Application of SPME-GC method for analysis of the aroma of white surface mould cheeses

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Summary
Solid-phase microextraction coupled to gas chromatography (SPME-GC) was used for the analysis of volatile aroma compounds in cheeses which had been ripened with a white surface mould *Penicillium camemberti*. These cheeses are of a characteristic appearance, taste and aroma due to the presence of sensory-active compounds formed during the ripening. The aim of this work was to compare aroma profiles of several types of these cheeses produced in Czech Republic and to follow changes of the aroma profile during the ripening. The method was simple and fast, extraction conditions affected minimally the thermal, mechanical or chemical changes of the samples. In total, 32 compounds were identified in the samples using this method, namely, 1 hydrocarbon, 3 aldehydes, 7 ketones, 11 alcohols, 2 esters, 5 fatty acids, 2 sulphur compounds and 1 nitrogen compound. As found, their proportion varied during ripening, but there was no significant increase in their content during experiments. Aroma profiles of the tested cheeses were similar in spite of the differences in the production technologies.

Keywords
aroma; mould cheese; SPME-GC

White surface mould cheeses are covered by a coating of white mycelia of the mould *Penicillium camemberti* or closely related *Penicillium caseicolum*. The presence of moulds gives them characteristic appearance, taste and aroma. Especially in the case of traditional cheeses, they are characterized by more complex maturation, because of the diversity of the microbial flora present and the extent of the enzymatic changes that occur. A typical example of surface mould ripened cheeses is Camembert, originated from France. Traditional Camembert is made from raw milk; the other surface mould cheeses are manufactured from raw or pasteurized milk. Many types, called for example Hermelin, Kamadet, Premium or Plesnivec, are made in the Czech Republic. They are produced from pasteurized milk using starter culture consisting of thermophilic streptococci or a mixture of streptococci and lactococci, and mould cultures. In order to obtain a more aromatic product, selected strains of yeasts, corynebacteria and yeast-like mould *Geotrichum candidum* can be added to milk.

The flavour of cheese originates from microbial, enzymatic and chemical transformations. The breakdown of milk proteins, fat, lactose and citrate during ripening gives rise to a series of volatile or non-volatile compounds: hydrocarbons, alcohols, aldehydes, ketones, esters, fatty acids (FA), lactones, sulphur- and nitrogen-containing compounds [1-3]. All of them may contribute to cheese aroma, but the exact contribution is largely unknown. Moulds have a much greater enzymatic potential than bacteria, consequently, the major processes of maturation are more marked in mould-ripened cheeses than in other types [4].

Lactose decomposition is caused primarily by lactic acid bacteria enzymes [5]. In the case of white surface mould cheeses, the surface fungal flora uses created lactic acid for its growth. There is, as a result, a marked increase in the external pH and an internal migration of lactate towards the surface of the cheese. These drastic pH changes have a marked effect on the maturation process, indicated by the development on the surface of aerobic and acid-sensitive flora, consisting of corynebacteria and micrococci. This flora contributes to the development of the final organoleptic properties and cannot develop without an increase in the surface pH [4].

Lipolysis is most important for blue cheese flavour. Maturation of white surface mould cheeses

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is shorter and the degree of lipolysis is also more restricted, reaching 3–6 % and sometimes 6–10 % in the well matured traditional products. It should be stressed that these high levels of free FA do not give rise to a rancid taste as they are in a dissociated form when the pH of the curd has increased [4]. Long-chain FA (> 12 carbon atoms) play a minor role in the flavour owing to their relatively high perception thresholds. Short and moderate chain length, even numbered FA (C4–C12) have much lower perception thresholds and characteristic notes (vinegar, sour). However, free FA are not only aroma compounds by themselves, but also serve as precursors of methyl ketones, alcohols, lactones and esters [6].

Ketones are common constituents of most dairy products, but they are intermediate compounds, which may be reduced to secondary alcohols [7]. Methyl ketones are derived from FA by β-oxidation or from β-ketoacids and are primarily known for their contribution to the aroma of mould cheeses [8]. They have typical odours (fruity, floral, mushroom or musty notes) and low perception thresholds [5, 8].

Esterification of FA with primary alcohols occurs by an enzymatic or a chemical pathway [9]. The microorganisms involved in ester formation are probably mainly yeasts, but some lactic acid bacteria can be responsible [7]. Most esters in cheeses are described as having sweet, fruity and floral notes. Some of them have a very low perception threshold and their contribution is heightened by synergistic effects. Further, they can contribute to the aroma of cheese by minimizing the sharpness and the bitterness imparted by FA and amines [6, 8].

