* used a panel of eight assessors with previous experience from GC-O analysis (Wu et al., 2014). They chose to consider odours perceived by fewer than four of these assessors to be noise. Three of these assessors were chosen to conduct a direct intensity analysis in addition to the detection frequency method. The sniffing conditions were the same expect that they had to assess the intensity on a four-point scale for each odour (Wu et al., 2014). Another study also used a panel of eight to perform a detection frequency method while studying the aroma-active compounds in rainbow trout (Selli et al., 2006). The people chosen to join the panel had previous experience with a GC-O and had trained odour detection and recognition. The signal to noise level was set at fewer than three panellists which is lower than the previously mentioned study in this chapter with the same number of panellists (Selli et al., 2006). The intensity was measured with a time intensity method that the same panellists from the detection method took part in. The intensity was rated on a nine-point scale that the panellists were trained to use (Selli et al., 2006). Frequency detection method was used in identification of odour-active compounds from the muscle of brown trout (Sérot et al., 2002). Nine panellists were chosen to participate and the noise to signal level was set at fewer than three panellists.

The studies mentioned in this chapter also requested that the panellists described the odours they detected with descriptions applicable to them such as green, mushroom, earthy, fishy, and so on. The description of odours helps in the identification of the odour-active compounds. The size of the panel participating in GC-O analyses, at least in the mentioned studies, is small but the need for a larger panel is not essential. One of the limiting factors for the panel size the preference for experience as there are not many people necessarily available for a GC-O analysis with the required experience. Panel training can be a process that takes some time and even experienced panellists should undergo some training before starting the main analysis.

1.5.4 Sensory panel training for GC-O

Before performing the GC-O analysis the panellists would need to be trained so that they can perform the analysis without significant variances between the panellists. Detection frequency methods that only account for odour detection do not necessarily require training but there is evidence that training improves the sensitivity of detection (Van Ruth & O'Connor, 2001). GC-O evaluation panel cannot be trained in a completely similar way as a sensory evaluation panel due to inherent differences in the two methods. Sensory evaluation techniques tend to be used to assess the intensity of flavour attributes from food and beverages. The panellist in sensory evaluation can take their time and assess an attribute several times before deciding on their score for that attribute. In a GC-O session the odours are only detectable for a few seconds at irregular intervals in an analysis session that can last up to 30 minutes. Odours being detectable for a few seconds requires the panellists to react quickly during the GC-O run (Vene et al., 2013). They will also have to be able to give descriptions of the odour and other attributes while they might already detect a new odour when describing an odour, they had detected a few seconds ago. 30 minutes is considered the maximum time that a GC-O session should last for since after this point the concentration of the panellist will certainly start to waver effecting the results. The GC-O analysis is focused on the specific odour-active compounds while sensory evaluation focuses on the overall flavour of the sample in question or other attributes such as the smell, visual qualities, or consistency. The specific odour-active compounds are separated and detected out of a sample that may contain hundreds of different compounds, in sensory analysis the detection of specific flavour compounds is very unlikely but that is also not generally the objective of the analysis (Vene et al., 2013).

When training panellists it is recommended to use compounds that are present in the product/sample of interest as standards. The standards are used to teach odour vocabulary and familiarise panellists with the scale of different intensities that they might detect during the GC-O. Performing additional tasks such as odour descriptions and intensity ratings are not recommended for inexperienced panellists in addition to just the detection of odours during the GC-O. Posterior intensity method can be used with a newly trained panel even though it is more difficult for the panellists (Vene et al., 2013). Training odour vocabulary with standard compounds, the usage of the intensity scale ratings and GC-O sessions with the real product/sample are the most effective training methods according to a previous study on GC-O panel training (Vene et al., 2013). During the GC-O session external disturbances should be minimized and there is no communication between other panellists or the person who is conducting the session during the analysis. Each individual panellists GC-O session time is arranged in advanced so that panellists are not in a hurry and that they are as calm as possible during the session. A panellists should not sniff more than six samples per day and the sessions should be as short as possible (Vene et al., 2013). While the panellist is speaking to record odour descriptions or intensities, they might miss an odour as they are not breathing in while speaking. Thinking about the odours description and intensity can also take longer than the duration of the odour from the GC-O (Vene et al., 2013). Training sessions with the final sample with the GC-O can help with this issue, panellists can also be told that there are tight intervals between odours in the sample so that they are as ready as possible in training. It is better if the training and the final GC-O evaluation are not spread over a period that is too long, as the learnings from the training sessions will be forgotten after enough time has passed and the motivation of panellists drops.

1.6 Volatile compounds found in other fish species that create off-flavours and odours

There are certain flavours which are species-related in fish and shellfish due to the environment the species live in and the food they consume. This leads to the accumulation of compounds that result in the formation of these specific flavours (Lindsay, 1990). The fatty acid composition of fish muscle is affected by the lipid composition of the diet that the fish eats (Sérot et al., 2002). Consumers prefer odours and flavours in fish that remind them of qualities relating to fresh fish, these are characterized as mild, green, and plant-like odours and flavours. Some of the volatile

compounds related to the fresh fish odour are hexanal, (E)-2-hexenal, (Z)-3-hexenal, 1-octen-3-ol, 1-octen-3-one, (Z,Z)-1,5-octadien-3-ol, (Z,Z)-1,5-octadien-3-one, (E,Z)-2,6-nonadienal, and (E,Z)-2,6-nonadienol. Some of these compounds are derived by enzymatic activity that breaks down long-chain PUFAs (Lindsay, 1990). (E,Z)-2,6-Nonadienal is formed by enzymatic degradation of n-3 PUFAs (Sérot et al., 2002), the compound is one of the main contributors of green (cucumber) odour notes in fresh fish and it degrades into (Z)-4-heptenal (Lindsay, 1990).

A study concerning four potent volatile compounds commonly found in fish or fish oil studied the effect that the combination of these compounds would have on off-flavours and odours. These four compounds were 1-penten-3-one, (Z)-4-heptenal, (E,E)-2,4-heptadienal, and (E,Z)-2,6-nonadienal (Venkateshwarlu et al., 2004). The study did find that the compounds interacted with each other enhancing and creating fishy or metallic odours and flavours. 1-Penten-3-one and (Z)-4-heptanal were found to have an interaction where heptanal enhances the formation of a metallic odour originating from 1-penten-3-one. 1-penten-3-one is also a product of n-3 PUFA oxidation and together with (E,Z)-2,6-nonadienal the increase in their concentrations contribute significantly to formation of off-flavours (Venkateshwarlu et al., 2004). The study illustrates the point that the way different volatile compounds interact with each other is important to the overall sensory perception.

From the volatile compounds detected in rainbow trout (*Oncorhynchus mykiss*) the most odour-active were aldehydes, such as (Z)-4-heptanal, (E,E)-2,4-heptadienal, octanal, nonanal, decanal, and (E,E)-2,4-decadienal. 1-Octen-3-ol had the strongest intensity value of the alcohol compounds and its odour is mushroom-like (Selli et al., 2006). These compounds will likely also influence the overall odour of Baltic herring. A study focusing on the effect of dietary lipids on odour-active compounds found in the muscle of brown trout (*Salmo trutta*) also discovered that most of the detected odour-active compounds were either aldehydes or alcohols (Sérot et al., 2002). Lipid-derived aldehydes have low odour detection thresholds which is why they generally contribute strongly to the overall odour of fish. The perception of most odour-active compounds seems to be more related to the PUFA concentration of fish muscle rather than the overall fatty acid composition. High concentrations of PUFAs initiate oxidative degradation of fatty acids more due to forming alkyl radicals easier than mono- or di-unsaturated fatty acids. After the radicals are formed a chain reaction starts that propagates the breakdown of other fatty acids (Sérot et al., 2002).

1.6.1 How different preparation/handling processes could affect the volatile compounds found in different fish

For silver carp (*Hypophthalmichthys molitrix*) a fishy odour develops when the muscle of the fish is chopped or homogenized (Fu et al., 2008). The formation of off-flavours is mainly caused by lipid oxidation. The lipid oxidation is thought to be catalysed by lipoxygenase, heme proteins, and irons (Fu et al., 2008). Baltic herring also has a high content of heme proteins (hemoglobin and myoglobin) due to it having a lot of dark muscle tissue, thus it also susceptible to lipid oxidation catalysed by heme proteins (Park, 2005).

When comparing different washing processes effects on silver carp using a saline or an alkaline solution proved to be much better at removing aroma-active compounds from silver carp mince than just water (Zhuo et al., 2016). The interactions between odours compounds and non-volatile compounds in the fish mince such as protein may play an important role when either binding or releasing odours compounds. The alkaline or saline solution dissolves fish proteins that leads to the releasing of volatile compounds that have bonded to the protein (Zhuo et al., 2016). This same effect is likely to happen in the pH-shift process using Baltic herring as raw material as it uses alkaline or saline solutions to increase or lower pH in the process, thus also removing aroma-active compounds from the remaining protein mass.

Considering smoking fish as an example all the parameters that can be altered were determined to influence the final products odour characteristics. These included parameters such as smoke generation, deposition method, smoking temperature, exhaust valve opening in the smokehouse or voltage applied in the electrostatic tunnel (Cardinal et al., 2005). Different preparation process can have many similar parameters that will affect the odour characteristics of the final product that one should consider.

