Analytical development and traceability in food chemistry. Examples of application to Swiss and foreign Emmental cheese

A THESIS
Submitted to the
UNIVERSITY OF NEUCHATEL
For the degree of
DOCTOR OF TECHNICAL SCIENCES

Presented by

LAURENT PILLONEL

Dipl. Chem. ETH
Born on 19th June, 1975
Citizen of Seiry (FR)

Accepted on the recommendation of
Prof Dr R. Tabacchi
Prof Dr T. Turlings
Dr J.O. Bosset
Dr J.F. Chamba
Dr J.L. Luisier
Dr E. Pfammatter

Neuchâtel, February 2004

Développement analytique et traçabilité en chimie alimentaire. Exemples d'application à des Emmental suisses et étrangers

Une thèse
Soumise à l'
UNIVERSITÉ DE NEUCHÂTEL
Pour le degré de
DOCTEUR EN SCIENCES TECHNIQUES

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IMPRIMATUR POUR LA THESE

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M. Laurent PILLONEL

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel, sur le rapport des membres du jury

MM. R. Tabacchi (directeur de thèse), T. Turlings, J.-O. Bosset (Liebefeld), J.-F. Chamba (La Roche/Foron F), J.-L. Luisier (Sion) et E. Pfammater (Sion)

autorise l'impression de la présente thèse.

Neuchâtel, le 4 mars 2004

La doyenne:

Martine Rahier

Acknowledgements

My warmest thanks go to Dr Jacques-Olivier Bosset (FAM, Berne) who gave me the idea for the project. His patience and goodwill as well as his huge knowledge of cheese and food chemistry were of great help throughout the thesis.

I would like to express my sincere gratitude to Prof Dr Raffaele Tabacchi (Université de Neuchâtel) for giving me the opportunity to carry out this thesis and for the confidence he had in this project.

I am grateful to Dr Christian Steffen, director of the FAM, for putting the infrastructure and the knowledge of all colleagues at my disposal.

I would like to thank the CTI program, the Swiss Federal Office of Public Health and Emmentaler Switzerland™ for their financial support of the project.

Furthermore I would like to thank:

Prof Dr Ted Turlings (Université de Neuchâtel), Dr Jean-François Chamba (ITFF, Foron/Roche, France), Dr Elmar Pfammatter (Laboratoire cantonal du Valais, Sion) and Dr Jean-Luc Luisier (HESVs Sion) for agreeing to be co-examiners of the thesis.

Jean-François Chamba (ITFF), Eric Dufour (ENITA, Lempdes, France), Giuseppino Fortunato (EMPA, St. Gallen), Pascal Froidevaux (IRA, Lausanne), Alfred Jakob (Labor Spiez, Spiez), Georg Haberhauer (ARC, Seibersdorf, Germany), Stephan Hölzl (Universität München, Germany), Peter Horn (Universität München), Elmar Pfammatter (Laboratoire cantonal du Valais), Umberto Piantini (HEVs), Daniel Picques (INRA, Grignon, France), Andreas Rossmann (Isolab, Schweitenkirchen, Germany), Emmanuelle Schaller (Büchi, Flawil), who kindly offered analyses from their laboratories. A project of this scale would never have been possible without the interest and the willingness of such scientific partners.

Thierry Zesiger (LDZ, Marin) for his technical support with electronic noses.

Gerda Urbach and Gérard Gremaud for reviewing the manuscripts as well as Robert Sieber for correcting the German summaries.

Ueli Bütikofer (FAM) and Werner Luginbühl (FAM) for their great help with the statistical exploitation of the data.

All colleagues at the FAM for the interesting discussions and the kindness I found everywhere.

My family and friends for their company in the alternative programs.

The Swiss chocolate industry for manufacturing the most important food product a Ph.D. student could ever need.

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SUMMARY

The current Ph.D. work is divided into four main parts. In the first one, the problematic of volatile compound preconcentration is dealt with to extend the application range of electronic noses (ENs). A review article highlights the advantages of portable equipments such as Solid Phase Micro-Extraction (SPME) and Solid Phase Dynamic Extraction (SPDE). Both showed comparable efficiencies when applied to the SMart Nose equipment, though the second one was less subject to ageing effects. Various canned processed cheese types stored deep frozen were tested for several years as control materials for gas chromatography (long term stability of volatile compounds in cheese-like matrices). These processed cheeses were then used to illustrate the data transferability between two ENs of same type, condition necessary to build usable databases for the daily practice.