Lipases in cheese originate from milk (in the case of cheese made from raw milk), rennet and microflora. Lactic acid bacteria present in starter cultures are generally only weakly lipolytic, most of the FA come from the triglycerides degradation by moulds [6].

Proteolysis in cheese during ripening contributes to the taste of cheese by the production of peptides and free amino acids. Large peptides do not contribute directly to cheese taste, but can be hydrolysed by proteinases to shorter peptides that may be bitter [10]. Free amino acids are the final products of proteolysis. Catabolism of free amino acids is a major process for aroma development. It can result in a number of compounds, all of which may contribute to cheese flavour. Proteolysis in cheese is catalysed by enzymes from coagulant, milk, microflora and by exogenous proteinases or peptidases [10].

The study of substances creating food aroma is nowadays of great interest in quality assessment. Several methods for extraction and concentration of them have been developed: e.g. steam distillation, extraction with organic solvents, surfactants and supercritical fluids, headspace techniques, dialysis and solid-phase extraction. However, these methods have certain drawbacks [5, 9]. The solid-phase microextraction (SPME) is a relatively new sample preparation technique that can eliminate some of them. This technique has been introduced by Arthur and Pawliszyn [11, 12] for the extraction of organic compounds from environmental samples, but has now gained a lot of interest in a broad field of analysis including food. Many authors describe analysis of flavour and off-flavour of some food, e.g. fruit [13], vegetables, meat [12], drinks [14, 15] and also dairy products [16-21].

The aim of this work was to compare aroma profile of several types of white surface mould cheeses produced in Czech Republic and to follow their changes during ripening. Volatile aroma compounds of cheeses were isolated using SPME and analysed by gas chromatography (GC).

MATERIALS AND METHODS

Chemicals

The following chemicals were used as standards: pentadecane, heptadecane, dimethyl disulphide, dimethyl sulphide, dimethyl trisulphide, benzothiazol, phenylacetaldehyde, hexanal, 8-nonen-2-one, decan-2-one, heptadecan-1-ol, heptadecan-2-ol, hexadecan-2-ol, myristic acid, benzoic acid, pentadecanoic acid, palmitic acid, phenylethyl-acetate, pentyl-benzoate (Sigma-Aldrich, Deisenhofen, Germany), phenylethanol, ethanal, propanal, hexanoic acid, isobutanoic acid, isopentanoic acid, capric acid, 3-hydroxybutan-2-one, nonan-2-one, pentan-2-one, undecan-2-one, heptan-2-on (Merck, Darmstadt, Germany), methanol, propan-1-ol, propan-2-ol, butanol, pentan-1-ol, pentan-2-ol, octan-1-ol, nonan-2-ol, 2-methylpropan-1-ol, 3-methylbutan-1-ol, acetone, propan-2-one, butan-2-one, butanoic acid, acetic acid, propanoic acid, ethyl-acetate (Lachema, Brno, Czech Republic), heptane, ethanol, heptan-2-ol, dodecan-1-ol, benzaldehyde (J. T. Baker, Deventer, Netherlands), oct-1-en-3-ol, butan-2,3-dione (Fluka, Buchs, Switzerland). All the chemicals were of chemically pure grade.

Samples

Three types of white surface mould cheeses were tested in this work: Hermelin is the medium fat cheese (dry matter 47–51%, fat in dry matter

50–54%), weight 120 g, cylindrical form. Block Hermelin is fatty cheese (dry matter 50–54%, fat in dry matter 60–64%), weight 1 kg, cylindrical form. Premium is fatty cheese (dry matter 51–55%, fat in dry matter 60–64%), weight 125 g, oval form.

All of them were produced by Pribina, Přibyslav, Czech Republic. Cheeses were sampled and analysed for volatile compounds after 5, 15, 25, 35, 45 and 55 days of ripening.

SPME-GC analysis

For analysis the grated cheese (1 g) was placed in a vial (4 ml), sealed by a septum-type cap and the vial kept in a water bath. During this time, the sample was sometimes shaken to homogenize and to increase the transfer of the analytes to the headspace. After an equilibration time (30 min), the SPME fibre was inserted in a vial for the sampling process. The fibre Carboxen^{TM}/polydimethylsiloxane 85μm was purchased from Supelco (Bellefonte, Pennsylvania, USA). The extraction of volatile aroma compounds was carried out by exposure to the headspace of the sample - 20 min 35 °C.

