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SENSORY ACTIVE SUBSTANCES CAUSING OFF-ODOR IN LIQUID WHEY DURING STORAGE

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ABSTRACT

Liquid whey is a nutritious product with high water activity and neutral pH. Therefore, it is very susceptible to microbiological spoilage that results in undesirable off-odors. Additionally, minimally processed foods are the recent trend so setting an appropriate shelf life is essential. The commonly used microbiological methods are lengthy and time-demanding, so a quick and early identification of microbial degradation would be a significant benefit. Here we tested a solid-phase microextraction, gas chromatography with mass spectrometry coupled with olfactometry analysis (SPME-GC-MS/O) on samples of sweet unpasteurized liquid whey stored at 6 °C, 12 °C and 25 °C for a week. We compared the common methods – plate methods, measurement of pH, and dry matter determination with our proposed SPME-GC-MS/O. We have identified seven sensory active compounds while octanoic acid and a compound not reliably identified by the MS detector (with main m/z observed 133 (100), 151 (65), and 135 (26)) being the most prominent. Microbiological methods proved irreplaceable for proper setting of storage conditions (with the growth of coliforms being significant (p <0.001) at 25 °C). However, SPME-GC-MS/O was able to identify volatile substances responsible for off-odors and can be used as a powerful tool to detect the cause of undesirable chemical and microbial changes in whey beverages.

Keywords: whey; SPME-GC-MS/O; off-odor; analysis

INTRODUCTION

Sensory characteristics, such as appearance, taste, and aroma, are the basic parameters for evaluating the quality of many products. While traditional sensory analysis continues to be a valuable method of food and beverage analysis, it is not without its limitations in the evaluation of certain defects. Recently, we found this to be the case when presented with the problem of identification of the off-odor in real samples of liquid whey.

Whey is the leftover liquid when coagulating milk to produce cheese or the released liquid after the fermentation of other dairy products, most often Greek-style yogurts or skyrs. After the coagulation of milk with enzymatic rennet in the production of cheeses, sweet whey is produced, while the use of lactic acid in the production of curd results in acid whey. These two types differ slightly in their composition **(Karagul-Yuceer, Drake and Cadwallader, 2003)**.

While both types mostly consist of water, lactose, proteins, minerals, and fat, acid whey contains more minerals (especially calcium) and less proteins and lactose than sweet whey **(Kilara, 2015)**. Lactose makes up more than 75% of the total solids and is also the main reason why whey is considered as one of the most polluting food streams. On the other hand, the contained proteins and peptides (mainly α-lactalbumin, β-lactoglobulin, serum albumin, and immunoglobulins) are of exceptional biological and functional value and thus offer a wide range of whey utilization **(Anand, Khanal and Chenchaiah, 2013; Smithers, 2008)**.

Dried or concentrated whey and products where some ingredients, especially proteins, are isolated or concentrated (whey protein hydrolysate (WPH), whey protein concentrate (WPC, 34 – 89% protein) and whey protein isolate (WPI, >90% protein)) are widely used as a food ingredient for human consumption **(Evans et al., 2009)**. Liquid whey drinks are gaining popularity, either native, demineralized, or further processed (fermented, carbonated) and flavored in various ways **(Francis, 1999)**.

Liquid whey beverages are very susceptible to microbial spoilage and associated undesirable qualitative deviations because of their rich nutritional content and high water activity. Their shelf life is ensured either by heat treatment (pasteurization or sterilization) or by fermentation and subsequent cold storage **(Lo et al., 2016)**.

Microorganisms generally spoiling whey are the ones that typically spoil milk. Raw and pasteurized milk, exposed to secondary contamination, is most often contaminated by gram-negative bacteria of the genus *Pseudomonas*, while pasteurized milk is most often spoiled by thermoduric spore-forming microorganisms of the *Bacillus* and *Paenibacillus* species. Since whey is easily subjected to lactose fermentation to produce ethanol, acetic, lactic, and propionic acid (which is formed from

