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Mono-, Di- and Trimethyl Benzene in Frozen Cheese Samples: Natural Metabolites or Environmental Pollutants?

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Introduction

Frozen storage of cheese samples may be necessary in the laboratory for the following reasons: i) in order to analyse samples delivered to the laboratory at different times (collected at different ages to investigate the ripening effect, or from different factories, technologies or even production batches within the same trial) under identical conditions or in batches to save time and money; ii) to store for further analysis valuable cheese samples already analysed using a common method; iii) to store reference samples for laboratory quality assurance/management (control charts) of gas chromatographs and their detectors.

Numerous analytical investigations into the effects of freezing dairy products have been carried out. *Emmons* et al. (1) dealt with the influence of variation of frozen temperature and wrappers on butter. Neither chemical nor microbial differences were found in frozen yoghurt samples after either two, four or six months storage (2). *Collomb* et al. (3) did not report any influence of frozen storage on the gross composition of cheese (dry matter, fat content, total nitrogen, water soluble nitrogen, non protein nitrogen and volatile free fatty acids) over 28 days. The physicochemical and sensory characteristics (pH, total solids, fat, protein, ash and salt) of several samples of defrosted goat cheese were also investigated by *Martin-Hernandez* et al. (4, 5) and *Alonso* et al. (6). In an optimisation of freezing conditions for semi-hard ewe cheese (7), no significant differences in mean fat and protein contents as part of TS (total solids) were found, whereas pH, the level of proteolysis and the viability of the microbial flora were altered. Further investigations dealt with microbial changes in ewe milk cheeses (8, 9) as well as in chemical, textural and

sensory properties of mozzarella cheese during storage (10–13), but none of these studies reported on the volatile components of the cheeses.

An investigation (14) into frozen butter focussed on the increase in the volatile carbonyl compounds as peroxidation products (β -oxidation) of unsaturated fatty acids. Carbonyls were found to increase over a period of 4.5 months, then partially decrease. No regular behaviour was reported for the yield of the lactone components during storage.

The starting point of the present study was the repetition of the GC-MSD&FID analysis of a highland Gruyère type (L'Etivaz) cheese. Analysis of this sample was repeated after 2.5 years storage at $-20\text{ }^{\circ}\text{C}$ to determine qualitatively and quantitatively the stability of the volatile organic components (VOC). Alcohols and terpenes decreased slightly and, as expected, aldehydes increased drastically due to autoxidation of the unsaturated fatty acids. The relative changes of these compounds will be further reported elsewhere.

Surprisingly, a few (partially new) aromatic hydrocarbons, namely mono-, di- and trimethyl benzenes occurred in a much higher concentration in the stored samples. The aim of the current work is to confirm this observation by reinvestigating numerous previously investigated cheese varieties at different ripening stages and stored under different conditions (duration, location, physical state and packaging material). All cheese samples tested were stored frozen at approx. $-20\text{ }^{\circ}\text{C}$ before analysis (the freezing point of Swiss cheese is approximately $-10\text{ }^{\circ}\text{C}$ (15)). Until now, storage under such conditions for up to months or years was generally considered to have no or only negligible effects on the chemical composition of dairy products.

Materials and methods

The present work consists of a post-run compilation of published or still unpublished results obtained within several studies carried out over many years. The results are considered here from a different point of view: the study of the generation of volatile compounds during frozen storage.

Origin of the samples and trial conditions

- a) Swiss Emmental cheese, one year old: one grated sample was stored frozen for 2–14 months in a gas tight glass container to compare different extraction, concentration and injection methods for analysis of VOC by GC/MS (16).
- b) Ripening trial of Swiss Emmental cheese: 30 samples aged six months were frozen for eight months; 26 further samples aged 12 months were stored for two to three months as pieces. Two grated samples aged 12 months were stored for 23 months in a gas tight glass container (paper in progress).
- c) Ripening trial of Swiss Emmental cheese wrapped in foil: Eight samples aged three, six, nine and 12 months were cut from the same blocks and stored for 14 to five months respectively as pieces (the longer the ripening, the shorter the storage time) before analysis (17).