Protein isolate received from different fish species using the pH-shift method has not previously been analysed with the GC-O. The pH-shift method has been studied as a potential process step to remove off-odours that are associated with some fish species. The studies have used other methods rather than GC-O to analyse the changes in volatile/odour-active compounds in the protein isolate formed with pH-shift. Instead of using GC-O to analyse the change in off-odour, it is common to use sensory analysis with trained panellists as an alternative. Comparing the fish sample in question before and after pH-shift and rating the intensities of some odours such as fishy, earthy, and

rancid between the two samples makes it possible to compare the samples on these parameters (Phetsang et al., 2021). Sensory evaluation can be used with many things such as the change in off-odour intensities during storage that focuses on certain odour characteristics such as fishy, earthy, and rancid (Phetsang et al., 2021). Sensory evaluation is easy to perform, and it does not require any extra machines that might not be available for the research. These are the main advantages over using a GC-O, however it is not able to provide a more detailed analysis on which and how many odour-active compounds are the ones influencing the overall odour the most. When the overall odour or certain parameters of the sample change after pH-shift or storage as an example, the change in the odour-active compounds behind the overall odour remains an unknown. Was there a new compound that was not previously detectable by the panellists in the altered sample or did a previously detected compound become more intense, which lead to the increasing intensity of a certain odour? These are questions that cannot be answered with just a sensory evaluation of the sample. The GC-O on the other hand is not helpful in determining the changes of the overall odour and sensory evaluation is needed when comparing samples before and after a certain process step. Combining the GC-O and sensory analysis can give a better idea of both the changes in individual odour-active compounds and in the overall odour.

1.6.2 Volatile compounds of Baltic herring

A previous study of volatile compounds found in Baltic herring discovered that 3-methylbutanal, 2-methylbutanal, hexanal, heptanal, pentanal and two octadienes were the most abundant volatiles in fresh, oven-baked Baltic herring (Aro et al., 2003). Aldehydes are the most common class of volatile compounds in Baltic herring. 2-Methylbutanal and 3-methylbutanal are branched chain aldehydes that are formed by Strecker degradation, where amino acids leucine and isoleucine react with α-dicarbonyl compounds (Aro et al., 2003). These two compounds were the most abundant compounds when measured by total peak are from the GC-MS chromatogram for the fresh fish. The two compounds were also the only volatile compounds that showed a decreasing trend compared to total volatiles during storage (Aro et al., 2003). The number of volatile compounds increased with the additional storage time of the fish.

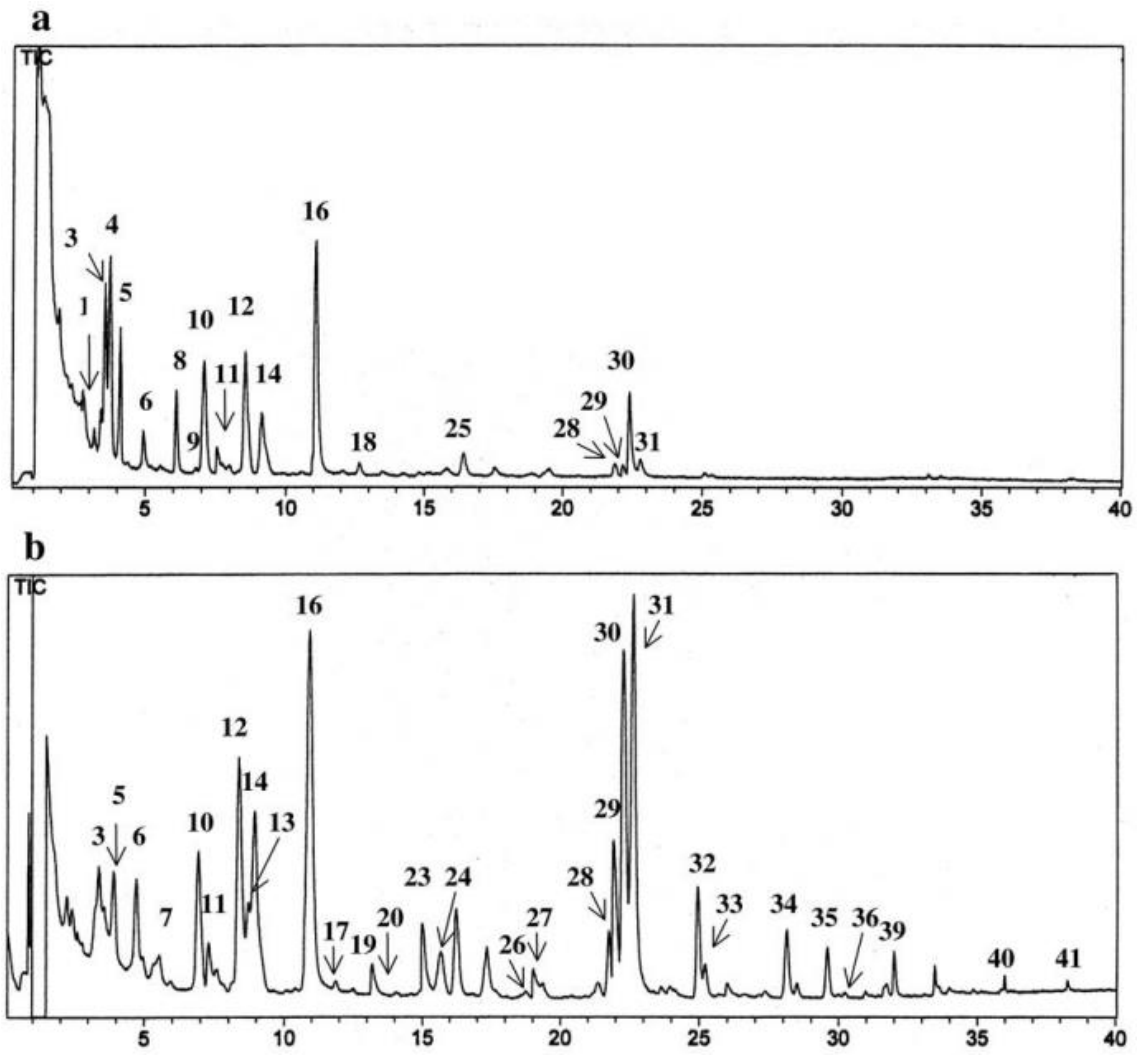

Figure 6. GC-MS chromatograms of baked Baltic herring comparing three-day storage (**a**) and eight-day storage (**b**) (Aro et al., 2003).

In **Figure 6.** the difference that a five-day storage at 6 °C has on the number and area of peaks when just comparing the chromatograms from GC-MS is noticeable. Even after only eight days of storage the formation of new volatile compounds has started in earnest. The sooner the fish is cooked/eaten after catching the better it is when trying to avoid the formation of potential off-odours. Storing herring at -80 °C keeps the sensory quality of the fish stable with only small changes even after 18 months. At -20 °C the odour and flavour characteristics start developing rancid qualities becoming unpleasant (Jónsson et al., 2007).

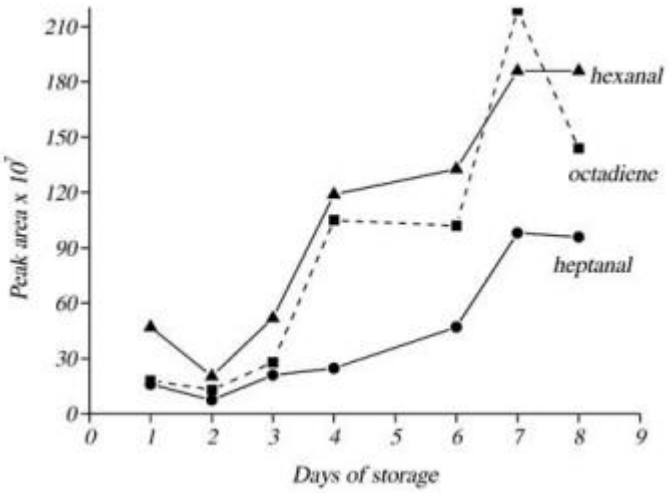

Figure 7. Change in the peak are of certain volatile compounds (hexanal, heptanal and octadiene) at different day intervals during storage (Aro et al., 2003).

The oxidation of long chain unsaturated fatty acids increased the concentration of aldehydes such as heptanal, hexanal, 2,4-heptadienal, 2,4-decadienal, propanal, nonanal and pentanal. Hexanal is a common volatile compound found in most other fish species (Sérot et al., 2002; Jónsdóttir et al., 2007; An et al., 2020). Hexanal was one the three major volatile compounds found in fresh fish that then it became the most abundant after eight days of storage (Aro et al., 2003). **Figure 7.** shows the development of hexanal, heptanal and octadiene during the storage measured by the peak area from GC-MS. The same study also found 2,5-octadienes in Baltic herring that have also been found in canned sockeye and pink salmon (Aro et al., 2003). The most likely source for the 2,5-octadienes is the oxidation of n-3 polyunsaturated fatty acids which is the same way straight chain alkanals are formed in the fish. 2,6-Nonadienal is also formed by the oxidation of n-3 fatty acids which is a precursor for (Z)-4-heptenal as mentioned previously in **Chapter 1.6**. After eight days of storage the most abundant volatiles had changed from 3-methylbutanal, 2-methylbutanal and hexanal to hexanal, octadienes, heptanal and 4-heptenal (Aro et al., 2003).

1.7 Aim of the study

The aim of the research is to use a fractionation method known as “pH-shift” or isoelectric precipitation to isolate the protein in the Baltic herring from its scales, skin, and bones. By doing this most lipids from the fish are left behind which are the main cause of the unpleasant aroma of the fish after oxidation. Thus, the aim of the study is to compare the raw Baltic herring and protein isolate without further processing using GC-FID/O and GC-MS systems to identify the compounds. The data collected from the GC-

O analysis of this study has been used in a published research paper that focused on the protein isolate produced from the Baltic herring with the pH-shift and its potential uses in food models (Kakko et al., 2022).