In the second part of the work, a screening of a great number of analytical methods was carried out to evaluate their potential for the authentication of the geographic origin of Emmental cheese. Twenty Emmental samples from six European regions were investigated on chemical, biochemical, microbiological, physical and sensory parameters. The most promising ones retained for the follow-up of the project were, volatile short-chain acids, chloride, pH-value, total nitrogen, 12%-TCA soluble nitrogen, water soluble nitrogen, copper, sodium, magnesium, zinc, enterococci, obligate heterofermentative Lactobacilli, *Lb helveticus*, L/D-lactate, succinate, pyruvate, L-leucine-aminopeptidase, δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ isotope ratios.

In the third part, the selected parameters were measured in 110 winter and 73 summer Emmental cheese samples collected in seven European regions, i.e. Switzerland, France Savoie, France Bretagne, France East-Central, South Germany, Austria and Finland. The analytical data was then processed using multivariate statistical analysis leading to pattern recognition and classification according to the geographic origin of the cheeses. Discriminant analysis (DA) and artificial neural networks delivered similar results. DA made it possible a reduction of the number of factors thanks to stepwise backward elimination. In a model including only eleven factors, 95% correct classification in the seven regions was achieved in the Jackknifed validation. Five Swiss Emmental samples out of 70 were misclassified. To improve the classification of the latters, a model with a pairwise approach (Switzerland *vs* a single foreign region at a time) was proposed. This procedure made it possible to recognise all Swiss samples correctly using fifteen parameters.

In the fourth and last part, a targeted screening was applied to two other cheese types, i.e. Raclette and Fontina cheeses. Raclette Suisse® and French Raclette cheese could be easily discriminated using the calcium content and the four stable isotope ratios δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. Fontina PDO is a raw milk cheese. Consequently its alkaline phosphatase content was much higher than in the Fontal cheeses, which are industrial imitations manufactured with pasteurised milk.

The last chapter is a guide line dedicated to the Swiss canton chemists for authenticating the Emmentaler Switzerland™. It discusses also the risks encountered by the most important Swiss cheese types toward mislabelling of origin.