- d) One grated 4.5-month ripe Swiss Emmental cheese sample was stored for 30 months in a gas tight glass container as reference material for quality assurance.
- e) One six month aged Swiss Emmental cheese was analysed without storage.
- f) Swiss Emmental UHT-processed cheese: Five samples were stored in gas tight aluminium cans at -20°C from one to 15 months as pieces (paper in progress).
- g) Characterisation of approx. three month ripened Fontina DPO cheese: 21 samples of various qualities and geographic origins were stored for up to two years as pieces at -20°C in two different freezers at the Institut agricole régional (I-Aosta) and then for another one to two months at the FAM before analysis using an electronic nose and a GC/MS-FID method (paper in progress).
- h) Two grated, Gruyere-type cheese samples aged 4.5 months were stored for 30 months in a gas tight glass container.
- i) Comparison of highland and lowland Gruyère type cheeses: 49 samples of cheese aged eight \pm one months were stored at -20°C for: i) three months (17 samples), ii) four months (17 samples), iii) five months (15 samples) and iv) 35 months (1 sample) as pieces. The shorter the ripening, the longer the storage before analysis. The mean value of the samples i) to iii) is indicated (18, 19).
- j) Comparison of Vacherin fribourgeois and Vacherin Mont-d'Or: Eight samples of four-months Vacherin fribourgeois and nine samples of one-month Vacherin Mont-d'Or were analysed after 11 months as pieces in the freezer (20).

Storage conditions

Table 1 indicates for all samples the trial considered the duration of storage at $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$, the number of samples analyzed as well as the packaging material used. The mass of the samples ranged approximately from 100 up to 500 g, except for processed cheese (approximately 30 g).

Reagents and chemicals

Milli-Q water was boiled for approx. 15 min with an electric heating device to strip off all residual volatile trace components under a continuous nitrogen flow.

Sample treatment

Shortly before analysis, a representative aliquot of approx. 20 g of fresh (trial e) or freshly defrosted sample material (all the other trials) was accurately weighed and finely dispersed in 100 ml water using a high speed homogeniser (Polytron PT 3000 equipped with a PT-DA 3020/2s cutting system, Kinematica) running at 10000 rpm for 1 min. Finally, 10.0 g of this mixture was carefully introduced into the 25-ml non-fritted sparger of the Purge & Trap extraction system.

Extraction of the volatile components

The Purge and Trap system LSC 2000 (Tekmar, Cincinnati, OH, USA) included a 25-ml non-fritted sparger (Schmidlin Co, art. no. 14-2333-4SL, CH-6345 Neu-

Table 1

Relative content of various benzene derivatives of different cheese types at various stages of ripening stored frozen under different conditions

Trial, conditions	a	b	b	b	c	c	c	c	d	e	f	g	h	i	i	j	j
Storage duration (mo)	2-14	2-3	8	23	5	8	11	14	30	0	1-15	25-26	30	4 ± 1	35	11	11
No. of samples	1	26	30	2	8	8	8	8	1	6	5	21	2	49	1	8	9
Packaging	gtc	aluf	aluf	gtc	aluf	aluf	aluf	aluf	gtc	no	aluc	aluf	gtc	aluf	aluf	aluf	aluf
Methyl benzene (au)	trace	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	10058	nd	9833	16267	1902	1107
1,3- and 1,4-dimethyl benzene (au)	trace	trace	3416	trace	10790	13603	21355	33907	trace	nd	trace	1362	nd	trace	15473	554	604
1,2-dimethyl benzene (au)	trace	trace	4349	trace	16396	20205	32076	50112	trace	nd	trace	3649	nd	trace	26073	trace	trace
1,2,4-trimethyl benzene (au)	trace	nd	907	nd	4551	4516	9915	16677	nd	nd	trace	1266	nd	nd	12981	nd	nd

Caption:

Trial, conditions: see text

nd = not detected/below detection limit (< 120 arbitrary units), trace < 360 arbitrary units (three times the detection limit)

gtc = gas tight glass container with a metallic lid, aluc = gas tight aluminium can, aluf = aluminium foil & minigrip, no: no packaging

au = FID peak height, mean value in arbitrary units

heim), a trap (No 8, containing a mixture of Carbosieve SIII (0.05 g) and Carbo-pack B60/80 (0.2 g)) as well as a cryofocussing unit. The moisture control module was not used. The operating conditions were as follows: purge gas: nitrogen; purge flow (vent): 30 ml/min; prepurge: 1 min; water bath: 45 °C; purge: 15 min; drypurge: 10 min; cap cool-down: -125 °C; desorb preheat to 210 °C; desorb: 4 min at 220 °C; inject: within 1.5 min from -125 to 200 °C; bake: 5 min at 260 °C; 6-port valve: 150 °C; line: 150 °C; capillary union heater (= transfer line from purge and trap to gas chromatograph): 150 °C.