2 Materials and Methods

2.1 pH-shift process

The gutted and beheaded Baltic herring (Martin Kala Oy, Turku, Finland) was stored at -80 °C and thawed at 4 °C overnight before starting the pH-shift protein isolation process. The theory behind the pH-shift method for Baltic herring protein isolation is described in **Chapter 1.4**, this version of the method was developed by Sofia Marmon (2012). The method had been previously tested and used with equipment at a laboratory in Åbo Akademi facilities that succeeded in producing the desired protein isolate (Aitta, 2019). The gutted and beheaded Baltic herring (bought from Martin Kala) was mixed with water in a 1:9 ratio and then blended (Raw mix 2 professional mixer, Rawmix Oy, Helsinki, Finland) into as homogenous mix as possible. 115.5 g of herring was blended with 924 g of water for the first mix and 131.1 g of herring was blended with 1048.8 g of water. Two batches were estimated to provide enough protein isolate for the requirements of this study. The mix was poured into a glass reactor (1 litre, 100 mm diameter, Lenz Laborglas GmbH & Co. KG, Wertheim, Germany) which had an internal cooling system set at 4 °C and the mixing speed was set at 800 rpm. An automatic pumping system (Alaris Asena GH MK III, Becton, Dickinson and Company, Franklin Lakes, U.S.A.) at volume of 30-40 mL/h with 1 M NaOH solution was used to increase the pH of the mix to 11.2. The increasing pH is meant to solubilize the proteins, 10-15 ml of 1M NaOH was the amount added in total and the time it took to reach the intended pH was about 15-20 minutes. The mix was then centrifuged (Cellsep 6/720R, MSE, Nuaillé, France) at 4000 times gravity for 20 minutes. The supernatant that formed after the centrifugation was filtered through a folded cheesecloth (Decola, Jyväskylä, Finland) placed in a colander separating it from insoluble material. The cheesecloth was used to separate a lipid layer that had been formed at the surface of the supernatant from the rest, the remaining precipitant was thrown away. The filtered supernatant was poured back into the reactor for the purpose of adding 1 M HCl until a pH of 5.4 is reached under the same conditions as before. To reach the pH target it took 12-17 ml of HCl solution added for 20-30 minutes. When the pH of 5.4 was reached, the remaining solution was still mixed in the reactor for an additional 10 minutes. The solution was then centrifuged again at 4000 g for 30 minutes, which formed three layers afterwards. The top layer (lipid emulsion) was removed with a spoon and the supernatant was poured away. The remaining bottom layer contained the precipitated proteins that were collected with a spoon to a decanter. The protein isolate was weighed

and 4 % of cryoprotectants (2 % of fructose and 2 % of sorbitol) was added based on the weight of the protein isolate. The pH of the protein isolate was adjusted to 7.0 with 4 M NaOH while mixing with the spoon, afterwards it was stored in a freezer at -80 °C.

2.2 Headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS)

The same batch of raw Baltic herring that was used in the pH-shift was minced in a meat grinder (Kenwood Limited, Havant, United Kingdom) for the second sample that would be compared to the protein isolate samples. 3 g ± 50 mg of either the protein sample or the raw herring was weighed in to 20 ml vials meant for HS-SPME-GC-MS. The GC-MS system consisted of a TRACE 1310 gas chromatograph coupled with a ISQ7000 single quadrupole mass spectrometer with a TriPlus RSH autosampler (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) The samples were stabilized at 40 °C for 20 min before the fibre meant to absorb the volatile compounds from the sample was injected into the vial. Absorption took place at 40 °C for 30 min before injection; the volatile compounds were in the headspace of the vial from where they get absorbed into the divinylbenzene/carbozen/polydimethylsiloxane fiber (1 cm, 50/30 μm film thickness, Supelco Inc., Bellefonte, Pennsylvania, U.S.A.). The initial oven temperature was set at 40 °C and it was held for 3 min, next an increase of 8 °C/min until 150 °C which was followed by another temperature ramp of 10 °C/min until 220 °C was reached and held for 10 minutes. The column used in the GC was a SPB-624 (30 m, 0.25 mm, 1.40 μm, Supelco Inc.). The mass spectrometer was operated in the electron ionization mode. Transfer line's temperature was set at 220 °C and the ion source was set at 250 °C. The spectra were collected in a mass range of 40-300 amu. The data was processed using a program called Chromeleon 7.0 (Thermo Scientific) and National Institute of Standards and Technology library (version 2.3, Gaithersburg, Maryland, U.S.A.).

2.3 Training of the panel for GC-O analysis

Six volunteers took part in the GC-O analysis of which four were women and two were men (ages between 24 to 38 at the time of the analysis). Six panellists were thought to be a suitable number for the analysis, a panel usually consists of at least four members up into the teens. Due to limited time available for panel training, panellist with previous experience with GC-O analysis were preferred for the panel. Out of the six panellists five had such experience and all members had previous experience from

sensory evaluations. The training was planned to include two sessions. The first training session was performed with the GC-O by smelling a raw minced Baltic herring sample. The goal was to familiarize the panellists with the GC-O and help them get used to describing the odours they recognize while also rating their intensity. As mentioned in **Chapter 1.5.4** the panellists sometimes will not have a lot of time to react between odours so this session helped them understand the reaction time they would have in the actual evaluations.

The second session was a group discussion where the panellists first discussed what they had learned from the first session, and then sniffed certain standard compounds found in the herring. The compounds used are listed in **Table 1.,** the training session was arranged in a way that the panellists would first sniff dilutions of the selected compounds (ten in total) once and then describe each compound's odour. If necessary additional sniffs can be done and between the different compound's panellists are encouraged to drink/smell water. They were then asked to discuss with each other what descriptions they thought best described each compounds odour. The standards were sniffed from 30 ml brown glass bottles that had a 1 cm x 1 cm paper slip placed in them. The paper slips had 1 µl of each standard compound and different dilutions of some of the standards pipetted onto the paper. The compounds were referred to with three-digit codes so that the panellists did not know what compound they were smelling. Three compounds (2-methylbutanl, hexanal & heptanal) were chosen to be diluted into a series of dilutions meant to familiarize the panellists with different odour intensities as the second part of the session. The panellists were asked to rate the intensities of the diluted compounds in question on a scale of 1-4 (1 = very mild, 2 = mild, 3 = fairly strong & 4 = strong) with an empty sample to give them an idea of the range of possible intensities (4 bottles per dilution series including an empty sample). The panellists were told that one of the bottles would not have any compound in it but not which one.

Table 1. Standard compounds used in the second session of panel training for GC-O, the compounds were named with three-digit codes to hide the name of the compounds from the panellists

Compounds and dilutions	Code
2-methylbutanal	959
2-methylbutanal 1:5	138
2-methylbutanal 1:10	835
Hexanal	044
Hexanal 1:5	230

Hexanal 1:10	415
Heptanal	887
Heptanal 1:5	942
Heptanal 1:10	774
3-methylbutanal	117
Hexanoic acid	473
2-ethylfuran	977
1-penten-3-ol	106
Nonanal	195
1-octen-3-ol	115
2,3-butanedione	890
Empty	607, 062 & 559

2.4 Gas Chromatography-Olfactometry

The same batch of Baltic herring that was used to isolate the protein isolate with the pH-shift was minced in a meat grinder for both GC-MS-SPME and the GC-O analysis. The same methods of detection frequency and posterior intensity (intensity scale of 1-4) used in the training were used in these GC-O sessions. The raw minced Baltic herring and the protein isolate were divided into zip-lock plastic bags and stored in -80 °C until the GC-O analysis. Dividing the samples into smaller portions in the bags helped thawing them before analysis and the portioning into roughly 10-15 g portions helped weighing the samples for analysis. The volatile compounds were extracted from the samples using the headspace solid phase microextraction technique with a Divinylbenzene/Carboxen/Polydimethylsiloxane fibre (2 cm, 50/30 µm film thickness, Supelco Inc., Bellefonte, Pennsylvania, U.S.A.). 10 g ± 0.1 g of either sample were weighed into 50 ml Erlenmeyer flasks (90 ml total volume) that was then immersed about halfway into a 40 °C water bath. The bath was heated with a heating plate and the flask was held in place with a clamp connected to a ring stand. The sample was then equilibrated for 20 minutes at that temperature so that the volatile compounds would evaporate from the sample and enter the headspace. The solid phase microextraction fibre was then inserted into the Erlenmeyer flask for 30 minutes to extract the volatiles from the headspace under the same conditions.

The GC-O used for the analysis of the samples was a HP 6890 Series with an FID (Hewlett Packard, Palo Alto, California, U.S.A.) and Chemstation software (Agilent Technologies, Palo Alto, California, U.S.A.). The column used was the same SPB-624 (30 m, 0.25 mm, 1.4 µm, Supelco Inc.) as in the GC-MS to separate the volatile

compounds. Initial oven temperature in the GC-O was set up to 40 °C that was held for 3 min, then increased at 8 °C/min until 150 °C when the ramp up was increased to 10 °C/min up to 220 °C, which is then held for 10 minutes. Helium was used as the carrier gas with a flow of 1.4 ml/min. Both samples were analysed twice by each panellist and each time they pressed a button to signal when they started smelling the odour and then stopped pressing when they no longer did, afterwards describing the odour and rating its intensity. The button for the signal was connected to a microphone so that the signal showed up in the audio recording. Otherwise, the entire session for each analysis was recorded and one session lasted for about 25 minutes. The recordings were reviewed using a program called Audacity 2.4.2 (Audacity). The SPB-624 column was connected directly to the olfactometry detection port (Gerstell) during the analysis; it was connected directly to the FID when testing how the GC settings would perform with the samples in question. When an odour achieves a NIF-% value of 50 % (detected in 6 out of 12 sessions) the odour-active compound could be considered significant. The average odour intensity for each compound was calculated from the ratings given meaning that if a panellist did not detect the compound/rate the intensity it was not included in the average.

Additionally, a DB-WAX column (60 m, 0.25 mm, 0.25 µm, Agilent Technologies, Santa Clara, California, U.S.A.) was used to help in the identification of the odour-active compounds. This was done after the previous GC-O analysis with two of the panellists who took part in the previous analysis. This time they were asked to only describe the odours while evaluating the same two samples but only once per sample. The panellists were also tasked to analyse a standard compound (listed in **Table 2.**) mixture that included different volatile compounds thought to be present in the Baltic herring. C7-C30 saturated Alkanes (Merck, New Jersey, U.S.A.) were run as a separate sample that was used to calculate retention indexes. These standard compounds were diluted with propylene glycol (Amresco, Ohio, U.S.A.) and Milli-Q (Merck, New Jersey, U.S.A.) purified water into dilution factors of 1:1000 when they were finally mixed. There was an area in the previous GC-O analysis where a couple of compounds were co-eluting close to each other, so the DB-WAX column was used to potentially improve the elution of this area. The oven parameters were changed to holding for 3 min at 40 °C from which the temperature was increased by 7 °C/min until 220 °C that was held for 10 min. This time the column was also connected simultaneously to the olfactometry port and FID with a Y-connector. Other parameters remained unchanged.