GC conditions: Gas chromatograph TRACE^{TM} GC (ThermoQuest Italia, Milan, Italy) equipped with flame ionization detection (FID) and split/splitless injection port, DB-WAX capillary column (30 m × 0.32 mm × 0.5 μm; J&W Scientific, Folsom, California, USA). The injector - 250 °C, splitless mode, the desorption time 5 min, linear purge closed for 5 min. The detector - 220 °C. The carrier gas (N_2) 0.9 ml.min^{-1}. The oven temperature program: 40 °C 1 min, 2 °C.min^{-1} to 120 °C, 5 °C.min^{-1} to 200 °C, 5 min.

Gas chromatography and mass spectrometry (GC-MS)

Gas chromatograph GC 8000 (Carlo Erba, Milan, Italy) coupled to a MS TRIO 1000 (Fisons Instruments, Valencia, California, USA). The ionizer temperature setting was at 150 °C, using electron impact (EI) mode, with electron energy at 70 eV. The carrier gas was He with a head pressure 150 kPa, the GC column and other operating parameters were the same as described.

RESULTS AND DISCUSSION

Identification of aroma compounds in cheeses

The identification of the individual compounds in sample is rather difficult, owing to their low concentrations in cheese and the relatively high concentrations of other compounds. Identification was carried out by GC-MS and confirmed by comparison of the retention times with those of standard substances. The mass spectra for all the compounds were compared with standard mass spectra provided by the database of the equipment.

Seven ketones were identified in surface mould cheeses tested: propanone, butan-2-one, pentan-2-one, nonan-2-one, undecan-2-one, butan-2,3-dione, 3-hydroxybutan-2-one.

One of the most important diketones is biacetyl (butan-2,3-dione) with its sweet buttery and vanilla aroma. This component is formed from lactose and citrate metabolism and its production is mainly due to the activity of lactic acid bacteria. It can be reduced to acetoine (3-hydroxybutan-2-one) with buttery aroma and the latter can be further reduced to butane-2,3-diol, which does not have a flavour impact [8]. In mould cheese methyl ketones are very important aroma compounds, the major are heptan-2-one and nonan-2-one [5, 16].

In total eleven alcohols were identified in cheeses tested: methanol, ethanol, propan-1-ol, propan-2-ol, 2-methylpropanol, butanol, pentan-2-ol, heptan-2-ol, octan-1-ol, oct-1-en-3-ol, phenylethanol. Primary alcohols are formed by the reduction of the corresponding aldehydes. They impart a fruity, nutty note to the cheese flavour, but in certain cheeses, high levels of these alcohols could be responsible for flavour defects. Ethanol comes from lactose fermentation. It has a limited role in the cheese aroma despite its high levels, but it contributes to the formation of esters [7]. Secondary alcohols are formed by enzymatic reduction of the corresponding methyl ketones. They have similar but heavier flavour notes than methyl ketones. 3-Methylbutan-1-ol is present at high concentration in mould cheeses. The principal secondary alcohols in mould ripened cheeses are heptan-2-ol and nonan-2-ol. These alcohols correspond to the high methyl ketone contents of the same cheeses. They have less influence on cheese flavour than methyl ketones, however, they may contribute indirectly because of their ability to form esters with FA [6].

Five FA were identified in the cheeses tested, namely ethanoic acid, butanoic acid, 2-methylpropanoic acid, hexanoic acid and 3-methylbutanoic acid. Fatty acids are important components of the flavour of many cheese types. They may originate from lipolysis, a lower proportion of short-chain FA originate from the degradation of lactose and amino acids and they can also be derived from ketones, esters and aldehydes by oxidation [8].

Two esters were identified in the cheeses tested, namely ethyl-acetate and phenylethyl-acetate. Esters are common cheese volatiles. Esterification reactions occur between short- to medium-chain FA and primary and secondary alcohols [8, 22].