ZUSAMMENFASSUNG

Die vorliegende Dissertation ist in vier Hauptteile gegliedert. Im ersten Teil wird die Problematik der Vorkonzentration von flüchtigen Verbindungen behandelt, um das Anwendungsfeld der elektronischen Nasen (ENs) zu erweitern. Ein Übersichtsartikel hebt die Vorteile von tragbaren Ausrüstungen wie Festphasen-Mikroextraktion (SPME) und Festphasen-Dynamischer-Extraktion (SPDE) hervor. Beide zeigten eine vergleichbare Effizienz in Anwendung mit dem SMart Nose-Gerät, obwohl die zweite weniger anfällig auf Alterungseffekte war. Verschiedene, in Aludöschen verpackte und tiefgefrorene Schmelzkäseproben wurden über einige Jahre als Kontrollmaterial für die Gaschromatographie getestet, um die Langzeitstabilität von flüchtigen Verbindungen in käseähnlichen Matrizen zu überprüfen. Diese Schmelzkäseproben wurden dann benutzt, um die Datenübertragbarkeit zwischen zwei ENs der gleichen Art zu veranschaulichen, eine notwendige Bedingung, um Datenbanken für die tägliche Praxis anwenden zu können.
Im zweiten Teil der Arbeit wurde ein Screening verschiedener analytischer Methoden durchgeführt, um deren Potential für den Nachweis der geographischen Herkunft des Emmentaler Käses zu evaluieren. Zwanzig Emmentalerproben aus sechs europäischen Regionen wurden auf chemische, biochemische, mikrobiologische, physikalische und sensorische Parameter untersucht. In den vielversprechendsten, die für die Fortsetzung des Projektes beibehalten wurden, zählen flüchtige kurzkettige Säuren, Chlorid, pH-Wert, totaler Stickstoff, 12%-TCA löslicher Stickstoff, wasserlöslicher Stickstoff, Kupfer, Natrium, Magnesium, Zink, Enterokokken, obligate heterofermentative Laktobacillen, *Lb helveticus*, L/D-Laktat, Succinat, Pyruvat, L-Leucin-Aminopeptidase, und das Verhältnis der stabilen Isotopen δ^2H, $\delta^{13}C$, $\delta^{15}N$ und $\delta^{34}S$.
Im dritten Teil wurden die ausgewählten Parameter bei 110 Winter- und 73 Sommer-Emmentalerproben aus sieben europäischen Regionen (Schweiz, Frankreich: Savoyen, Frankreich: Bretagne, Frankreich: Ost-Zentrum, Süden von Deutschland, Österreich und Finnland) gemessen. Die analytischen Daten wurden dann mit multivariaten statistischen Analysen ausgewertet, die zur Mustererkennung und Klassifikation der geographischen Herkunft der Käse führt. Die Diskriminanzanalyse (DA) und künstliche neuronale Netzwerke lieferten vergleichbare Resultate. Dabei ermöglichte es die DA, die Zahl der Faktoren dank "stepwise backward elimination" zu verkleinern. In einem Modell mit nur elf Faktoren, wurden mit der Jackknifed Crossvalidierung 95% der Proben aus den sieben Regionen korrekt klassifiziert. Fünf von 70 Schweizer Emmentalerproben wurden falsch zugeordnet. Um deren Klassifizierung zu verbessern, wurde ein Modell mit einer paarweisen Prozedur (die Schweiz einzeln gegen eine fremde Region) angewendet. Dies machte es möglich, alle Schweizer Proben mit Hilfe von fünfzehn Parametern richtig zu erkennen.
Im vierten und letzten Teil wurde ein gezieltes Screening an den zwei Käsesorten Raclette und Fontina durchgeführt. Raclette Suisse® und Raclette französischer Herkunft konnten mit dem Kalziumgehalt und dem Verhältnis der vier stabilen Isotopen δ^2H, $\delta^{13}C$, $\delta^{15}N$ und $\delta^{34}S$ leicht diskriminiert werden. Da Fontina GUB ein Rohmilchkäse ist, war die Aktivität der alkalischen Phosphatase viel höher als in den Fontal Käsen, bei denen es sich um industrielle Nachahmungen aus pasteurisierter Milch handelt.
Im letzten Kapitel werden Hinweise für die Kantonschemiker zur Beurteilung der Authentizität von Emmentaler Switzerland™ gegeben. Es bespricht auch die Gefahren, die für die wichtigsten Schweizer Käsesorten gegenüber einer falschen Ursprungsbezeichnung angetroffen werden.

RÉSUMÉ

La présente thèse de doctorat est divisée en quatre parties principales. Dans la première partie, la problématique de la préconcentration des composés volatils est traitée de manière à étendre le champ d'application des nez électroniques (ENs). Un article de revue fait ressortir les avantages des techniques à équipement portatif telles que la micro-extraction en phase solide (SPME) et l'extraction dynamique en phase solide (SPDE). Toutes deux ont montré une efficacité comparable avec l'équipement SMart Nose, bien que la SPDE soit moins sujette au vieillissement. Divers fromages fondus, conditionnés en capsules d'aluminium et congelés, ont été testés durant plusieurs années comme matériaux de contrôle pour la chromatographie en phase gazeuse (stabilité à long terme de composés volatils dans une matrice de type fromage). Ces fromages fondus ont ensuite été utilisés pour illustrer la transférabilité de données entre deux ENs de même type, condition nécessaire pour construire des bases de données pour la pratique quotidienne.
Dans la deuxième partie du travail, un criblage d'un grand nombre de méthodes analytiques a été effectué afin d'évaluer leur potentiel pour l'authentification de l'origine géographique de l' Emmental. Vingt échantillons de fromages provenant de six régions européennes ont été soumis à des analyses chimiques, biochimiques, microbiologiques, physiques et sensorielles. Les paramètres les plus prometteurs retenus pour la suite du projet étaient les acides volatils à courte chaîne, le chlorure, la valeur de pH, l'azote total, l'azote soluble dans le TCA à 12%, l'azote soluble dans l'eau, le cuivre, le sodium, le magnésium, le zinc, les entérocoques, les Lactobacilles hétérofermentatifs obligatoires, *Lb helveticus*, le lactate L et D, le succinate, le pyruvate, la L-leucine-aminopeptidase ainsi que les rapports isotopiques δ^2H, $\delta^{13}C$, $\delta^{15}N$ et $\delta^{34}S$.
Dans la troisième partie, les paramètres sélectionnés ont été mesurés dans 110 échantillons de fromage Emmental d'hiver et 73 d'été collectés dans sept régions, à savoir la Suisse, la France/Savoie, la France/Bretagne, la France/Est-central, le sud de l'Allemagne, l'Autriche et la Finlande. Les données analytiques ont été traitées par analyse statistique multivariée de manière à classer les échantillons selon leur origine géographique. L'analyse discriminante (DA) et les réseaux artificiels de neurones ont donné des résultats comparables. La DA permet une réduction du nombres de facteurs grâce à une "stepwise backward elimination". Dans un modèle incluant seulement onze facteurs, une classification correcte à 95% selon les sept régions d'origine a été obtenue avec une validation croisée de type "Jackknife". Cinq échantillons suisses sur 70 ont été mal classés. Pour améliorer ce score, un modèle avec une approche par paires (Suisse *vs* une seule région étrangère à la fois) a été proposé. Ce dernier a permis d'identifier tous les échantillons suisses en utilisant quinze paramètres.
Dans la quatrième et dernière partie, un criblage ciblé a été appliqué à deux autres sortes de fromage, la Raclette et la Fontina. La Raclette Suisse® et la raclette française ont facilement pu être discriminées grâce au contenu en calcium et aux quatre rapports d'isotopes stables δ^2H, $\delta^{13}C$, $\delta^{15}N$ et $\delta^{34}S$. La Fontina AOC est un fromage au lait cru. Par conséquent sa teneur en phosphatase alcaline était nettement plus élevée que celle des fromages de type Fontal qui sont des imitations industrielles fabriquées avec du lait pasteurisé.
Le dernier chapitre propose aux chimistes cantonaux des directives pour authentifier l'Emmentaler Switzerland™. On y discute également des risques potentiels auxquels sont exposées les principales sortes de fromages suisses quant à une éventuelle tromperie sur la déclaration d'origine.