Gas chromatography

A Hewlett-Packard (HP) 5890, Series II was used. The operating conditions were as follows: carrier gas: helium; inlet pressure: 40 kPa; flow: approx. 1.6 ml/min at 45 °C; transfer line (from GC to MS): 280 °C; interface: direct inlet; temperature programme: 13 min at 45 °C, heating rate: 5 °C/min up to 240 °C, and 5 min at 240 °C; capillary column: SPB1 sulfur (Supelco), 30 m × 0.32 mm id., film thickness: 4 µm.

Detection

Two detectors were mounted in parallel by splitting the flow at the end of the capillary column (split ratio: approx. 1:1 at 45 °C), i.e. a Hewlett-Packard flame ionisation detector (FID) and a mass-sensitive detector (MSD model HP 5972), operating in the scan mode (TIC) from 19 to 250 amu at 2.9 scan/s, ionisation by EI at 70eV by autotuning; MS-Scan after 3.5 min. The MSD was used for the identification of the VOC, the FID for their relative quantification.

Results and discussion

Table 1 summarises all results compiled from the previously mentioned investigations.

Independent of the packaging material, cheese variety and ripening grade, cheese samples analysed fresh (0 month, trial e) or after a short storage duration (\leq 3 months, trial b) did not contain any methyl benzene, dimethyl benzene or 1,2,4-trimethyl benzene derivatives, or contained only trace amounts of these.

Independent of the duration of frozen storage (up to 30 months), cheese variety, ripening grade and physical state (grated or in a piece), all frozen samples stored in gas tight containers (glass cup with a metallic lid or aluminium can, indicated with italic letters in table 1) were also almost or totally free of these derivatives (trials a, b, d, f and h).

On the other hand, cheese samples wrapped in partially permeable packaging material contained significantly more benzene derivatives after 35 months of storage than after four \pm one months (Gruyère type, trial i). This increase was particularly marked for dimethyl and trimethyl benzene. A similar increase was also found in samples of Swiss Emmental cheese (trial c) stored over five, eight, 11 and 14 months, respectively (fig. 1).

