

## Solid-Phase Microextraction for Analysis of Mould Cheese Aroma

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### Abstract

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Solid-phase microextraction coupled with gas chromatography was used for the analysis of volatile aroma compounds in Niva cheese. The extraction conditions were very mild, which minimises the thermal, mechanical, or chemical modification of the sample; the method is rapid, simple, and cheap. In total, 54 compounds were identified in Niva cheese using this method: 3 hydrocarbons, 5 aldehydes, 11 ketones, 18 alcohols, 3 esters, 10 fatty acids, and 4 sulphur compounds. These aroma compounds were quantified and subsequently the changes in the concentrations of them were studied throughout the ripening period. Most of the volatile compounds identified were present at all stages of the cheese ripening, their amounts changing significantly, however, in most cases the final concentration in the ripe cheeses was similar to the initial concentration in the unripe cheese.

**Keywords:** SPME; gas chromatography; mould cheese; aroma compounds

Aroma and flavour belong among the most important food quality criteria. They are major attributes that influence the selection and consumption of food. Cheese flavour results from the breakdown of milk proteins, fat, lactose, and citrate due to enzymes from microorganisms, coagulants, and milk. Many volatile compounds are potentially involved in cheese flavour: hydrocarbons, alcohols, aldehydes, ketones, esters, fatty acids (FA), lactones, sulphur- and nitrogen-containing compounds. To analyse the cheese flavour by gas chromatography (GC), it is necessary to extract these compounds from their matrix. Many of them are present only at very low concentrations and several methods for their extraction and concentration have been

developed: e.g. steam distillation, extraction with organic solvents, surfactants, and supercritical fluids, headspace techniques, dialysis, and solid-phase extraction (CARBONELL *et al.* 2002; FERNÁNDEZ-GARCÍA *et al.* 2002; QIAN *et al.* 2002). However, these methods have some drawbacks: they are time-consuming, require large volumes of samples or solvents, some volatile compounds can be damaged or lost, etc.

The solid-phase microextraction (SPME) is a relatively new sample preparation technique, based on the partition of the analyte between the extraction phase on the outside of a small fused-silica fibre, and the matrix. SPME was introduced by Arthur and Pawliszyn (PAWLISZYN 1997; KATAOKA *et al.*

2000) for the extraction of organic compounds from environmental samples, but now it has gained a lot of interest in a broad field of analysis including the analysis of food. Very interesting possibility is the use of SPME in the food aroma analysis. Many authors describe the analysis of flavour and off-flavour of some foods, e.g. fruit, vegetables, meat, drinks and dairy products (PÉRÉS *et al.* 2001; FRANK *et al.* 2004; MALLIA *et al.* 2005).

The aim of our work was to develop a simple, rapid, and cheap method for the extraction of the aroma compounds of cheese, based on SPME. Mould cheese was used to evaluate our method and the optimised method was used for monitoring the changes throughout Niva cheese ripening.

## 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, 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-one (Merck, 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, propionic acid, ethylacetate (Lachema, CR), heptane, ethanol, heptan-2-ol, dodecan-1-ol, benzaldehyde (J.T. Baker, the Netherlands), oct-1-en-3-ol, butan-2,3-dione (Fluka, Switzerland). All the chemicals were of chemically pure grade.

**Samples.** Niva cheese (50% dry matter, 55% fat in dry matter) was manufactured according to the traditional procedures in a dairy in Český Krumlov. The cheeses were sampled at 5, 15, 25, 35, 45, and 55 days of ripening. The cheese samples were stored at  $-12^{\circ}\text{C}$  before use.

For analysis, grated cheese was placed in a vial (4 ml), sealed by a septum-type cap and held in a water bath. During this time, the sample was sometimes shaken to homogenise and to increase the transfer of the analytes to the headspace. After

the equilibration time, the SPME fibre was inserted in a vial for the sampling process.