lactic acid by the bacteria of the genus *Propionibacterium*), the naturally present lactic acid bacteria – LAB (e.g, *Streptococcus*, *Lactobacillus* and *Lactococcus*) form a variety of aromatic active compounds such as 2,3-butanedione (diacetyl), acetoin, acetaldehyde or acetic acid from pyruvate, an intermediate in lactose fermentation **(Lo et al., 2016)**. When preparing alcoholic whey beverages, mainly yeasts of the genus *Kluyveromyces (K. fragilis* and *K. marxianus*) are used. These beverages are characterized by the presence of volatile compounds, including higher alcohols (mainly isoamyl alcohol, isobutanol, and 1-propanol), ethyl esters (mainly ethyl acetate), as well as acids and acetals **(Dragone et al., 2009)**. All of these substances contribute to the natural aroma of whey, but at higher concentrations to an undesirable odor. However, the main contributors to the off-odor of both liquid and dry whey are lipid oxidation products namely aldehydes, ketones, alcohols, and alkanes **(Carunchia Whetstine et al., 2003)**.

Sensory properties have traditionally been described and evaluated via sensory analysis, which can be loosely divided into two groups: discriminant methods and descriptive methods. The purpose of discrimination testing is to indicate whether a tested sample is perceived as being significantly different from a standard one (e.g. Triangle or Duo-Trio test). Descriptive methods, such as the flavor profile method or quantitative descriptive analyses, are more similar to chemical analysis in that they aim to determine the presence or intensity of a particular characteristic **(Kilcast, 2010)**. The problem is that while descriptive methods can characterize a particular off-odor, they are not able to link it to the specific compound, or compounds, responsible for certain occasional defects. That is where instrumental methods come in.

However, instrumental methods are best used in combination with sensory analysis. For example, GC-MS is able to identify the most abundant volatile compounds in a sample but cannot provide clear information on whether the substances are sensorially active. And the most sensitive physical detectors (MSD, ECD, FID) only have detection limits ranging from 1 to 10 pg, whereas human noses can readily detect to 0.05 pg **(Muñoz et al., 2010)**. Gas chromatography with an olfactometric detector (GC-O) combines the high resolution of capillary gas chromatography with the high selectivity and sensitivity of the human nose to detect and identify the compound, or compounds, responsible for an off-odor. The assessors sniff the eluate from the gas chromatograph using a special olfactory port to detect the presence of sensory-active compounds. Recently, solid-phase microextraction with gas chromatography/mass spectrometry coupled with olfactometry (SPME-GC-MS/O) has been used to identify substances in a variety of matrices, including coffee, cheeses, milk powders, orange juice, cashew apple (*Anacardium occidentale*) juice, yogurt, and even chocolate. **(Zellner et al., 2008; Gocmen et al., 2005; Semmelroch and Grosch, 1995; Zepka et al., 2014)**

Scientific hypothesis

The determination of sensory active substances allows for quick and early identification of microbial degradation and lipid oxidation.

MATERIAL AND METHODOLOGY

Samples

Samples of unflavoured, unpasteurized sweet liquid whey, with a fat content of up to 1%, sold in 1 liter PET bottles were purchased for the analysis. Recommended storage at a temperature from 4 °C to 8 °C and up to 4 days. Individual whey samples in the original packaging were analyzed (for pH, dry matter, and microbiology) 1 day after production (at time T0) and after 1 week stored in thermostats at 6 °C, 12 °C and 25 °C. This shelf-life study was conducted in two batches, and each sample was stored in each temperature in duplicates. Therefore, we obtained 4 sets of data for each storage temperature.

Methods

Microbiological

Samples were analyzed using plate methods **ISO 7218 (2007)** for coliforms according to **ISO 4832 (2006)** using VRB agar (Merck) and yeasts and molds according to **ISO 21527-1 (2008)** using YGC agar (Merck).

pH

The pH was measured using an Inolab pH meter (Thermo Scientific).

Dry matter

Samples were dried to a constant weight at 105 °C.

Volatile compounds

Volatile compounds were measured by SPME-GC-MS for samples T0, 6 °C and 25 °C under the same conditions as sensory active compounds.

Sensory active compounds

For the evaluation of sensory active compounds, the evaluators were first tested and trained by sniffing sticks (Olfasense GmbH), and the samples T0, 6 °C, and 25 °C were subsequently analyzed by SPME-GC-MS/O.