RIASSUNTO

Il presente lavoro di dottorato è suddiviso in quattro parti principali. Nella prima parte è discussa, la problematica della pre-concentrazione dei composti volatili, in modo da estendere il campo d'applicazione dei nasi elettronici (ENs). Un articolo riassuntivo elenca i vantaggi delle tecniche portabili come la micro-estrazione in fase solida (SPME) e l'estrazione dinamica in fase solida (SPDE). Ambedue le tecniche hanno mostrato un'efficacia comparabile nell'applicazione dell'attrezzatura SMart Nose, benchè la SPDE sia meno soggetta agli effetti d'invecchiamento. Diversi formaggi fusi inscatolati e congelati, sono stati esaminati durante alcuni anni come materiale di controllo per la cromatografia in fase gassosa (stabilità a lungo termine di composti volatili in una matrice tipo formaggio). In seguito, questi formaggi fusi sono stati utilizzati per illustrare la trasferibilità dei dati tra due ENs di stesso tipo, condizione necessaria allo scopo di stabilire delle basi di dati per l'impiego quotidiano.
Nella seconda parte del lavoro, una scelta di numerosi metodi analitici è stata effettuata per valutare il loro potenziale per l'autenticazione dell'origine geografica del formaggio Emmental. Venti campioni di formaggi provenienti da sei regioni europee sono stati sottoposti ad analisi chimiche, biochimiche, microbiologiche, fisiche e sensoriali. I parametri più promettenti, considerati per il seguito del progetto sono: acidi volatili a corta catena, cloruri, pH, azoto totale, azoto solubile nel TCA al 12%, azoto solubile nell'acqua, rame, sodio, magnesio, zinco, enterococchi, Lactobacilli heterofermentativi obbligati, *Lb helveticus*, L/D-lattato, succinato, piruvato, L- leucine aminopeptidasi, rapporti isotopici δ^2H, δ^{13}C, δ^{15}N e δ^{34}S.
Nella terza parte, i parametri scelti sono stati misurati su 110 campioni di formaggio Emmental d'inverno e 73 d'estate raccolti in sette regioni, Svizzera, Francia Savoia, Francia Bretagna, Francia Est-Centrale, Germania del Sud, Austria e Finlandia. I dati analitici sono stati trasformati mediante analisi statistica multivariata in modo da classificare i campioni secondo la loro origine geografica. L'analisi discriminante (DA) e la rete artificiale di neuroni hanno fornito risultati comparabili. La DA permette una riduzione dei numeri di fattori grazie ad una "stepwise backward elimination". In un modello che include soltanto undici fattori, una classificazione corretta al 95% nelle sette regioni di origine è stata ottenuta con una validazione incrociata tipo "Jackknife". Cinque campioni svizzeri su 70 sono stati mal classificati. Per migliorare la classificazione di quest'ultimi, è proposto un modello con un approccio per paia (Svizzera, *vs* una sola regione straniera alla volta). Questa procedura ha permesso di riconoscere tutti i campioni svizzeri utilizzando quindici parametri.
Nella quarta ed ultima parte, una scelta puntuale è stata applicata ad altri due tipi di formaggio, il formaggio Raclette e la Fontina. Il Raclette Suisse® ed il raclette francese hanno facilmente potuto essere discriminati tramite il contenuto di calcio ed i quattro rapporti degli isotopi stabili δ^2H, δ^{13}C, δ^{15}N e δ^{34}S. Il Fontina DOP è un formaggio al latte crudo. Il suo tasso di fosfatasi alcalina attiva è conseguentemente nettamente più elevato di quello dei formaggi di tipo Fontal che sono imitazioni industriali fabbricate con latte pastorizzato. L'ultimo capitolo costituisce una linea direttiva dedicata ai chimici cantonali per l'autenticazione dell' Emmental Switzerland™. I rischi incorsi dai principali tipi di formaggio svizzero in relazione ad una dichiarazione infedele d'origine sono pure discussi.