**SPME.** The SPME fibre Carboxen™/polydimethylsiloxane 85  $\mu\text{m}$  was purchased from Supelco (Bellefonte, PA, USA). The extraction was carried out by HS-SPME mode.

**Gas chromatography and mass spectrometry (GC-MS).** Gas chromatograph TRACE™ GC (ThermoQuest, I) equipped with flame ionization detection and split/splitless injection port, DB-WAX capillary column (30 m  $\times$  0.32 mm  $\times$  0.5  $\mu\text{m}$ ; J. & W. Scientific, Folsom, CA).

The injector –  $250^{\circ}\text{C}$ , splitless mode, the desorption time 5 min, linear purge closed for 5 min.

The oven temperature program –  $40^{\circ}\text{C}$  for 1 min,  $40$ – $200^{\circ}\text{C}$  at  $5^{\circ}\text{C}/\text{min}$ ,  $200^{\circ}\text{C}$  for 7 min. The detector  $220^{\circ}\text{C}$ . The carrier gas ( $\text{N}_2$ ) 0.9 ml/min.

GC-MS analyses – gas chromatograph GC 8000 (Carlo Erba, I) coupled to a MSTRIO 1000 (Fisons Instruments, USA). The carrier gas He, the GC column and other operating parameters were the same as described.

**The reproducibility, linearity and detection limits.** The reproducibility of the method was determined by five replicate extractions of five standard compounds chosen under optimal conditions. The standards were added to the matrix of the ripe cheese. The results were expressed as relative standard deviations (RSD) of the peak area counts.

The method of standard addition was used for the assessment of the linearity and detection limits. The standards at different concentrations (within the range 0.003–20  $\mu\text{g}/\text{g}$ ) were added to the matrix (ripe cheese sample).

## RESULTS AND DISCUSSION

There are two possibilities of SPME extraction: direct immersion SPME (DI-SPME), where the fibre is exposed to the liquid sample, and headspace SPME (HS-SPME), where the fibre is exposed to the headspace above the sample. The HS-SPME mode is preferred for volatile compounds because it provides greater selectivity, sensitivity, and rapidity, and an elongated fibre lifetime.

Selectivity can be altered by changing the phase type and thickness according to the characteristics of the analytes – volatile compounds require a thick phase coat. Various fibres were tested and finally Carboxen™/polydimethylsiloxane 85  $\mu\text{m}$  fibre was chosen as most suitable for the extraction of

volatile aroma compounds as confirmed by many authors (PÉRÉS *et al.* 2001; FRANK *et al.* 2004).

Altering the sample conditions can optimise the extraction yield. The following parameters were optimised: equilibration time, extraction time, temperature, and sample weight, with the aim to maximise the compound recovery while keeping a reasonably short total analysis time. Also desorption parameters were optimised. SPME is an equilibrium process, hence under equilibrium conditions precision and sensitivity are expected to be optimal. However, it is not necessary to reach full equilibrium, if constant extracting conditions are maintained. The optimal SPME parameters selected for the subsequent analyses were:

- equilibration time 30 min;
- extraction time 20 min;
- extraction temperature 35°C;
- sample amount 1 g;
- desorption temperature 250°C;
- desorption time 5 min.

The reproducibility was good, RSD of the standard analyses were in the range of 2–11%. Detection limits differed for the various compounds analysed, with small volatile molecules they were higher owing to their bad affinity to the fibre, and were in the range of 0.003–0.2 µg/g. The linearity was also good, the correlation coefficients were all over 0.98.

#### Identification of aroma compounds in Niva cheese

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 and non-volatile compounds which may contribute to the cheese flavour. Several degradation types occur simultaneously and the ultimate result will be a very wide range of compounds. The factual contribution of them to the flavour of cheese is largely unknown.