Testing by assessors

Ten assessors underwent two sets of tests. In the first one, the assessors were asked to match a sniffing stick to an odor written on the list. The second one was to describe the odor of each sniffing stick without using any prompts.
The batch of sniffing sticks included the following standards:

1. (E,E)-nona-2,4-dienal (fatty, rancid odour)
2. non-2-enal (paper, carton)
3. dimethyl disulphide (garlic, sulphur)
4. acetoin (yogurt)
5. methional (boiled potatoes)
6. δ-decalactone (floral, fruit)

Six assessors out of ten who showed low detection limits, low recognition thresholds and were in particular accurate in verbal identification of the unknown aroma of the standard compounds of sniffing sticks were subsequently involved in the olfactometric detection.

SPME-GC-MS/O

1 gram of sample was placed into a 10 mL vial. Determination of the volatile profile and sensory active substances was performed using Agilent GC 7890B, MS

5977A with DB-5 capillary column (30 m x 250 µm x 0.25 µm) and SPME fiber 50/30 µm DVB/CAR/PDMS (Supelco). The injector was operated in split mode 1:1, with He 5.5 flowing at 1.4 mL.min^{-1}. The temperature conditions were as follows: incubation for 60 s at 50 °C, with 1500 s sorption injector temperature was set at 260 °C with 360 s long desorption at 260 °C. GC system was set to 60 °C for 2 min followed by a temperature rise of 10 °C/min to a final temperature of 290 °C. NIST integrated library and its retention indices were used for the identification **(NIST MS Search 2.0)**.

The eluate was split 1:1 between the MS detector (MSD 230 °C, quadrupole 150 °C) and the olfactometer (JAS, 180 °C, capillary diameter 150 µm with the airflow rate 40 mL.min^{-1} of the humidifier) at the outlet of the GC column. Nasal impact frequency (NIF) technique with the posterior evaluation of odor intensity (1 – lowest intensity to 3 – highest intensity) was used. NIF value equals the number of assessors detecting a compound dived by the total number of assessors **(Plutowska and Wardencki, 2008)**.

Statistical analysis

The R Program (R Core team, 2017, version 3.5.2.) for Statistical Computing was used for statistical evaluation namely ANOVA and t-test. Results are presented as mean ±standard deviation

RESULTS AND DISCUSSION

The storage experiment was designed to copy the recommended storage conditions while promoting and accelerating the development of sensory active substances indicative of undesirable changes. For the samples stored at 6 °C for 1 week, there were no significant changes in coliforms, yeast and mold count, dry matter, and pH, as expected, because the declared storage conditions were 4 – 8 °C with a shelf life of 4 days (Figure 1 shows the average values of the 4 measurements). The whey dry matter content did not change significantly (p >0.05) with the storage temperature. At the same time in samples stored at 12 °C and 25 °C, the pH dropped significantly (p <0.001) by 35%. This pH drop was caused by an increased content of organic acids (probably produced by lactic acid or acetic acid bacteria and yeasts) **(Campbell et al., 2011; Sattin et al., 2016)**, which was confirmed by a subsequent chromatographic analysis of the volatile compounds. The number of coliforms and yeasts in the sample stored at 25 °C increased significantly (p <0.05) (coliforms one hundred times, yeasts almost five thousand times). No molds were detected in any samples.

The profile of volatile substances of fresh whey was very poor. Only hexane and octanoic acid had a peak area higher than 10^4. Other peaks were either not identified or were contaminants from the chromatographic system (siloxanes, higher hydrocarbons, etc.). The limited sensitivity of the MS detector is unfortunately due to a compromise of possible simultaneous application of olfactometry (see batch separation conditions and split between detectors).