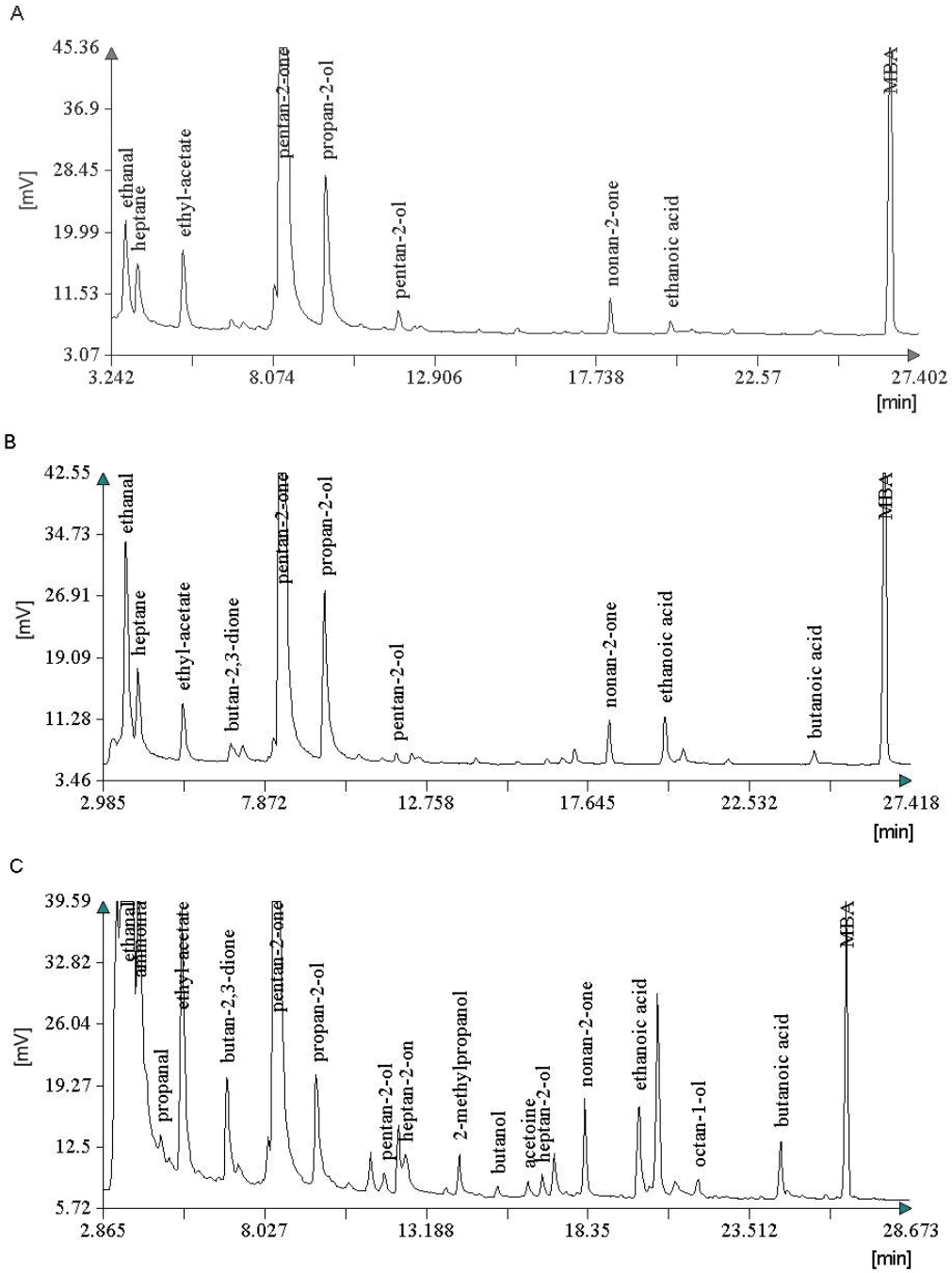

Fig. 1. Typical chromatograms of the most significant aroma compounds identified in cheeses.
A - Block Hermelin, B - Hermelin, C - Premium. MBA – methylbutanoic acid.

Three aldehydes were identified in the cheeses tested: ethanal, propanal, and phenylacetaldehyde. Straight-chain aldehydes may result from β-oxidation of unsaturated FA or from amino acids by the Strecker degradation. This reaction is simple and can occur without enzymatic catalysis during ripening. Branched-chain aldehydes probably originate from amino acid degradation via enzymatic as well as non-enzymatic, e.g. Strecker degradation, processes [8, 9]. Aldehydes are transitory compounds in cheese because they are rapidly reduced to primary alcohols or oxidised to the corresponding acids [7]. They are characterized by green-grass or herbaceous aroma and can be very unpleasant when their concentrations exceed certain value.