PREAMBLE

What do electronic noses and Emmental cheese have in common? In 2000, the Ph.D. thesis of E. Schaller, conducted at the FAM and dedicated to the application of MS-based electronic noses to dairy products, highlighted the limits of this analytical tool. The major drawback lay in the system's poor sensitivity to low and middle volatile compounds. There was therefore a need to develop fast preconcentration techniques coupled to electronic noses. A new Ph.D. project was started by J.O. Bosset and funded by the CTI in collaboration with SMart Nose Ltd. (Marin-Epagnier, Switzerland) selected as industrial partner. I saw a chance to combine my interest in analytical chemistry and the dairy sector. In fact I was anxious to supplement my scientific education as a chemist with the practice of cheese making in the highlands during the summer months, where I found a healthy balance to my scientific activity. In September 2000, I started by writing a review article on preconcentration techniques. The use of canned processed cheeses as standard materials in volatile analysis was the subject of a second article. These processed cheeses were then used for testing data transferability between two electronic noses of the same type. In the last publication of part I, two recent preconcentration techniques were compared for application to the SMart Nose.

Parallel to this CTI-project, another project dealing with the authenticity of Swiss cheeses had also been launched by J.O. Bosset. It was funded by the Swiss Federal Office of Public Health (SFOPH) in collaboration with the Swiss Federal Dairy Research Station (FAM). I was extremely interested in this project and as no candidate had been found, I decided to modify my Ph.D. topic. This explains the abrupt change of subject between part I and the rest of the thesis, which is mostly dedicated to the geographic origin of Emmental cheese. However, I found that both subjects could be related and I tried to integrate the work done during the first 6 months into this new subject.

Volatile compounds (project CTI) are not only responsible for the aroma of a cheese sample, but they could also be used as indicators of origin (project SFOPH). The research carried out on preconcentration techniques for the electronic nose should therefore be applied for the authentication of Emmental cheese, as shown in Chapter 5. An attempt was made to analyse the samples with both an electronic nose coupled to a new preconcentration technique (Annex A) and a new gas chromatograph equipped with a thermodesorption system (Annex B). Unfortunately this work could not be finished due to technical and time problems.

I naturally invested most of my time in parts II, III and IV. Food authentication has gradually become a topic of great interest in Western Europe and many research groups are currently working on various foods. The scientists generally work in specialist laboratories with one or two high-tech analytical instruments and try to apply their techniques to a given problem of authenticity. Working at the FAM gave me the opportunity to use a totally different strategy. The FAM does not possess the instruments for such typical analyses of origin authentication, e.g. Isotope Mass Ratio Spectrometry (IRMS), Nuclear Magnetic Resonance (NMR) or Inductively Coupled Plasma Mass Spectrometry (ICP-MS), but it does have an immense knowledge of cheese . Instead of focusing the research on one or two methods, a screening test of more than 20 methods was preferred (Part II). Only the excellent collaboration and confidence gained as part of this work with external laboratories, in Switzerland and abroad, made it possible to carry out a project with so many facets. The information obtained from the screening test was crucial for selecting the methods to be used in the final study. The results from the latter were then integrated into various multivariate statistical models for origin assignment (Part III). In part IV, a feasibility study for the authenticity of Raclette Suisse® and Fontina PDO was carried out in the form of a targeted screening of methods. The last

chapter, acting as conclusion, was dedicated to the development of a real case scenario for the food control laboratories and suggests guidelines for the authentication of Emmentaler Switzerland™. A discussion was also held on the risk of origin mislabelling for other cheese types.