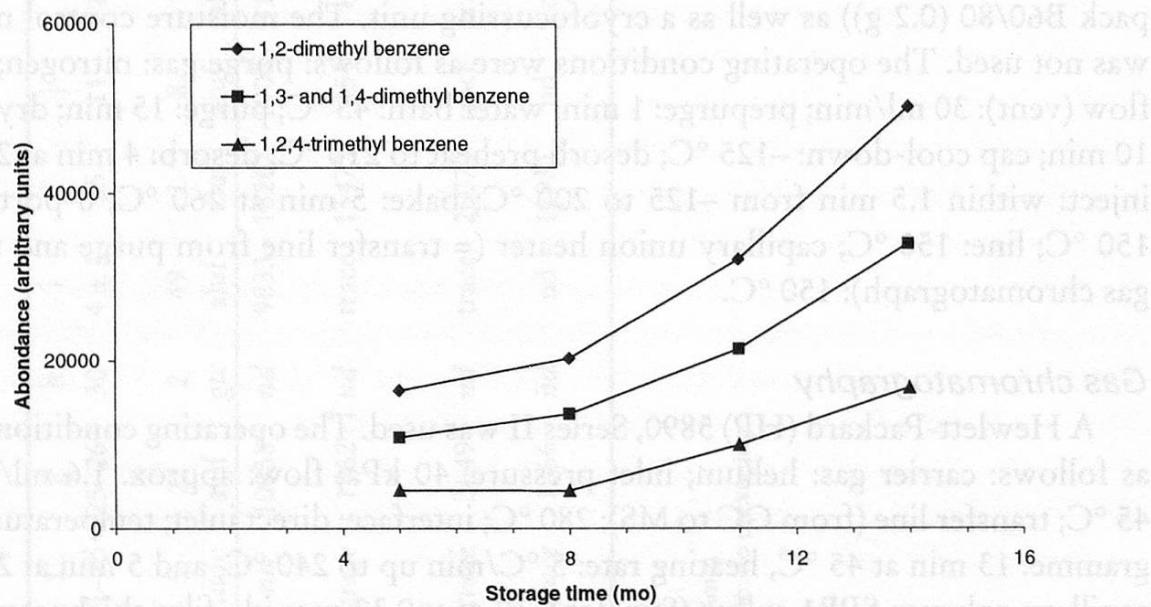


Figure 1 Relative content (arbitrary units) of various xylene and trimethyl benzene of frozen Emmenthal cheese samples vs storage time (trial c)

Caption: The samples were collected from the same cheese blocks during ripening in the cellar. Methyl benzene (toluene) was not detected in these samples

All the cheese samples mentioned above were stored frozen and analysed at the Swiss Federal Dairy Research Station in Liebefeld (FAM). Fontina PDO cheese samples, also analysed at the FAM but stored frozen in two different freezers at the Italian Institut agricole régional in Aosta, contained comparable amounts of these substances (trial g).

For these 21 Italian Fontina PDO cheese samples (trial g), figure 2 shows some interesting correlations between these different benzene derivatives: none ($R^2 = 0.002$) between 1,2-dimethyl benzene and toluene, a weak correlation ($R^2 = 0.574$) between 1,2,4-trimethyl benzene and 1,3- & 1,4-dimethyl benzene – the two latter components were not resolved chromatographically using a SPB-1 sulfur column – a higher correlation ($R^2 = 0.735$) between 1,2,4-trimethyl benzene and 1,2-dimethyl benzene, and a strong correlation ($R^2 = 0.942$) between 1,2-dimethyl benzene and 1,3- & 1,4-dimethyl benzene. These well-differentiated correlations could indicate that these various derivatives have diverse origins, except for the highly correlated 1,2- and 1,3- & 1,4-dimethyl benzenes.

Benzene, another ubiquitous air pollutant generally associated with dimethyl and trimethyl benzene, always occurred in the analysed frozen cheese samples. This volatile component is however not discussed within the current study since it is poorly resolved, and consequently poorly quantified, from its neighbouring broad peak (i.e. 1-butanol). Both components are barely separated with the SPB-1 sulfur