One hydrocarbon was identified in cheeses tested: heptane. Hydrocarbons are secondary products of lipid autooxidation. They do not make

a major contribution to aroma, but may serve as precursors for the formation of other aroma compounds [23]. Hydrocarbons have been frequently reported in many cheeses, although usually at low concentrations [7].

Two sulphur compounds were identified in cheeses tested, namely dimethyl disulphide and-dimethyl trisulphide. Sulphur compounds originate from methionine and cysteine degradation. These components are described as having strong garlic, onion or very ripe cheese odours. Their perception thresholds are very low and they are probably involved in the final aroma of mould cheeses [8].

Ammonia was the only nitrogen compound identified in the tested cheeses. Nitrogen compounds (N compounds) come from amino acids.

Typical chromatograms of most significant aroma compounds identified in cheeses are presented in Fig. 1.

Comparison of aroma profiles of ripe cheeses

SPME-GC procedure with FID detection was used for quantification of aroma compounds, the method of standard addition was chosen, the standards were added to the grated cheese in a vial. The reproducibility was good (RSD in range 2–11%). Detection limits were in the range of 0.003-0.2 $\mu g.g^{-1}$. The linearity was tested within the range of 0.003–30 $\mu g.g^{-1}$, the correlation coefficients were all over 0.99.

The aroma compounds of three different types of white surface mould cheeses produced in the Czech Republic were compared, namely Hermelin, Premium and block Hermelin. These cheeses differ in their size, shape and composition. Production technology is rather different, mainly used microbial cultures.

Comparison of these cheeses reveals differences in the content of aroma compounds. They can be ascribed primarily to the differences in the production processes, e.g. different starter cultures are used, and also to variously long ripening time. Chemical composition of cheeses, first of all the fat content, also significantly influences final aroma. The comparison of aroma profiles of ripe Hermelin, Premium and block Hermelin cheeses is graphically presented in Fig. 2.

The highest concentrations of ketones were found in block Hermelin. Propanone was quantitatively the most important ketone in all three cheese types, the other ketones identified were present only in trace amounts. The highest concentrations of alcohols were also found in block Hermelin. Ethanol was found in the significantly high amounts in all three cheese types followed by propan-2-ol. The other alcohols identified were present only in trace amounts. The highest concentrations of FA were also found in block Hermelin. Butanoic and ethanoic acids were the most significant acids in all cheese types, with the exception of Hermelin, which does not contain ethanoic acid at all. The amount of esters was similar in all cheese types, they were present only in trace amounts. The by far highest concentration of aldehydes was found in Hermelin; they were present only in trace amounts in Premium.

KARAHADIAN et al. [24] suggest that the high concentrations of secondary alcohols along with methyl ketones contribute to the flavour of the mould surface ripened cheese. The concentration of short chain free FA in Brie cheese indicates that these compounds could contribute to the flavour of mould surface ripened cheeses, although, the elevated pH (about 6.8 to 7.2) of well ripened cheese would cause a significant suppression of their flavours.

SABLÉ et al. [6] introduce the homologous series of odd-chain methyl ketones, from C3 to C15, as some of the most important compounds of white mould ripened cheese. Among volatile FA, the ethanoic, butanoic, 3-methylbutanoic and octanoic acids are the most potent odorants of Camembert cheese. Oct-1-en-3-ol, 2-phenylethanol and 2-phenylethyl acetate were quantitatively important in Camembert type cheese. These molecules together with sulphur compounds and probably lactones are reported as the key aroma substances in Camembert cheese. Sulphur containing components are considered indispensable to achieve the characteristic aroma of Camembert cheese [23]. Coryneform bacteria, especially *Brevibacterium linens*, are probably the key producers of sulphur compounds in cheeses. This explains the formation of significant concentrations of them in white mould cheese. Methanethiol appeared to be one of

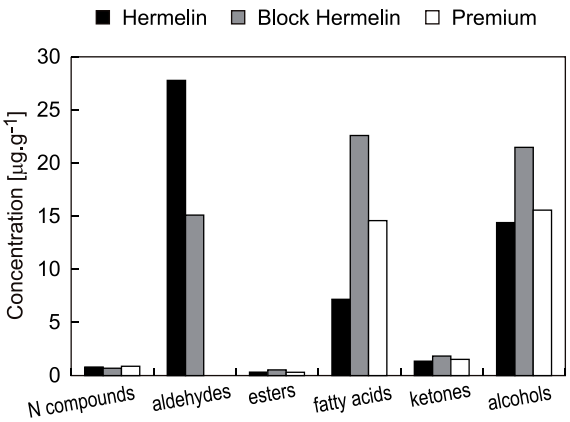

Fig. 2. Comparison of aroma profiles of three tested white surface mould cheeses.

the characteristic flavour compounds in soft white mould cheeses. Methional, dimethyl sulphide and methanethiol were also detected in significant quantities in Camembert cheese [6].