Proteolysis in cheese during ripening plays an important role in the development of texture. However, it also contributes to the taste of cheese by the production of peptides and free amino acids. Large peptides do not contribute directly to the cheese taste, but can be hydrolysed to shorter peptides that may be bitter. Free amino acids are the final products of proteolysis. Very extensive proteolysis occurs in blue-mould cheeses (SØRENSEN & BENFELDT 2001).

Lipolysis is most important for the blue cheese flavour. Lactic acid bacteria present in starter cultures are generally only weakly lipolytic, most of the FA coming from the triglycerides degradation by moulds. Compounds generated by lipid metabolism predominate among the aroma compounds identified in blue cheese (QIAN *et al.* 2002).

Niva is soft blue-veined cheese manufactured from pasteurised cow milk. It has a crumbly texture, white to light beige interior with blue veining and a pleasant salty, piquant flavour. It is aged at least two months to achieve the typical appearance and flavour. It is known that free FA, odd-carbon chain methyl ketones, and secondary alcohols are the major contributors to the characteristic flavour of blue cheese, so the flavour development during the cheese ripening is dependent on milk triacylglycerols hydrolysis by *Penicillium roqueforti* lipases and subsequent oxidation of FA to methyl ketones. However, a large number of other volatile compounds have been identified in blue cheeses (SABLÉ & COTTENCEAU 1999; UR REHMAN *et al.* 2000; QIAN *et al.* 2002).

The identification of the individual compounds in the sample is difficult, owing to their low concentrations in cheese and the relatively high concentrations of other compounds. The 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. In total, 54 compounds were identified in Niva cheese: 3 hydrocarbons, 5 aldehydes, 11 ketones, 18 alcohols, 3 esters, 10 fatty acids, and 4 sulphur compounds.

Eleven ketones were identified in Niva cheese: acetone, propan-2-one, butan-2-one, pentan-2-one, butan-2,3-dione, heptan-2-one, 3-hydroxybutan-2-one, nonan-2-one, 8-nonen-2-one, decan-2-one and undecan-2-one. Ketones are common constituents of most dairy products, which may be reduced to secondary alcohols (CARBONELL *et al.* 2002). Methyl ketones are derived from FA by  $\beta$ -oxidation or from  $\beta$ -ketoacids and are primarily known for their contribution to the aroma of mould cheeses. They have typical odours (fruity, floral, mushroom, or musty notes) and low perception thresholds (QIAN *et al.* 2002). One of the most important diketones is biacetyl (butan-2,3-dione) with sweet buttery and vanilla aroma. It is formed through lactose and citrate metabolism and its

production is mainly due to the activity of lactic acid bacteria. It can be reduced to acetoin (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 (CURIONI & BOSSET 2002). Biacetyl was identified as very important to blue cheese, in which acetoin was also detected. As mentioned previously, in mould cheese methyl ketones are the most abundant aroma compounds, the major ones being heptan-2-one and nonan-2-one (CARBONELL *et al.* 2002; QIAN *et al.* 2002; FRANK *et al.* 2004).

Eighteen alcohols were identified in Niva cheese: ethanol, propan-2-ol, propan-1-ol, 2-methylpropan-1-ol, pentan-2-ol, butanol, 3-methylbutan-1-ol, pentan-1-ol, methanol, heptan-2-ol, oct-1-en-3-ol, octan-1-ol, nonan-2-ol, phenylethanol, dodecan-1-ol, heptadecan-1-ol, hexadecan-2-ol and heptadecan-2-ol. Primary alcohols are formed by the proper aldehydes reduction. They impart a fruity, nutty note to the cheese flavour, and in certain cheeses high levels of them can cause flavour defects. Secondary alcohols are formed by enzymatic reduction of the corresponding methyl ketones. They have similar but heavier flavour notes than methyl ketones. 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 (CARBONELL *et al.* 2002). 3-methylbutan-1-ol was found at high concentrations in mould cheeses. The principal secondary alcohols in mould ripened cheeses are heptan-2-ol and nonan-2-ol, which correspond to the high methyl ketone contents. They have less influence on the cheese flavour than methyl ketones, however, they may indirectly contribute to it because of their ability to form esters with FA (SABLÉ & COTTENCEAU 1999).