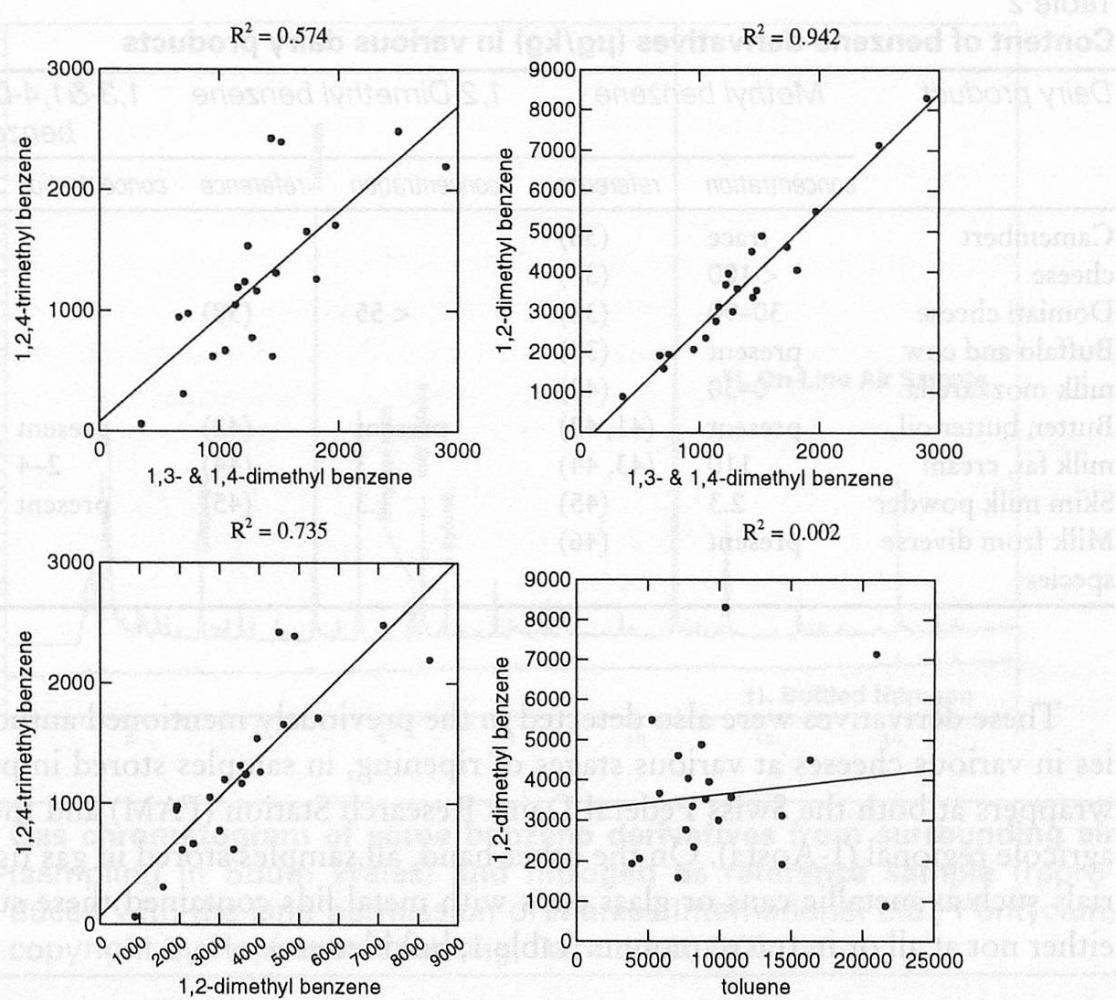


Figure 2 Correlation between methyl benzene (toluene), dimethyl benzene (xylene) and trimethyl benzene concentrations within the same set of 21 Fontina PDO cheese samples

capillary column used for the previously mentioned trials (a to j). This overlapping effect is made worse by using trap no 8 of the Tekmar which contains a mixture of Carbosieve SIII (0.05 g) and Carbopack B60/80 (0.2 g). The use of tenax would e.g. drastically reduce the trapping of alcohols, and consequently improve the separation of benzene.

Low concentrations of these components were also found in Vacherin fribourgeois and Vacherin Mont-d'Or cheese samples (trial j) wrapped in good but not completely gas tight material.

Methyl benzene (toluene), 1,2-methyl benzene (o-xylene), 1,3-methyl benzene (m-xylene) and 1,4-methyl benzene (p-xylene) are well known volatile hydrocarbons reported in many foods. In the TNO's library (21), toluene e.g. is quoted in 159 products, 1,2-dimethyl benzene in 79 products, 1,3-dimethyl benzene in 74 products, and 1,4-dimethyl benzene in 70 products. Data concerning dairy products are reported in table 2.

Table 2

Content of benzene derivatives (µg/kg) in various dairy products

Dairy product	Methyl benzene	1,2-Dimethyl benzene	1,3-&1,4-Dimethyl benzene			
	concentration	reference	concentration	reference	concentration	reference
Camembert cheese	trace < 100	(36) (37)				
Domiati cheese	30-90	(38)	< 55	(38)		
Buffalo and cow milk mozzarella	present 0-30	(39) (40)				
Butter, butter oil, milk fat, cream	present 110	(41, 42) (43, 44)	present 3	(42) (44)	present 2-4	(42) (43, 44)
Skim milk powder	2.3	(45)	1.3	(45)	present	(45)
Milk from diverse species	present	(46)				

These derivatives were also detected in the previously mentioned authors' studies in various cheeses at various stages of ripening, in samples stored in permeable wrappers at both the Swiss Federal Dairy Research Station (FAM) and the Institut agricole régional (I-Aosta). On the other hand, all samples stored in gas tight materials such as metallic cans or glass cups with metal lids contained these substances either not at all or in trace amounts (table 1, bold letters).