Oct-1-en-3-ol, in combination with oct-1-en-3-on, gives rise to a characteristic mushroom-like sensation of surface mould ripened cheese. Their presence might in part mask the effect of methyl ketones, also present in surface mould ripened cheeses. Sulphur compounds are key odorants of the sulphurous, garlic note in the Camembert. Butan-2,3-dione and δ-decalactone cause the buttery note. The pungent and sweaty character is mainly caused by ethanoic and butanoic acids [4].

The amount of N compounds, especially ammonia, was smaller, and similar in all three cheese types. Conversely, ammonia reaches high concentrations in the case of traditional well matured surface mould cheeses. This extensive degradation of protein is, in the main, due to the high proteolytic activity of *Penicillium* [4].

To summarize, FA, alcohols and aldehydes were quantitatively the most important compounds in cheeses tested. Block Hermelin contained the highest amounts of FA and alcohols, Hermelin contained significantly high concentrations of aldehydes and alcohols. Generally the highest amount of volatile aroma compounds was found in block Hermelin (as can be seen in figure 2), however, the difference from other cheeses is not really significant. Consequently it is possible to say, that none of cheeses investigated have stronger aroma than the others. In spite of the differences in producing technology, aroma profile (and flavour) of these cheeses is similar.

Changes of aroma compounds identified during ripening of cheeses

As mentioned before, cheese ripening includes microbiological and enzymatic processes contributing to the unique flavour and textural characteristics. Only for a few kinds of cheese have these changes been described in depth. In the case of three tested surface mould cheeses most of the identified volatile compounds were present in all stages of cheese ripening, however their relative amount changed significantly.

The concentration of ketones underwent during ripening similar changes in all cheese types tested with the maximum at about 10 days. Many authors describe the increase in the ketones concentration during ripening of various cheeses which is linked to lipolysis [7]. Alcohols are quantitatively the main chemical family in various cheese types and their concentration during ripening increases significantly although at different rates [7]. In our case no significant increase in alcohols concentration was found, their content did not change significantly. Only ethanol was present at very high concentration at the beginning of the ripening and then decreased sharply. Changes in the concentration of short-chain fatty acids were similar in all cheeses tested. Some authors note the increase in concentration of volatile FA during ripening of various kinds of cheeses [6, 16]; in our case FA reached maximum at about 20–30 days of ripening. Only ethanoic acid was present in the Premium cheese at highest concentration at about 10 ripening days and then decreased sharply. As reported by some authors, in the various kinds of cheese the concentration of esters can decrease or increase during ripening [25]. The esters in cheeses tested reached maximum at about 20–30 days of ripening. Aldehydes mostly increase during ripening of various cheeses [25]. They were not present in the Premium cheese at all or only in trace amounts, whilst in other two cheeses they appeared at the end of ripening period at relatively high concentrations. Sulphur compounds identified in cheeses tested were present only in trace amounts, although sulphur containing components are considered indispensable for achieving the characteristic aroma of Camembert cheese and were found in significant concentrations in white mould cheeses [23]. Ammonia was identified in cheeses and its concentration almost did not change during ripening.

CONCLUSIONS

Some important aroma compounds of white surface mould cheeses produced in the Czech Republic were identified and quantified using SPME coupled with GC. This method is simple and fast, minimizes thermal, mechanical, and chemical modifications of the sample. Consequently, it is suitable for the characterization of the cheese aroma.

Important changes of identified aroma compounds took place during ripening, but surprisingly no significant increase in concentration was found.

Fatty acids, alcohols and aldehydes were quantitatively the most important compounds in cheeses tested. The block Hermelin cheese contained the highest amounts of FA and alcohols, while Hermelin brand contained significantly higher concentrations of aldehydes and alcohols. Generally the highest, but not significantly higher, amount of volatile aroma compounds was found in block Hermelin. Consequently, it is possible to say, that none of the investigated brands of cheese

has stronger aroma than others. In spite of the differences in the production technology, aroma (and flavour) of these cheeses remain similar, as can also be noticed during consumption.

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