The global approach strategy and the originality of the work aroused great interest in the scientific world. I had the opportunity to present my results in different European countries (Annex C) and I hope the reader will enjoy reading my thesis as much as I enjoyed working on it during these three years of research.

Laurent Pillonel

Un voyage se passe de motif, il ne tarde pas à prouver qu'il se suffit à lui-même. On croit qu'on va faire un voyage mais bientôt c'est le voyage qui vous fait... ou vous défait.

Nicolas Bouvier

01.09.2000-29.02.2001: CTI, project 4614.1
01.03.2001-31.12.2004: SFOPH, contract no. 00.001493/01.001323/02.001425

CHAPTER 1

Rapid preconcentration and enrichment techniques for the analysis of food volatiles: a review

L. Pillonel, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Food Science and Technology, 35, 1-14 (2002)

Preconcentration and enrichment techniques for volatile compounds are reviewed. Only rapid and cheap methods, applicable to food analysis are considered. The methods are grouped into two sampling modes: (i) dynamic headspace or purge-and-trap (solid, liquid, cold or solvent trap and direct thermal desorption), and (ii) SPME (including SBSE, HSSE and SPDE). All these extraction methods can be coupled to either gas chromatographs or electronic noses. The description of these techniques is made in the sense of their historical development. Where comparative studies have been carried out, advantages and disadvantages of the methods have been cited. In the last part, two tables give a representative but not exhaustive list of the preconcentration methods used for the analysis of dairy products.

Keywords: volatile compounds; headspace analysis, preconcentration; food analysis

Introduction

The industrial world is characterised by the omnipresence of consumer goods, especially of manufactured food products. The very high production rates and the need for a standard product stable in its aroma require the use of efficient analytic tools for the characterisation of such products. Prior to the analysis of volatile compounds in food, it is most of the time necessary to preconcentrate them as they are diluted in the surrounding headspace. The preconcentrated analytes are then commonly injected in a gas chromatograph. For industrial applications such as quality control, process monitoring, shelf-life and authenticity investigation the use of a rapid and cheap pattern recognition analysis such as is delivered by a sensor array (electronic nose) may be more appropriated (Schaller *et al.*, 1998). The common electronic noses developed within the last few years have however turned out to have some limitations; they are non specific and may suffer from poisoning effects and lack of sensivity (Schaller *et al.*, 1999). The new generation of electronic noses based on a mass selective detector is claimed to overcome the main limitations of the previous models using chemical sensors. Although the MS-based technology has already been applied to different food products (Vernat and Berdagué, 1995, Dittmann *et al.*, 1998; Marsili, 1999*a*, 2000; Schaller *et al.*, 1999; Dittman *et al.*, 2000; Feldhoff *et al.*, 2000; Schaller *et al.*, 2000; Ampuero *et al.*, 2001), the sensivity of the current MS detector seems too low for certain applications such as cheese discrimination (Schaller *et al.*, 1999). The development of a reliable preconcentration step would be decisive for a breakthrough of this technology onto the market.

The objective of this paper is to review automated, rapid, cheap and easy-to-use preconcentration techniques adequate for volatile compounds as quoted in the literature. Most of them have already been applied to food analysis (dynamic headspace with trapping on solid sorbent or cold trap, SPME). Others such as dynamic headspace with trapping into liquid polymer have not yet found application in flavour analysis, but present promise for the future. The newest technologies SBSE (Stir Bar Sorptive Extraction), HSSE (Headspace Sorptive Extraction) and SPDE (Solid Phase Dynamic Extraction) are also discussed. So far, except for a single assay with dynamic headspace, only SPME has been tried as a preconcentration technique for MS-based discrimination.

Dynamic Headspace

Principle

The dynamic headspace technique is a very popular method for analysing volatile compounds in food (Canac–Arteaga, 2001), as well as in other products

†To whom correspondence should be addressed. E-mail:. jacques-oliver.bosset@fam.admin.ch

such as airborne pollutants (Burger, *et al.*, 1991) and VOCs in water (Noij *et al.*, 1987). When an inert carrier gas is bubbled through a liquid, the technique is also referred to as 'purge-and-trap'.