Table 2. Standard compounds used with DB-WAX column that were analysed together as a mixture.

Compound	CAS	Manufacturer
6-methyl-5-hepten-2-one	110-93-0	Aldrich
Hexanoic acid	142-62-1	Sigma-Aldrich
1-Penten-3-ol	616-25-1	Fluka
2,2,4,6,6-Pentamethylheptane	13475-82-6	Tokyo Chemical industry
2-ethylfuran	18,698-8	Aldrich
3-methylbunal	590-86-3	Aldrich
2,4-heptadienal	4313-03-5	Sigma-Aldrich
2,3-butanedione	431-03-8	Sigma-Aldrich
Nonanal	124-19-6	Aldrich
2-methylbutanal	123-72-8	Acros organics
Octanal	124-13-0	Sigma-Aldrich
Butanal	123-72-8	Acros organics
1-Octen-3-ol	3391-86-4	Aldrich
2-methylpropanal	78-84-2	Acros organics
Propanal	123-38-6	Aldrich
2,3-pentadione	600-14-6	Sigma-Aldrich
Heptanal	111-71-7	Sigma-Aldrich
Hexanal	66-25-1	Aldrich

3 Results and Discussion

3.1 HS-SPME-GC-MS results

Table 3. Volatile compounds identified with GC-MS that could also be odour-active. PI: protein isolate sample, Raw BH: raw Baltic herring sample & SPB-624 RI: calculated retention index for the compounds

SPB-624 RI	Compound	Detected in
< 700	butanal	PI
< 700	3-methylbutanal	Raw BH
705	2-methylbutanal	Raw BH
721	2-ethylfuran	PI
738	1-penten-3-ol	Both
741	2,3-pentadione	Both
844	hexanal	PI
949	heptanal	PI
1053	octanal	PI
1070	(E,E)-2,4-heptadienal	PI
1086	(E,E)-2,4-heptadienal	PI
1156	nonanal	PI
1168	(E,E)-3,5-octadien-2-one	PI

The compounds were identified by comparing them to the National Institute of Standards and Technology library with the Chromeleon 7.0 program as mentioned previously in **Chapter 2.2**. The program shows how well the detected compounds match with the identification data from the library. The compounds that match well with the data can be considered to being identified. Retention indexes for the compounds were calculated with the use of C7-C30 Saturated Alkanes and they can be used to confirm the identification of the compounds with the retention index results from the GC-O. Many more volatile compounds could be identified from the protein isolate sample than from the raw Baltic herring sample with the HS-SPME-GC-MS. 3-methylbutanal and 2-methylbutanal were identified in the raw Baltic herring sample but not in the protein isolate sample. This is also consistent with the GC-O analysis results in **Chapter 3.3** where the two compounds have their NIF-% values and intensity ratings drop off noticeably from the raw Baltic herring to the protein isolate. 3-methylbutanal still has a NIF-% value of ≥ 40 % in the protein isolate but 2-methylbutanal has not been detected by the panellists in the GC-O analysis from the protein isolate. 1-penten-3-ol, 2,3-pentadione, hexanal, octanal and nonanal have also been detected in the GC-O analysis, the retention indexes of the compounds match between the GC-O and HS-

SPME-GC-MS and the odour descriptions of the panellists are like those of other studies (An et al., 2020; Zhou et al., 2016), online databases of odour descriptions (The Good Scents Company, 2023) or online databases of retention indexes for the DB-WAX column (NIST Chemistry WebBook, 2023). The retention indexes for (E,E)-2,4-heptadienal and (E,E)-3,5-octadien-2-one do not quite match with the GC-O results of the odour-active compounds that comply with the ≥ 40 % NIF threshold. The compounds could be present in one or both samples but many of the panellists did not detect their odour if this is the case. One of the 2,4-heptadienals detected in the HS-SPME-GC-MS is probably (E,Z)-2,4-heptadienal which a different isomer but it resembles the (E,E)-2,4-heptadienal in the library data. This same issue concerns the heptanal compound, the actual compound in question is very likely to be (Z)-4-heptenal instead of heptanal but there is some issue regarding the library data. (Z)-4-heptenal is detected in the GC-O analysis by all panellists in both samples and the GC-O retention index matches the retention index of heptanal in HS-SPME-GC-MS. Butanal and 2-ethylfuran were not detected in the GC-O analysis at least so that their NIF-% value would be above the ≥ 40 % threshold.

3.2 Aromagrams for raw Baltic herring and protein isolate samples made from the GC-O data

The audio recordings from the GC-O were edited with the Audacity program to add labels to the signals from the button presses that show the duration and retention time when the panellists perceived the odour. The labels also included the intensity ratings and descriptions for the odours. The recording microphone is switched on before the volatile compound collecting fibre was injected into the GC-O, when the fibre is injected the signal button is pressed. The labels with descriptions and ratings are then pulled from the program into text data that show how long the button pressing lasted.

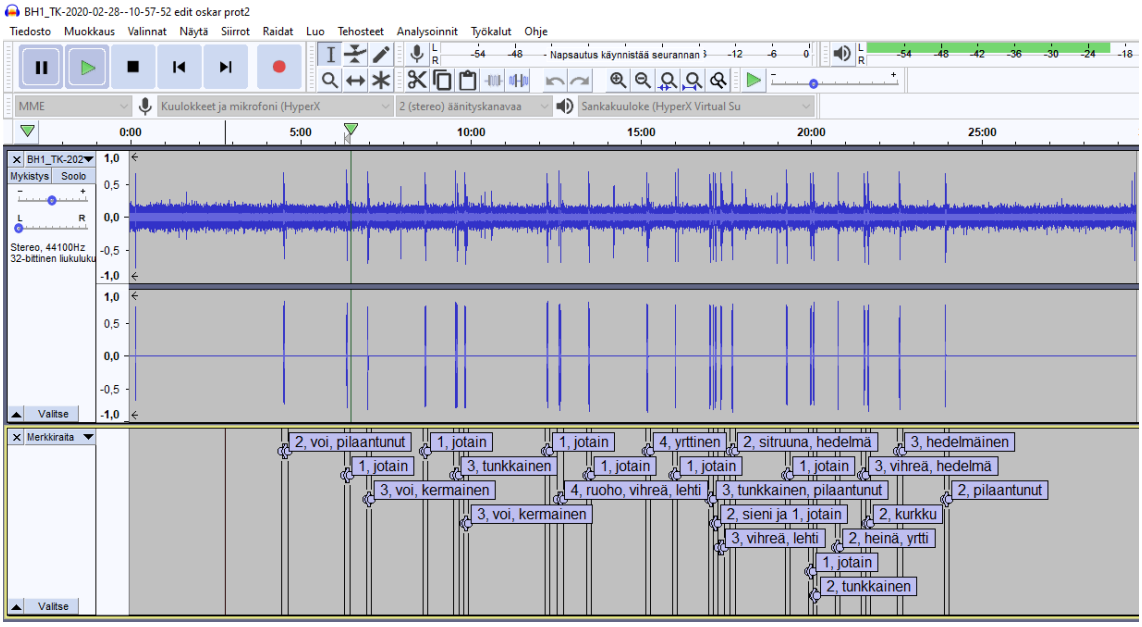

Figure 8. Audacity voice and signal editing program view for the GC-O panellists' recordings.

In **Figure 8.** the Audacity program is shown and the editing of the voice recording, button press signals and panellists' answers are shown in three sections. At the top row is the individual panellist's voice recording which also records the static background noise due to the microphone being on throughout the recording session. The second row contains the signals received from the button presses, when the panellist detects an odour after which they will describe the odour and rate its intensity that is recorded in the track at the top. The first button press is meant to signal the injection of the sample into the gas chromatograph that is used as the starting point for the recording. The descriptions of odours and intensity ratings for the detected odour are recorded in the bottom section according to what the panellist says, and the labels track length is adjusted to the timing of the button press. The time data of the label which is adjusted to the button press signal is then imported to Excel. This data is used to make an aromagram by multiplying the frequency percentage (panellists detecting an odour and pressing the signal button) by duration which results in the SNIF value as described in **Chapter 1.5.1**.