Conclusion

Such preliminary results lead to the conclusion that these VOC should be ubiquitous environmental pollutants. Due to their high lipophilic properties, these compounds accumulate in the fatty matrix of dairy products such as cheese and butter during frozen storage. A similar effect was observed by *Biedermann* et al. (22, 23) with olives and olive oil in which the same pollutants, as well as some others, were detected originating from the surrounding air (24, 25) (fig. 3), and even at higher concentration when these foodstuffs were stored and treated in the room where the combustion engines operated. Benzene and toluene were also frequently reported in several food (26-28). At these concentrations (< 100 µg/kg), such components have no toxicological interest.

Nevertheless, several questions still remain open such as:

- Why was methyl benzene not found in trials b and c carried out with a smearless Swiss Emmental cheese over a long frozen storage while it occurred in significant concentrations in trials g, i and j carried out with four smeared cheese varieties (Fontina, Gruyère/L'Etivaz, Vacherin Mont-d'Or and Vacherin fribourgeois)?
- Are other benzene derivatives such as ethyl benzene and methyl ethyl benzene also detected in milk fat, butter (42, 43) and cheese (29-31) as well as benzene,

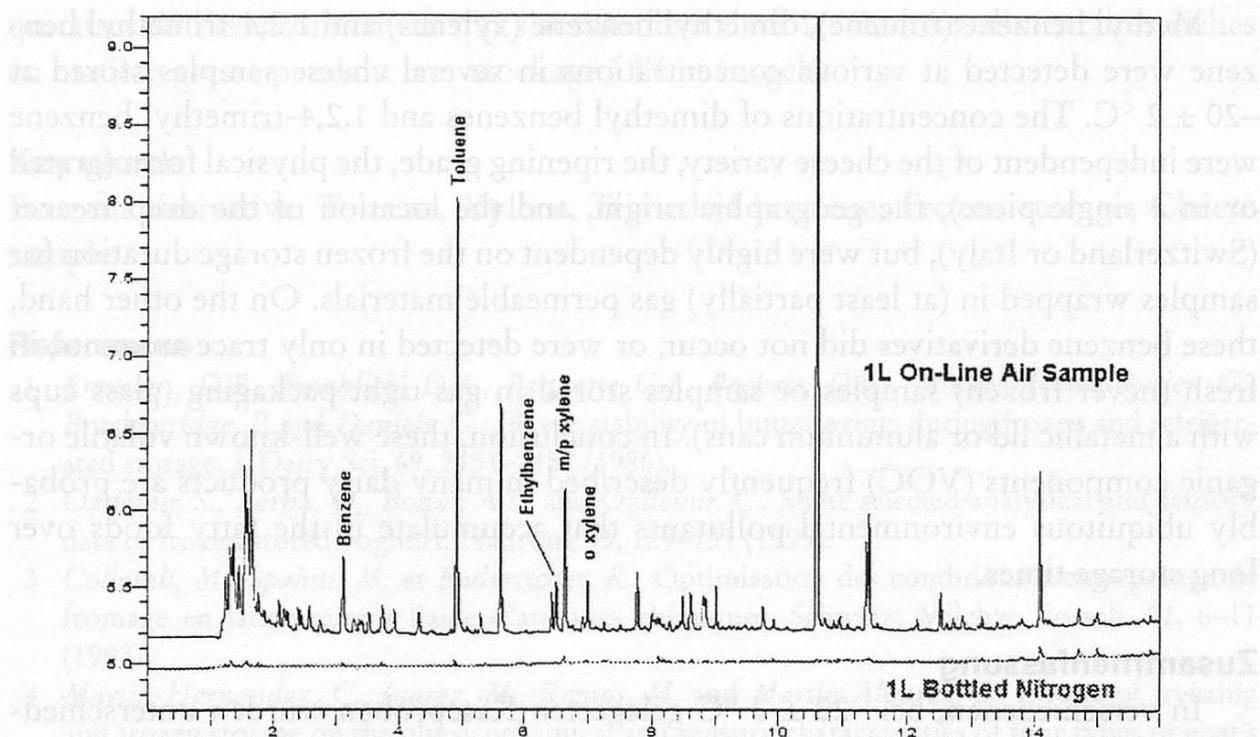


Figure 3 Gas chromatogram of some benzene derivatives from surrounding air (sampling in South Wales) and nitrogen as reference sample (reproduced with the kind permission of Markes International Ltd., Pontyclun, copyright by Hoppenstedt-Verlag, Darmstadt) (47)

- isopropyl benzene, methylpropyl benzene, t-butyl benzene, naphthalene (32–35) further environmental pollutants?
- Could some of these VOC originate from both microbial activity of cheeses (especially those with a smeared surface) and environment?