Ten FA were identified in Niva cheese: acetic, capric, isobutanoic, propionic, isopentanoic, butanoic, hexanoic, myristic, benzoic, pentadecanoic, and palmitic acids. 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 (CURIONI & BOSSET 2002). Long-chain FA (> 12 carbon atoms) play a minor role in the flavour owing to their relatively high perception thresholds. Short and moderate-chain, even-numbered FA (C4-C12) have much lower perception

thresholds and characteristic notes (vinegar, sour). Moreover, free FA also serve as precursors to methyl ketones, alcohols, lactones, and esters (SABLÉ *et al.* 1999; VÍTOVÁ *et al.* 2004). On the other hand, higher concentrations of free FA can cause off-flavours (e.g. rancid). Short-chain free FA acids are important contributors to the characteristic flavour of blue cheeses and were identified by many authors at high concentrations (SABLÉ & COTTENCEAU 1999; QIAN *et al.* 2002).

Three esters were identified in Niva cheese: ethyl-acetate, phenylethyl-acetate and pentyl-benzoate. Esters are common cheese volatiles. Esterification reactions occur between short- to medium-chain FA and alcohols. Most esters in cheeses are described as having sweet, fruity, and floral notes. Some of them have very low perception thresholds and their contribution is heightened by synergistic effect. Further, they can contribute to the aroma of cheese by minimising the sharpness and the bitterness imparted by FA and amines (CURIONI & BOSSET 2002). GONZALES DE LLANO *et al.* (1990) found high proportions of methyl and ethyl esters in ripe blue cheese, QIAN *et al.* (2002) consider ethyl butanoate and ethyl hexanoate to be important compounds contributing to the blue cheese aroma.

Five aldehydes were identified in Niva cheese: propanal, ethanal, hexanal, phenylacetaldehyde, and benzaldehyde. Straight-chain aldehydes may result from  $\beta$ -oxidation of unsaturated FA or from amino acids by Strecker degradation. Branched-chain aldehydes probably originate from amino acid degradation via enzymatic as well as non-enzymatic, e.g. Strecker degradation, processes (CURIONI & BOSSET 2002). Aldehydes are transitory compounds in cheese because they are rapidly reduced to primary alcohols or oxidised to the corresponding acids (CARBONELL *et al.* 2002). They are characterised by green-grass or herbaceous aroma and can be very unpleasant when their concentrations exceed certain values. QIAN *et al.* (2002) consider 2-methylpropanal and 3-methylbutanal to be important compounds contributing to the blue cheese aroma.

Three hydrocarbons were identified in Niva cheese in trace amounts: heptane, pentadecane, and heptadecane. Hydrocarbons are secondary products of lipid autooxidation. They do not make a major contribution to the aroma, but may serve as precursors to other aroma compounds (ORTIGOSA *et al.* 2001). Hydrocarbons have been frequently

reported in many cheeses, although usually at low concentrations (CARBONELL *et al.* 2002).

Four sulphur compounds were identified in Niva cheese: dimethyl sulphide, dimethyl disulphide, dimethyl trisulphide, and benzothiazol. 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. ORTIGOSA *et al.* (2001) consider them to be indispensable to achieve the characteristic aroma of white mould cheese, QIAN *et al.* (2002) introduce methional and dimethyl trisulfide as important compounds contributing to the blue cheese aroma.

### Changes of aroma compounds during ripening of Niva cheese

SPME-GC procedure was also used to study the volatile compounds evolution during the ripening of Niva cheese, the method of standard addition was chosen for quantification, the standards were added to grated cheese in a vial.