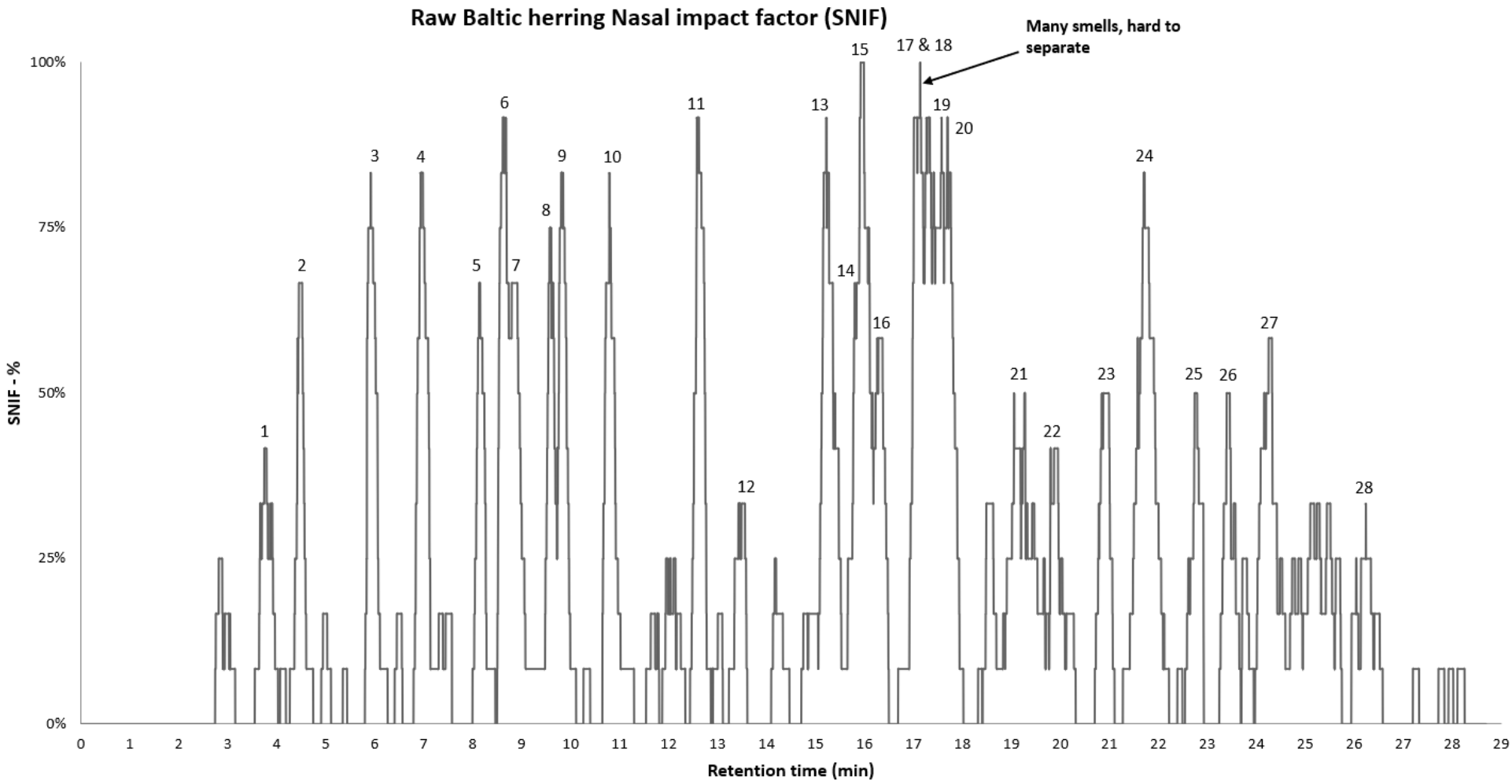

Figure 9. SNIF aromagram made from the raw Baltic herring sample GC-O data.

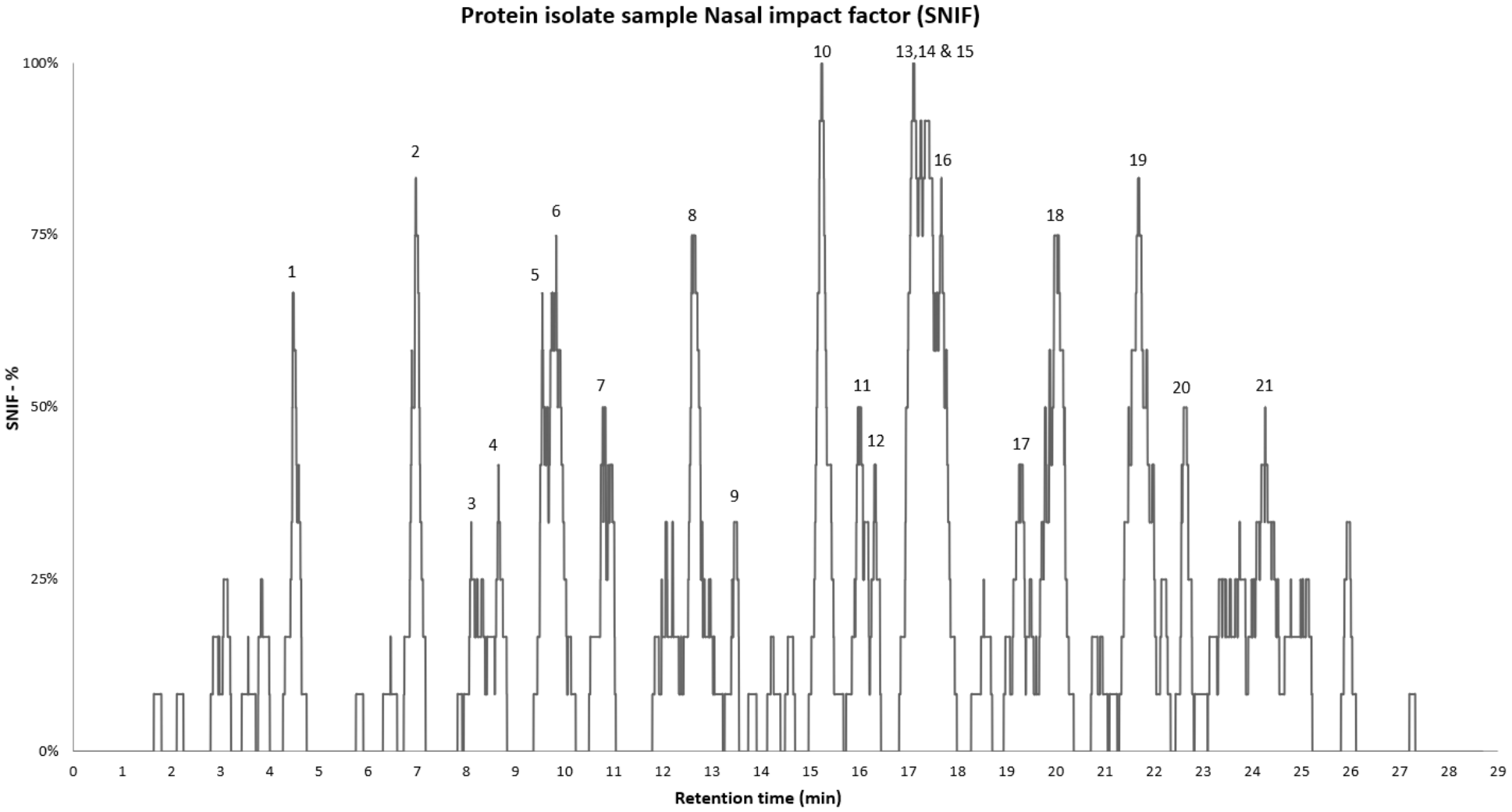

Figure 10. SNIF aromagram made from the protein isolate sample GC-O data.

The aromagrams for the raw Baltic herring and protein isolate shown in **Figures 9. & 10.** visualize the detection of odour-active compounds by the panellists**.** The peaks that are numbered in the two figures refer to the numbers in **Tables 4. and 5.** for the corresponding compounds, **Figure 9.** and **Table 4.** are connected to each other while **Figure 10.** and **Table 5.** are connected. The connection comes from the two sample materials: raw Baltic herring and protein isolate from the Baltic herring received from the pH-shift. And the results in the tables and figures are from the same GC-O analysis data. The Baltic herring sample had 28 odour-active compounds with a NIF rating of 50-40 % or higher and the protein isolate had 21 odour-active compounds with a NIF rating of 50-40 % or higher. These compounds are the numbered peaks in the tables and figures mentioned earlier in this chapter. 40 % NIF values were included in the significant odour-active compounds since there were a few compounds that were detected in at least six sessions out of twelve but the button pressing signals did not quite overlap. An odour might have been detectable for a short period and the panellists' button pressing techniques varied a bit which affected the timing of the signal. Asking the panellists to perform other tasks than just the button pressing may have resulted in the poor timing of the button presses. Sometimes there were different odours present in a long continuous sequence resulting in long signals after which the panellist described the different odours and rated their intensity. There are odours that were detected by six panellists (NIF 50 %) but that do not present the data correctly on the aromagram due to the issues with the signal timing. In **Figure 9.** peaks 1, 12 and 28 are examples of such odours that had a NIF rating of 50 % but in the aromagram they shown to be under the 50 % value based on peak height. Peak number 12 is (Z)-3-hexenal and the other two compounds were not identified. In **Figure 10.** the peaks that do not match the 50 % value in the aromagram are 3, 4, 12 and 17. Out of these four peaks number 4 was 3-methylbutanal and peak number 12 was 1-octen-3-one, the other two were not identified.

3.3 Odour-active compounds detected in the samples

The 28 odour-active compounds with NIF values of ≥ 40 % detected from the raw Baltic herring sample and the 21 detected from the protein isolate sample were analysed more closely. The compounds were identified by their odour descriptions and retention indexes by comparing them to the standard compound sample mixture's equivalent data.

Additionally, the retention index was compared to the retention index data found in the NIST Chemistry WebBook and to certain other studies retention index results (An et al., 2020; Wu et al., 2014; Hartvigsen et al., 2000; Sérot et al., 2002; Phetsang et al., 2021; Salum et al., 2017).

3-methylbutanal and 2-methylbutanal have been detected in Baltic herring before and their concentrations have been shown to decrease during storage (Aro et al., 2003). In this study the detection frequency and intensity of both compounds fell when comparing the protein isolate with the raw fish sample. The compounds were identified by comparing the odour description and retention index to the standard samples. When 3-methylbutanal was detected in the raw herring it was described as stuffy and pungent but when the intensity lowered in the protein isolate the odour was closer to chocolate. 2-methylbutanal had a sweeter chocolate odour in the raw herring and was not detected in the protein sample. 2-methylpropanal was described having a chocolate, almond and cognac like odour, the compound was also only detected having a significant odour in the raw herring. 2-methylpropanal has been detected before in cod and its odour was described as sweet and flowery malty (Jónsdóttir et al., 2007) and it has been detected in Baltic herring (Aro et al., 2003). 2,3-butanedione and 2,3-pentadione were detected in both samples and had high intensity ratings. These compounds had their odours described as butter, cream, and caramel by the panellists. These two compounds could be considered to have quite pleasant odours and thus they might not contribute to the commonly experienced unpleasant fishy odour of the herring. The two compounds have been detected in other fish such as the Alaska pollock and Pacific whiting and they were described as buttery (An et al., 2020).