After the storage experiment at 25 °C, the profile of volatiles changed drastically both in the number of peaks as well as in their area. A number of ketones (acetone, 2,3-butanedione, 2-butanone, acetoin, 2,3-pentandione, 2-heptanone, 2-nonanone), sulphur compounds (dimethyl sulphide dimethyl disulphide, dimethyl trisulphide, 2,4-dithiapentane), carbonyl compounds (heptane, hexane), an aldehyde (nonanal), alcohols (1-butanol 1-hexanol, 1-octanol) and organic acids (acetic acid, hexanoic acid, and octanoic acid) were found. We have not identified as many aldehydes as **Croissant et al. (2009)** but a wider range of compounds more similar to **Leksrisompong, Miracle, and Drake (2010)** and **Liaw et**

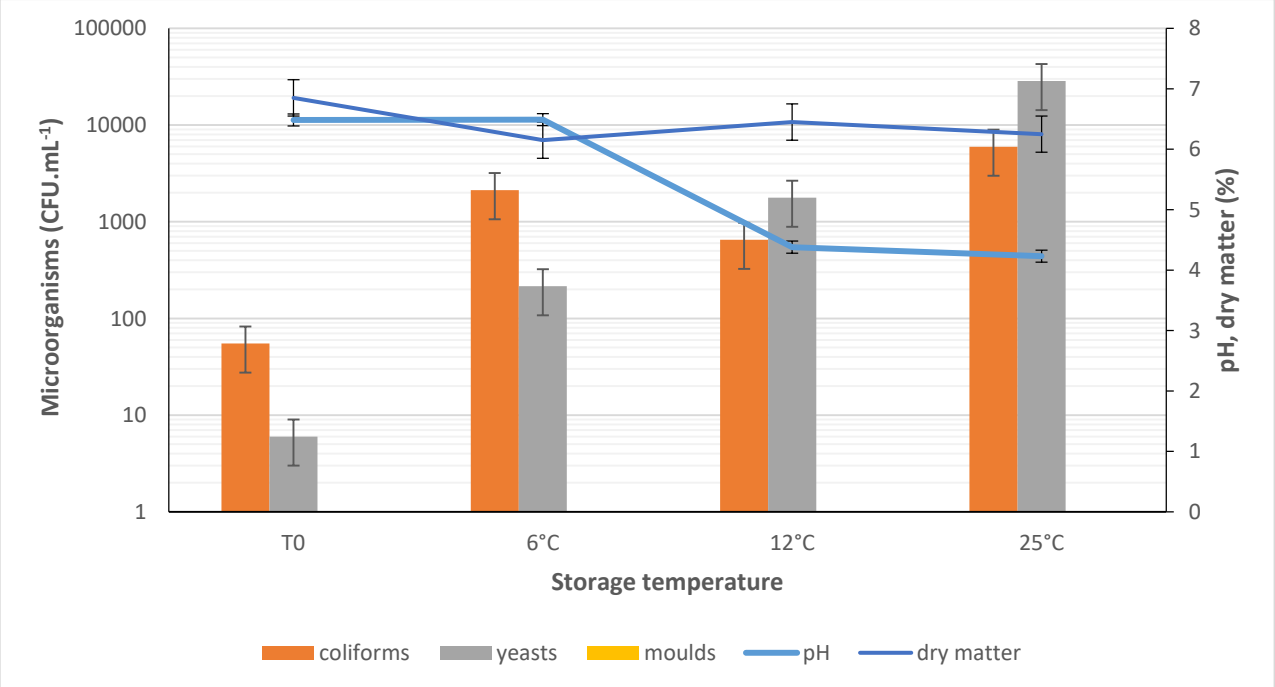

Figure 1 Microbiological, pH and dry matter analysis results obtained from 4 replicates of whey samples analysed at the time of purchase (T0) and after storage in 6, 12 and 25°C for 7 days.

Table 1 Sensory active substances detected by at least 2 assessors out of 6.

Rt	RI (NIST)	Compound	Odour (labelled)	Odour (perceived)	NIF*		
					T0	6 °C	25 °C
1.88	534	Dimethyl sulphide	Cabbage, onion, sulphur	Pungent	-	-	0.50
2.17	593	2,3-butanedione	Butter, caramel, cream, sweet	Butter, sweet, milk	-	-	0.67
5.16	801	Hexanal	Green, fatty, leafy	Fresh, grass, butter	-	-	0.67
7.43		NI		Cabbage, fatty, cheesy	1.00	1.00	1.00
8.52	982	Dimethyl trisulphide	Sulphur, onion, cooked	Cabbage, sulphur, cheese	-	-	0.83
8.71	1000	Hexanoic acid	Cheese, fatty, acid, sweet	Mushroom, fruity	-	-	0.67
11.89	1191	Octanoic acid	Cheese, fatty, sweet, rancid	Milky, musty	0.67	0.67	1.00

Note: *NIF value equals the number of assessors detecting a compound divided by the total number of assessors.

al. (2011) findings, including the not commonly found acetoin (a product of LAB metabolism) **(Nadal et al., 2009)**.