As mentioned before, cheese ripening includes microbiological and enzymatic processes contributing to the unique flavour and textural characteristics. Knowledge of these changes could allow standardisation of the cheese manufacturing and a better control of the process. But only in a few kinds of cheese have these changes been described in depth. In the case of Niva cheese, most of the volatile compounds identified were present at

all stages of the cheese ripening, however, their amounts changed significantly. The changes of the most important and most abundant compounds chosen are graphically expressed in Figures 1–4. Each value represents the mean of three replicate determinations, RSD was in all cases to 10%. Ketones, alcohols, and FA were quantitatively the most important compounds present.

The changes of ketones in Niva cheese are presented in Figure 1. As can be seen, their concentrations increased during ripening with maximum after about 40 days. Only acetone was present at a high concentration in unripe cheese and then its amount sharply decreased. The increase in the ketones concentrations during ripening is characteristic for many kinds of cheese as confirmed by many authors, this fact being linked to lipolysis (CARBONELL *et al.* 2002).

Ethanol and pentan-2-ol were the most abundant alcohols in Niva cheese. GONZALES DE LLANO *et al.* (1990) describe the evolution of methyl ketones and 2-alkanols in blue cheese as parallel: the contents of both increased during the first part of the ripening and then decreased after reaching their maximum after 60 days. Also in other types of cheeses, alcohols are quantitatively the main chemical family and their contents increase significantly, but at different rates during ripening (CARBONELL *et al.* 2002). In our case, no significant increase was found in alcohols concentrations in Niva cheese (Figure 2), with the exception of ethanol.

To changes of short-chain fatty acids in Niva cheese are presented in Figure 3. No significant increase in their amount was found (except acetic