1-penten-3-ol and dimethyl disulphide both had odours that were described as pungent and solvent-like. Similar descriptions were given to propanal and acetic acid, they were most described as having a solvent-like odour. 1-penten-3-ol, dimethyl disulphide and acetic acid have been previously detected in studies regarding Baltic herring volatile compounds (Aro et al., 2003, 2002). Hexanal and (E,Z)-2,6-nonadienal had their odour described as green, additionally hexanal was often described as smelling like grass and (E,Z)-2,6-nonadienal was described as cucumber. Hexanal has also previously been detected in Baltic herring (Aro et al., 2003) and it is commonly found in other fish species, it is often described having a green or grassy odour (Sérot et al., 2002; Jónsdóttir et al., 2007; An et al., 2020). (E,Z)-2,6-nonadienal has been detected in mayonnaise enriched by fish oil and other fish species, it is described having an odour

reminiscent of a cucumber (Hartvigsen et al., 2000; An et al., 2020). Octanal and (Z)-3-hexenal were described as fruity, citrus, and sweet. Both compounds have been detected in Baltic herring before (Aro et al., 2003) and in other fish such as silver carp (An et al., 2020). Octanal was described having a citrus odour and (Z)-3-hexenal had a leafy odour in the silver carp. (Z)-4-heptenal had a very intense fatty and fishy odour and as such the compound could be one of the major sources behind the unpleasant fishy odour of the Baltic herring. It has been detected in Baltic herring (Aro et al., 2003) and other fish (Jónsdóttir et al., 2003; Selli et al., 2006; An et al., 2020). It has been described as having a fishy or boiled potato-like odour in these studies.

1-octen-3-one, (Z)-1,5-octadien-3-ol and 1-octen-3-ol were described as having a mushroom-like smell with (Z)-1-octadien-3-ol being mostly described as having a green, forest and carrot-like odour. 1-octen-3-one has been detected in silver carp, Pacific Whiting and Alaskan pollock while 1-octen-3-ol was detected in silver carp and Pacific whiting (An et al., 2020). They were described to have a mushroom-like odour. Both have also been detected in mayonnaise enriched with fish oil as has (Z)-1,5-octadien-3-ol (Hartvigsen et al., 2000). 1-octen-3-one had its odour described as mushroom while 1-octen-3-ol was described as pungent, soil and fruity and (Z)-1,5-octadien-3-ol was described as citrus and green. (Z)-1,5-octadien-3-ol has been detected in brown trout and its odour was described to be mushroom-like (Sérot et al., 2002). Methional has been detected in Pacific whiting, Alaskan pollock and brown trout while its odour was described as potato-like (An et al., 2020; Sérot et al., 2002). The panellists described the odour of methional to be potato and mushroom-like in the GC-O sessions of this research. Nonanal has been detected in silver carp, Alaskan pollock, Pacific whiting and Baltic herring, its odour has been described as soapy, oxidated oil, green and fatty-like in these fish (Aro et al, 2002; Aro et al, 2003; Fu et al., 2009; Zhou et al., 2016; An et al., 2020). The panellists in this study described it to have a forest, egg, and mushroom-like odour.

Table 4. Odour-active compounds in raw Baltic herring sample with ≥ 40 % NIF values. Compounds that were only tentatively identified are written in *italics*. MF-%: calculation explained in **Chapter 1.5.2**, SPB-624 & DB-WAX IR: calculated retention indexes.

	SPB-624 IR	Odour Descriptions	Intensity	MF-%	DB-WAX IR	Compound
1	n.d.	Fish, Stuffy	1.67	45.7 %	n.d.	unknown
2	< 600	Glue, Solvent	1.33	49.9 %	793	propanal
3	< 600	Chocolate, Almond, Cognac	2.2	67.7 %	812	2-methylpropanal
4	< 600	Butter, Cream, Caramel	2.91	81.7 %	973	2,3-butanedione
5	n.d.	Solvent	1.63	52.1 %	n.d.	*acetic acid*
6	693	Stuffy, Pungent	3.18	85.4 %	917	3-methylbutanal
7	703	Sweat, Sweet	2.38	63 %	913	2-methylbutanal
8	730	Solvent, Pungent, Plant	2.3	69.2 %	n.d.	1-penten-3-ol
9	739	Cream, Butter, Caramel	3.09	84.2 %	1055	2,3-pentadione
10	785	Solvent, Pungent, Plastic	2.1	66.1 %	n.d.	dimethyl disulphide
11	844	Green, Grass	2.92	85.4 %	1081	hexanal
12	892	Fruit, Sweet	1.5	43.3 %	1131	(Z)-3-hexenal
13	947	Fatty, Fish	3.25	90.1 %	1238	(Z)-4-heptenal
14	977	Sweat	2.5	55.9 %	n.d.	unknown
15	983	Potato, Mushroom	2.92	85.4 %	1470	methional
16	998	Mushroom	2.13	59.6 %	1308	*1-octen-3-one*
17	n.d.	Mushroom	3	86.6 %	n.d.	*1-octen-3-ol*
18	1037	Mushroom, Metallic	-	-	1443	*1-octen-3-ol*
19	1049	Green, Forest, Carrot	3.38	75.1 %	1481	(Z)-1,5-octadien-3-ol
20	1067	Fruit, Citrus	2.27	72.9 %	1285	octanal
21	1151	Forest, Egg, Mushroom	1.71	49.9 %	1392	nonanal
22	n.d.	Dusty, Malt	1.8	43.3 %	n.d.	unknown
23	n.d.	Forest, Malt	1.38	48 %	n.d.	unknown
24	1295	Cucumber, Vegetable, Green	2.67	81.7 %	1594	(E,Z)-2,6-nonadienal
25	1374	Sweat, Stuffy, Pungent	2.43	59.5 %	n.d.	unknown
26	1408	Sawdust, Mushroom	1.57	47.8 %	n.d.	unknown
27	1495	Sweet, Forest	2	57.7 %	1748	unknown
28	1598	Mushroom, Forest	1.57	47.8 %		unknown

The issues regarding the compounds that were only tentatively identified and written with *italics* are explained in more detail in the final paragraph of **Chapter 3.3**.

Table 5. Odour-active compounds in protein isolate sample with ≥ 40 % NIF values. Compounds that were only tentatively identified are written in *italics*. MF-%: calculation explained in **Chapter 1.5.2**, SPB-624 & DB-WAX IR: calculated retention indexes.

	SPB-624 RI	Descriptions	Intensity	MF-%	DB-WAX RI	Compound
1	< 600	Glue, Solvent	1.82	64.6 %	793	propanal
2	< 600	Cream, Butter, Caramel	2.27	72.1 %	973	2,3-butanedione
3	n.d.	Pungent, Solvent	1.71	49.9 %	n.d.	*acetic acid*
4	693	Chocolate, Almond, Stuffy	1.5	43.3 %	914	3-methylbutanal
5	730	Stuffy, Solvent, Mushroom	2.1	66.1 %	n.d.	1-penten-3-ol
6	739	Cream, Butter, Caramel	3.25	86.6 %	1055	2,3-pentadione
7	n.d.	Glue, Plastic, Solvent	2.11	62.9 %	n.d.	dimethyl disulfide
8	843	Green, Grass	3.25	90.1 %	1081	hexanal
9	n.d.	Fruit, Sweet	1.4	38.2 %	1131	(Z)-3-hexenal
10	947	Rotten, Fish, Mushroom	3.5	93.5 %	1238	(Z)-4-heptenal
11	n.d.	Earth, Mushroom, Potato	1.56	54.1 %	1470	methional
12	n.d.	Mushroom	1.5	45.7 %	1308	*1-octen-3-one*
13	1025	Stuffy, Pungent, Fatty	2.83	84.1 %	n.d.	*1-octen-3-ol*
14	1036	Mushroom	3	61.2 %	1443	*1-octen-3-ol*
15	1049	Green, Carrot, Forest, Plants	2.89	73.6 %	1489	(Z)-1,5-octadien-3-ol
16	1067	Fruit, Citrus	2.4	70.7 %	1285	octanal
17	1151	Mushroom	1.25	45.6 %	1390	nonanal
18	1224	Fresh, Citrus	1.5	55.9 %	n.d.	*(E,E)-3,5-octadien-2-one*
19	1299	Green, Cucumber, Fruit	2.64	77.8 %	1594	(E,Z)-2,6-nonadienal
20	n.d.	Stuffy, Pungent	2.25	61.2 %	n.d.	unknown
21	1494	Sweet, Licorice	1.65	52.1 %	n.d.	unknown

The issues regarding the compounds that were only tentatively identified and written with *italics* are explained in more detail in the final paragraph of **Chapter 3.3**.

In **Tables 4. and 5.** the odour-active compounds with NIF values of 40 % or higher are listed based on their retention time when using the SPB-624 column during the GC-O analysis. The first of these compounds is given the number 1 and the rest are numbered in an ascending order. Retention indexes for both columns (SPB-624 & DB-WAX) were calculated using C7-C30 Saturated Alkanes as the standard based on the retention times of the compounds' peaks in the FID detector of the GC-O. The SPB-624 retention indexes were calculated from the main GC-O session results with the six panellists. DB-WAX retention indexes were calculated based on a separate GC-O analysis with two out of the six panellists using the DB-WAX column with the same samples from the main GC-O analysis. The most common odour descriptions given by the panellists for the odour-active compounds in question that were detected in the GC-O sessions are written in the two tables. The average posterior intensity rating is listed in the tables. Each panellists gives an odour they detect an intensity rating from a scale of one to four, based on these ratings the average is calculated. The average does not include panellists who did not detect the odour and thus did not give it an intensity rating either. The MF-% value was calculated as the square root of detection frequency (%) multiplied by intensity rating (%) out of the maximum intensity rating possible, as mentioned in **Chapter 1.5.2**.