The sample may be warmed by an electrical heater (in most cases) or by microwaves (Conte *et al.*, 1996) to increase the fugacity of volatile compounds. The stripped volatiles are then trapped on a solid or liquid sorbent, in a cold-trap or in a solvent. This step can be carried out in an open- or a closed-loop (Nùñez *et al.*, 1984). In the open-loop configuration, the nontrapped molecules are eliminated. In the closed-loop method, the gaseous phase flows through the sample and the trap in a closed circuit (Grob, 1973).

After adsorption onto a sorbent, the trapped compounds are desorbed by heating and then cryofocused at the head of the GC-column (for cryofocusing methods, see Kolb, 1999). The analytes can also be eluted with a solvent (Olafsdottir *et al.*, 1985; Burger and Munro, 1987; Rizzolo *et al.*, 1992; Krumbein and Ulrich, 1996), but recovery is not always satisfactory (Boren, 1985). In addition, thermal desorption shows the following advantages: (i) analysis of 100% of the trap content (instead of an aliquot part), (ii) no solvent peak, (iii) no waste and (iv) no contamination from solvent. Moreover, elution with a solvent is difficult to automate (Hallama *et al.*, 1998).

Adsorption on solid sorbents

The stripped volatile compounds are trapped on a porous material packed in a cartridge or in short columns. Some common sorbents such as activated charcoal and silica gel are less suitable for 'purge-and-trap' due to their high surface activity. The high temperatures required for the desorption can lead to sample degradation (Hori *et al.*, 1989). The most commonly used sorbents for trapping airborne volatiles have been reviewed in Harper (2000). They belong to one of those four main groups: Tenax TA (2,6-diphenyl-p-phenylene oxide polymer), Chromosorb 106, graphitised carbons (Carbotrap B, C) and carbon molecular sieves (e.g. Carbosieve S-III, Carboxen 1000, Carboxen 1003, Spherocarb, Anasorb). Nùñez *et al.* (1984) described in detail the adsorbent materials available.

In a study into water adsorption capacities (Helmig and Vierling, 1995), it was found that Tenax TA, Tenax GR and Carbotrap had a very low water uptake of < 1–3 mg of water/g of adsorbent. This property is very important for aqueous samples.

The four commonly used materials, Tenax TA, Tekmar No.8, Vocarb 3000 and Vocarb 4000, have been compared using the volatiles from whey protein concentrate (Laye *et al.*, 1995). Tenax TA was most effective for recovering aldehydes, esters, aliphatic hydrocarbons and aromatic hydrocarbons whereas Vocarb 3000 was most effective for trapping alcohols and ketones. Tenax TA is currently the porous polymer of choice for the analysis of volatile flavours because of (i) its high thermal stability (up to 450 °C), (ii) its relatively low water retention and (iii) its low bleed.

Breakthrough volumes. A crucial point of the adsorption is the loading capacity of the trap. When the latter is saturated, volatiles will break through. The volume of gas which causes the trap to be overloaded is called the breakthrough volume. This breakthrough can however be partially avoided by carefully choosing the most adequate trapping material and working conditions (Nùñez *et al.*, 1984; Laurens and Rohwer, 1997). With porous adsorbents, highly volatile compounds mostly have a small breakthrough volume, which can be problematic for some specific applications. References relating to breakthrough characteristics on different sorbents are quoted by Feng and Mitra (1998). For many compounds, breakthrough volumes on Tenax TA, Tenax GR, Carbotrap, Carbotrap C, Carboxen 569, Carbosieve SIII and glass beads can be found at http://www.sisweb.com/index/referenc/resins.htm.

Multi-bed adsorbents. For trapping volatile compounds with very different properties, multi-bed adsorbents can be helpful (Hastenteufe and Betz, 1992). Typical combinations include Tenax TA or graphitised carbon and carbon molecular sieve (Harper, 2000). The weaker sorbent (Tenax TA) is placed first to trap the heavier molecules and the lighter compounds are retained on the stronger sorbent located in second position. Desorption always takes place in the reverse direction to the adsorption step (Guillot *et al.*, 2000).

Commonly used equipment. Several automated dynamic headspace systems incorporating a solid sorbent are commercially available (e.g. Rektorik, Tekmar, HP). The Rektorik system desorption is by a microwave oven, which requires the use of one of two available electrically conducting adsorbents: graphite powder or charcoal. The desorption is so rapid that cryofocusing is no longer necessary at the head of the separating column. The system was developed and described by the inventor (Rektorik, 1985).