Compared to other dairy products, whey is not very rich in sensory active substances **(Fox et al., 2016; Qian and Reineccius, 2003)**. This finding is in an agreement with our measurements of fresh whey at T0 where only 2 sensory active compounds were detected – not identified (NI) compound and octanoic acid (Table 1). Octanoic acid was found to have a very low odor threshold in air (0.86 $\mu g.kg^{-1}$) **(Cometto-Muñiz and Abraham, 2010)** as opposed to, for example, hexanal (having 0.14 $\mu g.L^{-1}$ therefore 119 $\mu g.kg^{-1}$ in the air at 25 °C **(Ömür-Özbek and Dietrich, 2008)**. This explains why it was detected at T0, along with the fact that hexanal is an oxidation product from linoleic acid, therefore its concentration increases during storage. The other compound that could not be reliably identified by the spectra NIST library (the probability match was less than 50%) had 133 (100), 151 (65), and 13 5 (26) m/z ions as the largest. Detection of such a compound confirms the higher sensitivity of a human nose compared to a mass detector **(Muñoz et al., 2010)**. In total, seven compounds, mostly with an unpleasant odor, were detected by at least two assessors in the sample stored at 25 °C their odor was compared to literature ("The Good Scents Company," n.d.).

Dimethyl sulphide is usually associated with a cabbage-like odor produced by cooking certain vegetables and cereals, formed along with dimethyl trisulphide by bacterial degradation of sulphur amino acids **(Franco-Luesma and Ferreira, 2016; Luo et al., 2018; Nishibori et al., 2014)**. 2,3-butanedione, or diacetyl, is a natural by-product of the fermentation of lactic acid by the oxidative decarboxylation of α-acetolactate **(Hugenholtz et al., 2000)**. It may also be formed as an intermediate in high-temperature treatment with non-enzymatic Maillard browning and may later be involved in Strecker degradation with other free amino acids **(Smit, Smit and Engels, 2005)**. It is responsible together with acetoin for the characteristic taste of butter **(Karagul-Yuceer, Drake and Cadwallader, 2003)**. Hexanal is a lipid oxidation product and has been proposed as a potential quality marker. Free fatty acids (hexanoic and octanoic) are formed by microbial hydrolysis of fats **(Panseri et al., 2011)**.

Based on the assessor's results, dimethyl trisulphide produced the highest intensity odor, even though one assessor did not detect it. There are a number of factors that could have caused it, for example, selective anosmia to sulphur compounds (as dimethyl sulphide was not detected either) or the assessor's fatigue **(Brattoli et al., 2013)**. Dimethyl trisulfide had a peak approximately 20 times smaller than octanoic acid but since the peak area does not correlate with the odor intensity **(Högnadóttir and Rouseff, 2003)**, olfactometry was able to mark it as one of the main compounds responsible for off-odor.

CONCLUSION

When storing whey samples at elevated temperatures (12 °C and 25 °C), there was a significant increase (p <0.001) in the number of coliform bacteria and yeasts which led to an increased amount of organic acids and alcohols, causing undesirable off-odors. However, the scientific hypothesis (Determination of sensory active substances allows for quick and early identification of microbial degradation and lipid oxidation) is only partially confirmed. Since the slight (by 1 – 2 order) increase in the number of pathogenic and spoilage microorganisms at low

storage temperature was not yet reflected in the sufficient production of secondary volatile metabolites (and therefore on the sensory properties of the product) the SPME-GC-MS/O method was not able to detect it.

Thus we conclude that the traditional microbial testing is irreplaceable for a proper setting of storage conditions and shelf life Olfactometers can then play a significant role in detecting the causes of major product odor changes(as we have shown on the sample stored at 25 °C) and therefore spotting specific signs of microbial and chemical degradation.

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