The names of the odour-active compounds that were identified are given in the two tables. The odour-active compounds that could not be identified are marked as "unknowns". The compounds that have some issues regarding their identification are written in *italics*. The odour descriptions form the GC-O can match online data or other studies but then retention indexes do not match, or retention indexes could not be calculated. It can of course be the other way around that the retention index matches but the odour description does not. These compounds included in this group were acetic acid, 1-octen-3-one, 1-octen-3-ol and (E,E)-3,5-octadien-2-one. Acetic acid has been detected in Baltic herring before and its proportion of total volatile compounds increased during storage when measured by peak area (Aro et al., 2002). It has also been detected in other fish species and its odour was described as vinegar-like and it eluted between 2,3-butanedione and 3-methylbutanal in that study, which matches where the compound eluted in this study (An et al., 2020). The area where 1-octen-3-one and 1-octen-3-ol elute is very narrow which made it so that retention indexes could not be calculated for some of the odour-active compounds in that area. There can be another compound besides those two in the same area making it difficult to assess which

compound eluted first, second and so on. With the DB-WAX column 1-octen-3-one and 1-octen-3-ol are separated from each other well and they can be more easily identified and have their retention indexes calculated. The situation of (E,E)-3,5-octadien-2-one is explained in **Chapter 3.5** with more detail, it was only detected in the protein isolate with at least having a NIF-% value of 40 % or higher.

3.4 Odour-active compounds with significant modified frequency-% values

The raw Baltic herring had 19 odour-active compounds with MF-% values higher than 50 %. The protein isolate had 16 odour-active compounds with MF-% values higher than 50 %. A couple of compounds had MF-% values close to the 50 % cut-off point, two in the raw herring sample and one in the protein isolate sample. 2,3-butanedione, 3-methylbutanal, 2,3-pentadione, hexanal, (Z)-4-heptenal, 1-octen-3-ol, (E,Z)-2,6-nonadienal and methional had MF-% ratings higher than 80 % in the raw Baltic herring. The protein isolate had four compounds higher than the 80 % value: 2,3-pentadione, hexanal, (Z)-4-heptenal and 1-octen-3-ol. 2,3-butanedione and (E,Z)-2,6-nonadienal had their MF-% below the 80 % cut-off point in the protein isolate but both were still higher than 70 %, 3-methylbutanal had a much more notable drop to 43.3 %. Methional had its MF-% decreased from 85.4 % to 54.1 % which is also a major change. It can be considered that the six odour-active compounds besides 3-methylbutanal and methional are the most important odour-active compounds detected in the Baltic herring, when looking at both the raw herring and protein isolate sample results.

The remaining odour-active compounds between the 50 % and 80 % MF range were propanal, 2-methylpropanal, acetic acid, 2-methylbutanal, 1-penten-3-ol, dimethyl disulphide, 1-octen-3-one, (Z)-1,5-octadien-3-ol, octanal, nonanal and three unknown compounds. 2-methylpropanal, 2-methylbutanal and one unknown compound had MF-% ratings higher than 50 % in the raw herring but none of them were detected in the protein isolate. Other compounds dropped below the 50 % cut-off point in one sample but were higher than it in the other sample. 1-octen-3-one was one these compounds, it had a 59.6 % % rating in the raw herring but a 45.7 % rating in the protein isolate. More details about the differences in odour-active compounds between the samples was discussed in **Chapter 3.5**.

3.4.1 How some of the different odour-active compounds could have been formed

Hexanal, (Z)-3-hexenal, 1-octen-3-ol, 1-octen-3-one, (Z)-1,5-octadien-3-ol and (E,Z)-2,6-nonadienal are formed by lipoxygenase catalysed oxidation from long-chain PUFAs. It used to be difficult to separate which compounds were formed by autoxidation and what was the result of enzymic activity (Lindsay, 1990). Fish species that contain 12-lipoxygenase have shown to lead to the formation (E,Z)-2,6-nonadienal from eicosapentaenoic acid (EPA) and to the formation of other compounds. 12-lipoxygenase targets the position of n-9 hydroperoxide on the EPA and that will be then cleaved with the help of a lyase enzyme (Lindsay, 1990). Hexanal and (Z)-3-hexenal are formed through 15-lipoxygenase by first forming appropriate hydroperoxide intermediates that are then cleaved by lyase or dismutation. If the precursor for this reaction is an n-3 PUFA it will form (Z)-3-hexenal or if the precursor is an n-6 PUFA it will form hexanal (Lindsay, 1990). Eight-carbon volatile alcohols and ketones have been detected in most seafoods. These compounds have individual odours that are described as mushroom-like or green leaf-like aromas but in seafoods they seem to contribute to plant-like and metallic odours (Lindsay, 1990). (Z)-1,5-octadien-3-ol is formed from EPA by lipoxygenase siting the 12-hydroperoxide which is then cleaved by a lyase, ketones are formed by dehydrogenase action on the alcohol compounds. Alcohols are found in higher concentrations in fresh seafood, but carbonyl (aldehydes and ketones) compounds have much lower odour detection thresholds that results in them having a larger impact in the overall fresh seafood aroma (Lindsay, 1990). This also holds true for most pairs of alcohols and carbonyls than for just eight-carbon alcohols and carbonyls. Thus, aldehydes and ketones tend to be the compounds that have a large impact on the intensity and general aroma of fresh fish. The results of this Baltic herring odour-active compound study also support the conclusion that aldehydes and ketones are the most important compounds attributing to the aroma of fish in general. Adding bisulphite to fish slurries proved to substantially supress the aroma of the fish by forming bisulphite adducts with the carbonyl compounds (Lindsay, 1990).

Autoxidation of fish oil starts with the formation of green and cucumber-like aromas which occur from the formation of (E)-2-hexenal, (Z)-3-hexen-1-ol and (E,Z)-2,6-nonadienal (Lindsay, 1990). (E,Z)-2,6-nonadienal can be formed by both autoxidation or lipoxygenase catalysed oxidation. There are many carbonyl compounds that result from the dismutation of hydroperoxides in autoxidised fish oil. These compounds

contribute to oxidised, paint-like and rancid aromas that are also common in plant-based polyunsaturated oils (Lindsay, 1990). (Z)-4-heptenal has been discovered to form by water-mediated retro-aldol degradation of (E,Z)-2,6-nonadienal in cod. As (E,Z)-2,6-nonadienal degrades into (Z)-4-heptenal the green and cucumber-like aromas it provides are lost and it is replaced by a compound that promotes fishy odours. (Z)-4-heptanal has a low odour detection threshold that enables it to contribute to the overall aroma of fish even at small concentrations and potentially overpower other contributing odour-active compounds (Lindsay, 1990).

3.5 Differences between the two samples in odour-active compounds

3-methylbutanal was still detected in six of the twelve sessions in the protein isolate but 2-methylbutanal was not detected at all by the panellists. The average intensity rating fell from 3.18 to 1.5 and the MF-% value from 85.4 % to 43.3 % for 3-methylbutanal when comparing the two samples. The protein isolate was made from the same batch as the raw fish sample without extra storage at lower than -80 °C, thus the two compounds should not have started falling in concentration as it did in a previous study during storage longer than 3 days (Aro et al., 2003). This means that the reason why they were no longer detected by the panellists in the protein isolate relates to something that was affected by the pH-shift process. 2-methylpropanal was another compound that was detected in the raw Baltic herring, but it was not detected in the protein isolate.

Propanal and hexanal are odour-active compounds that have higher odour intensity ratings and MF-% values in the protein isolate than in the raw Baltic herring. Propanal's average intensity rating increases from 1.33 to 1.82 and the MF-% value increases from 49.9 % to 64.6 %, the number of panellists which detected the odour increased from nine to eleven and the eleven panellists even gave it a higher intensity rating on average. Hexanal had a less significant change particularly because it was already detected by all panellists in both samples. The average intensity rating increased from 2.92 to 3.25 thus also increasing the MF-% from 85.4 % to 90.1 %. The reason why these two compounds appear to have a stronger odour in the protein isolate is likely to be that lipid oxidation has formed more hexanal and propanal during the pH-shift process. The pH-shift was run under alkaline conditions that as discussed in **Chapter 1.4** causes more lipid oxidation than running the pH-shift under acidic conditions, thus enhancing the formation of lipid oxidation products in the protein isolate. 2,3-butanedione, 1-octen-3-one and methional are compounds that had stronger average intensity ratings and MF-%

values in the raw Baltic herring than in the protein isolate. Out of these three compounds methional had the most noticeable decrease in the two values, average intensity decreased from 2.92 to 1.56 and the MF-% from 85.4 % to 54.1 %. Methional is a compound that is relatively unstable and as such it can easily degrade; this might be the cause for the difference between the two samples in these recorded values. For 2,3-butanedione the average intensity decreased from 2.91 to 2.27 and the MF-% value from 81.7 % to 72.1 %. For 1-octen-3-one the same values decreased from 2.13 and 59.6 % to 1.5 and 45.7 %. Both compounds are ketones so one might think that the pH-shift is affecting ketones in some way but then there are no notable changes in the two values concerning 2,3-pentadione, so there is no effect on all ketones.

There were six odour-active compounds in the raw Baltic herring with NIF-% values of ≥ 40 % that were not detected at all or had a lower NIF-% value than the threshold in the protein isolate sample. These compounds were not able to be identified in this study and thus they are listed as unknowns in **Table 4.** These compounds are numbered 1, 14, 22, 23, 26 and 28 in the previously mentioned table. Number 1 was described as fishy/stuffy, number 14 as sweat, number 22 as dusty/malt, number 23 as forest/malt, number 26 as sawdust/mushroom and number 28 as mushroom/forest. In the protein isolate sample there was one compound that was over the ≥ 40 % NIF threshold which did not appear in the raw Baltic herring. The compound is numbered 18 in **Table 5.** with the other notable odour-active compounds from the protein isolate. The compound was described as fresh/citrus-like and it was thought to be (E,E)-3,5-octadien-2-one but there are factors which point to it possible being some other compound. The retention index from HS-SPME-GC-MS does not match with the GC-O retention index when using the SPB-624 column. The additional GC-O analysis with the DB-WAX column did not help with identification as an odour that matched the description was not detected with a retention index that matches the retention index data for DB-WAX columns.