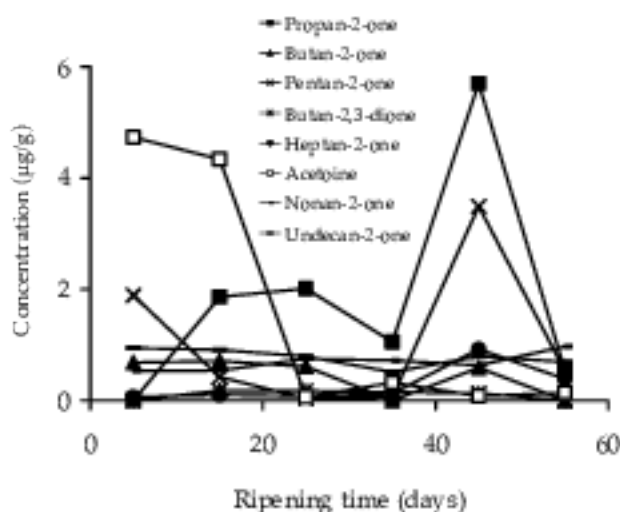


Figure 1. Changes of ketones during ripening of Niva cheese

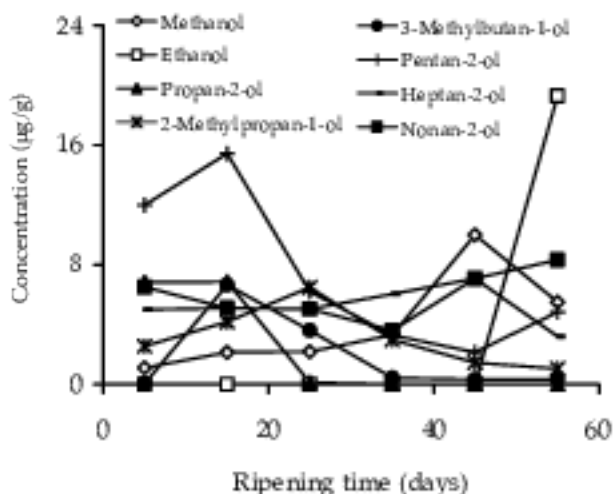


Figure 2. Changes of alcohols during ripening of Niva cheese

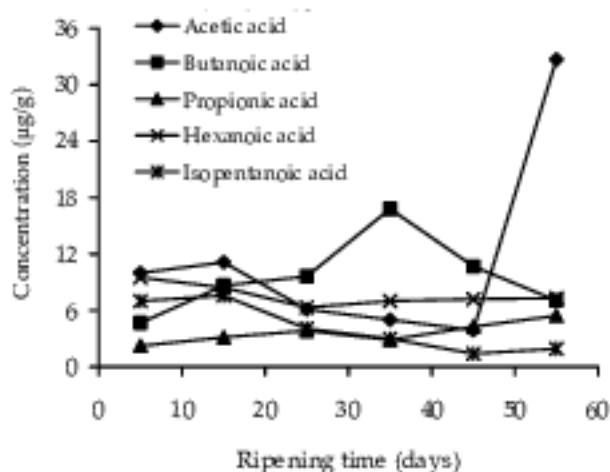


Figure 3. Changes of fatty acids during ripening of Niva cheese

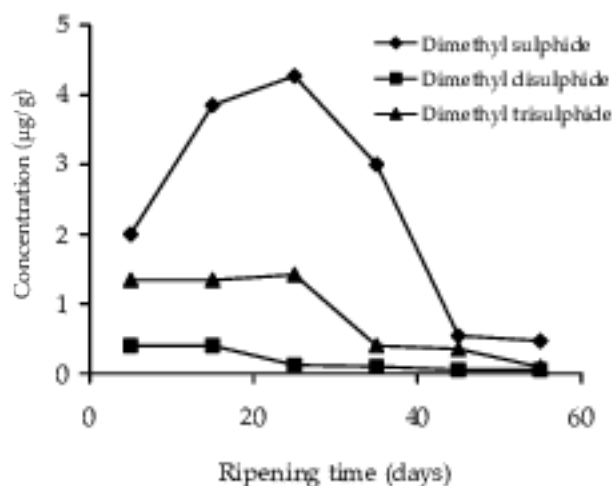


Figure 4. Changes of sulphur compounds during ripening of Niva cheese

acid), although some authors noticed an increase in the concentrations of volatile FA during the ripening of various kinds of cheese (MULET *et al.* 1999; VÍTOVÁ *et al.* 2004).

As reported by some authors, the concentrations of esters can decrease or increase during the ripening of various kinds of cheese (MULET *et al.* 1999; CARBONELL *et al.* 2002). GONZALES DE LLANO *et al.* (1990) describe the evolution of esters in blue cheese as similar to those of ketones and alkanols. The formation of them was slower, attaining the maximum after 180 days (end of ripening). Three esters identified in Niva cheese reached maximum concentrations (only about 0.1 µg/g) in about 40 days of ripening.

The contents of aldehydes mostly increase during the ripening of various cheeses (CARBONELL *et al.* 2002). In the case of Niva cheese, the contents of the aldehydes identified reached maximum in about 40 days, the ethanal content decreased during ripening. The maximum concentrations of them were in the range of 0.4–0.8 µg/g.

The contents of sulphur compounds identified in Niva cheese decreased during ripening, dimethyl sulphide was present in the highest concentration (Figure 4).

To summarise, the analytical method based on SPME combined with GC is a simple and rapid method for the analysis of the volatile compounds in cheese. It minimises thermal, mechanical, and chemical modifications of the matrix, it is consequently suitable for the characterisation of the cheese aroma, but not only cheese as it can be

applied to various foods. Some important aroma compounds in Niva cheese were identified and quantified using this method. Although important changes of them took place during ripening, in most cases, the final concentrations in ripe cheeses were similar to the initial concentrations in the unripe cheese.

#### List of symbols

FA	– fatty acid
GC	– gas chromatography
GC-MS	– gas chromatography-mass spectrometry
SPME	– solid-phase microextraction
DI-SPME	– direct immersion SPME
HS-SPME	– headspace SPME
RSD	– relative standard deviation

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