Further investigations are necessary to answer such questions. A trial will be carried out consisting of a triglyceride sample stored frozen, such as puriss tricaprin, under a given geometry (e.g. in an open glass Petri disk filled with a 5 mm film thickness) over one year in the same freezer in order to concentrate the surrounding pollutants before their analysis with the analytical method as mentioned above. In this way it should be proved which pollutants occur and at what level.

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Summary

Methyl benzene (toluene), dimethyl benzene (xylenes) and 1,2,4-trimethyl benzene were detected at various concentrations in several cheese samples stored at -20 ± 2 °C. The concentrations of dimethyl benzenes and 1,2,4-trimethyl benzene were independent of the cheese variety, the ripening grade, the physical form (grated or in a single piece), the geographic origin, and the location of the deep freezer (Switzerland or Italy), but were highly dependent on the frozen storage duration for samples wrapped in (at least partially) gas permeable materials. On the other hand, these benzene derivatives did not occur, or were detected in only trace amounts, in fresh (never frozen) samples or samples stored in gas tight packaging (glass cups with a metallic lid or aluminium cans). In conclusion, these well-known volatile organic components (VOC) frequently described in many dairy products are probably ubiquitous environmental pollutants that accumulate in the fatty foods over long storage times.

Zusammenfassung

In verschiedenen, bei -20 ± 2 °C gelagerten Käseproben wurden unterschiedliche Konzentrationen von Methyl- (Toluol), Dimethyl- (Xylol) und 1,2,4-Trimethylbenzol gefunden. Die Konzentrationen von Dimethyl- und 1,2,4-Trimethylbenzol waren nur von der Lagerungsdauer abhängig, nicht aber von der Käsesorte, vom physikalischen Zustand (geriebene oder blockförmige Probe), vom Reifungsgrad und von der geografischen Herkunft der Laibe, wenn die Proben in gasdurchlässigen Materialien eingepackt waren. Diese Benzolderivate waren hingegen nicht oder nur in Spuren vorhanden, wenn die Käseproben nach einer Lagerung in gasdichten Packungen (Glasgefäß mit Metalldecke, Aluminiumdosen) oder frisch (ohne Einfrieren) gemessen wurden. Man kann daraus schliessen, dass diese gut bekannten flüchtigen organischen Komponenten (VOC) wahrscheinlich ubiquitäre Umweltkontaminanten sind, die sich während der Tieftemperaturlagerung in den fettreichen Lebensmitteln akkumulieren.

Résumé

On a trouvé du méthylbenzène (toluène), des diméthylbenzènes (xylènes) et du triméthylbenzène en concentrations variables dans diverses sortes de fromages conservées à -20 ± 2 °C. Les concentrations de diméthylbenzenes et de 1,2,4-triméthylbenzene mesurées ne dépendaient ni de la sorte de fromage considérée, ni de l'état physique de l'échantillon (râpé ou en un bloc), ni du degré d'affinage ou de l'origine géographique de la meule, mais seulement de la durée du stockage lorsque les échantillons étaient conservés dans des emballages non parfaitement étanches aux gaz. Inversement, ces dérivés benzéniques n'étaient pas présents ou qu'en trace lorsque lesdits emballages étaient parfaitement étanches (bocaux de verre avec couvercle métallique, boîte d'aluminium) ou lorsque les échantillons ont été mesurés frais (sans congélation). On peut en conclure que ces composés organiques volatils

(VOC) souvent cités dans les produits laitiers semblent être des contaminants ubiquitaires de l'environnement qui s'accumulent dans les denrées alimentaires riches en matière grasse pendant leur stockage à l'état surgelé.

Key words

Benzene derivative, Toluene, Xylene, Trimethyl benzene, Frozen storage, Cheese sample

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