3.6 Ways to improve the performance of GC-O

The GC-O in this study was sometimes connected to a flame-ionization detector in addition to the olfactory port. A GC-MS/O system could have been used instead to improve the identification of odour-active compounds. However, such a system was not available in the university premises and so a separate GC-MS and GC-O was used instead. The GC-O session lasted for about 25 minutes in this study which is around the time limit that a GC-O analysis session should last for. The ramp up speeds could be

lowered from 8 °C/min and 10 °C/min to make the total run time longer for the GC-O. The total run time of the GC-O will increase but the analysis session the panellists participate in can be split in two to manage the time increase. Other studies have split their GC-O runs for the panellists into two parts so that they do not start to suffer from fatigue because of a long session (Selli et al, 2006 & Wu et al., 2014). Lowering the ramp up speed can also improve the separation of some odour-active compounds when eluting, helping with areas in the GC-O where many compounds eluate close to each other.

3.7 Sensory evaluation between the raw Baltic herring and protein isolate produced with the pH-shift

Performing a sensory evaluation between the raw Baltic herring and the protein isolate samples was not a part of the scope of this study. Performing a sensory evaluation to compare the overall odour between the samples is a valuable addition to a GC-O analysis which focuses on the specific odour-active compounds as discussed in **Chapter 1.6.1**. The GC-O data from this study was used in a research publication that also performed a sensory evaluation between the two samples (Kakko et al., 2022). The results of that sensory evaluation utilizing a check-all-that-apply test can be seen in **Figure 11.**, the panellists were asked if they noticed certain odours from the two samples that were preselected based on the most common odour descriptions of the GC-O analysis. This data was used to form a frequency (%) detection result based on the number of times panellists detected a preselected odour description from the samples divided by the maximum possible detections. If the panellists detected a certain odour matching the given descriptions, they were also asked to rate the odours intensity on the same scale from the GC-O analysis (1 to 4, 0 was gives for no detection) (Kakko et al., 2022). The panellists that performed the GC-O in this study and then this sensory evaluation as a part of the research publication were the same six panellists and both were performed in duplicate. The odour description of raw fish had a statistically significant change between the two samples concerning the frequency (%) results, which can be seen on the **A** section of **Figure 11** (Kakko et al., 2022). Other descriptions did not have a statistically significant between the two samples on the frequency (%) data, but the fish oil description was close. The total intensity and fish-like odour intensity of the samples had statistically significant differences between the samples, musty odour was almost statistically significant (Kakko et al., 2022). The intensity ratings are shown in section **B** of **Figure 11**. Both were lower in the protein

isolate when comparing to the raw Baltic herring. According to these results it can be said that the protein isolate smells less like fish than the raw Baltic herring and its overall odour is less intense.

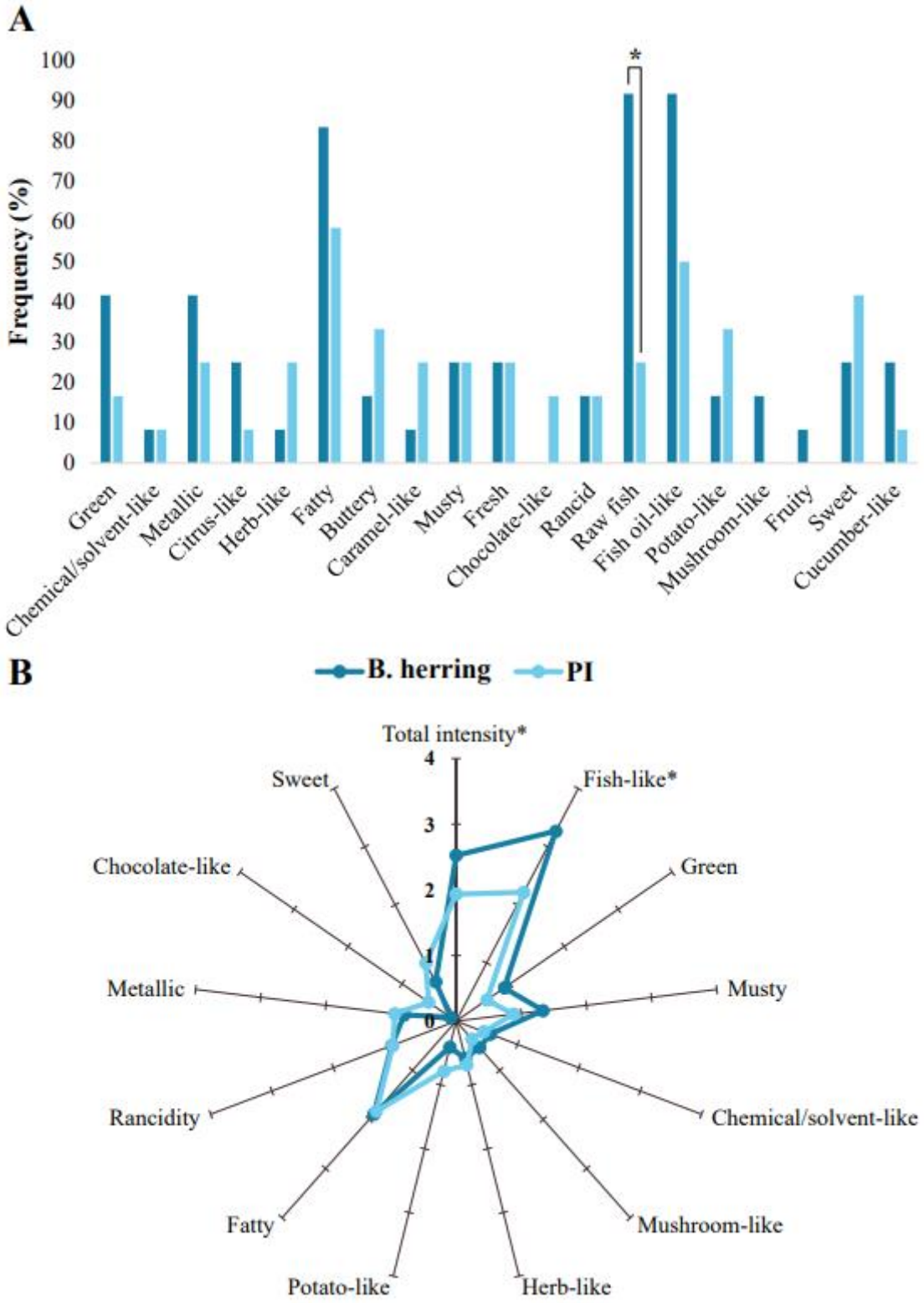

Figure 11. Sensory evaluation results comparing raw Baltic herring with the protein isolate using the check-all-that-apply test. Nineteen different odour attributes were chosen for comparison for their frequency (**A**) of appearance and twelve for odour intensities (**B**) in

addition to the total intensity of the samples. The asterisk marks statistically significant differences ($p < 0.05$) between the two samples (Kakko et al., 2022).

4 Conclusions

There was a noticeable difference between the protein isolate produced with the pH-shift and the raw Baltic herring samples regarding the changes in odour-active compounds and overall odour profile. The pH-shift was conducted using alkaline solubilisation, but more recent literature has confirmed that acidic solubilisation may lead to a lower rate of lipid oxidation, which might yield better results when trying to remove or inhibit odour-active compounds from the Baltic herring. Nevertheless, even when using the pH-shift under alkaline conditions there were notable differences between the two samples. Adding antioxidants in the process could make an even larger difference between the protein isolate and raw fish in odour-active compounds and that effect could be studied with GC-O in more detail in further studies.

The odour-active compound identification results can be improved by using a GC-MS/O system instead of using the GC-O and GC-MS systems separately if such a machine is available, which it was not for this study. The HS-SPME-GC-MS system did provide valuable information for the identification of certain odour-active compounds. Additional training sessions for the panellists might have improved the individual panel members ability to detect more odours than they did or the consistency of their performance. 28 odour-active compounds that had NIF rating of 40 % or higher were detected in the GC-O from the raw Baltic herring and 21 odour-active compounds for the protein isolate. Out of the 28 compounds 19 were identified and out of the 21 compounds 18 were identified. The identification was much better with the protein isolate mainly due to many of the unknown compounds from the raw Baltic herring not being present in the protein isolate.

The odour-active compounds with the highest (≥ 80 %) MF-% values or in other words the most impactful odour-active compounds were 2,3-butanedione, 3-methylbutanal, 2,3-pentadione, hexanal, (Z)-4-heptenal, 1-octen-3-ol, (E,Z)-2,6-nonadienal and methional in the Baltic herring. For the protein isolate the compounds that are above the same threshold were 2,3-pentadione, hexanal, (Z)-4-heptenal and 1-octen-3-ol. The changes in MF-% values for individual compounds showed which compounds changed from being important to the overall odour in the Baltic herring to less relevant in the protein isolate. 3-methylbutanal and methional had the most significant change out of the eight compounds between the raw Baltic herring and protein isolate samples. Their MF-% values decreased from 85.4 % and 85.4 % to 43.3 % and 54.1 % respectively.

The raw Baltic herring had eight odour-active compounds with high impact while the protein isolate had six. There were five other odour-active compounds that had higher MF-% values than 50 % in the raw Baltic herring but fell below it in the protein isolate. (E,E)-3,5-octadien-2-one was the only compound that had the reverse effect in the protein isolate.

Performing a sensory evaluation between the two samples was not a part of the scope of this study but it was performed in another research publication that used the same GC-O data of this study. That publication concluded that there is a difference in the fish-like odour between the overall odour profile of the two samples and that the overall odour is less intense. It can be concluded that the pH-shift does impact the overall odour of the raw Baltic herring and certain odour-active compounds by either removing them or lessening their impact. Some odour-active compounds were enhanced by the alkaline pH-shift such as propanal and hexanal.

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