This system is however less reproducible, less sensitive and more subject to artifacts than the others (Imhof and Bosset, 1991). The latter allow many different materials to be used for the trap thus making it possible to combine two or more different trapping materials as described above.

Alternative equipment. Sucan and Russell (1997) and Villaseñor *et al.* (2000) used a Tenax trap identical in size to a GC injector splitless liner. After off-line adsorption, the trap was placed directly into the injector for desorption, thus eliminating any transfer line leading to possible memory effects (Hartman *et al.*, 1991). However it required cryofocusing for a sharp injection. Capillary tubes have also been packed with a sorbent (Mitra *et al.*, 1996). If the trap is connected directly to the head of a GC column, it is called 'on-line microtrap' (OLMT). OLMT produces narrow peaks without any cryofocusing. However microtraps are prone to break through as they contain only a small quantity of adsorbent. Larger diameter traps with more adsorbent

would reduce break through by increasing the loading capacity but they also would generate a broader injection band. To avoid this problem, Feng and Mitra (1998) used a two-stage microtrap packed with Carbopack C. First the volatiles were trapped on a larger diameter tube (1.1–1.3 mm i.d.) called 'retention trap'. The latter was desorbed and the volatiles were refocused onto the second microtrap with a smaller diameter called 'injection trap'. After a few seconds' delay, the injection trap was desorbed to generate a sharp band injection.

Adsorption on solid traps has some serious problems such as the occurrence of artifacts due to catalytic activity of the adsorbent, discrimination and displacement effects due to selective adsorption and irreversible adsorption of higher mw and/or polar molecules (Bicchi *et al.*, 1989). A trapping-desorption system based on partition chromatography can significantly reduce all these problems.

Sorption into liquid coatings

The distinction between adsorption and sorption (absorption, dissolution) is not always clearly drawn in the literature. However the difference in mechanism is fundamental. In the case of absorption, the analytes are sorbed (dissolved) into the bulk of a liquid phase, while in adsorption, the analytes will remain on the surface of the porous material. Preconcentration by sorption into a film of liquid polymer has a number of advantages over adsorption onto an active packing material. When e.g. the surface of a porous adsorbent is already occupied by a heavy substance, no lighter molecules have any chance of being adsorbed. In the absence of heavier molecules, small molecules are retained, but are displaced as soon as heavier substances enter the trap. On the other hand, apolar liquid coatings are not subject to competition. Moreover, high molecular weight components can still be desorbed at mild temperatures and no catalytic activity is observed.

Another important advantage of the liquid polymer is that the equilibrium distribution coefficient (K) of an analyte between the gaseous phase and the coating can be accurately estimated by its retention index on a column coated with the same polymer as the fibre (Pawlyszin, 1977). The coating/water distribution can be calculated from the coating/gas distribution constant and the gas/water distribution constant (Henry's constant). This is particularly useful for calculating breakthrough volumes, thus eliminating the need to determine the breakthrough volume for each individual component as is the case with classical adsorbents. For packed polydimethysiloxane (PDMS) traps, it was demonstrated that breakthrough volumes of alkanes can be calculated from theoretical equations (Baltussen *et al.*, 1997*a*).

Coated capillary traps. Grob and Habich (1985) were pioneers in developing open tubular columns coated with a film of liquid stationary phase (almost exclusively cross-linked polydimethylsiloxane, PDMS) for trapping volatiles. Their idea was to create a trap that permits a very rapid desorption, showing good blanks (the degradation products formed from PDMS can easily be identified by MS-detector), not subject to the formation of artifacts (no catalytic activity) and having a flow compatibility with capillary columns. Also, the water uptake of PDMS is so low that no additional water management is needed. The trapped material can be released either by thermal desorption or liquid extraction (Blomberg and Roeraade, 1987).

As pointed out by Grob and Habich (1985), the weakness of these traps is their low loading capacity due to the reduced amount of trapping material. However the loading capacity can be improved either by using a longer trap or a thicker coating (very thick-film capillary).

The first variant was effected by coating long capillary traps (up to 3 m) in a static mode and using them for the determination of plant volatiles (Bicchi *et al.*, 1989). Such capillaries, however, become impractical as they are not easy to handle and cryofocusing is necessary. These traps were desorbed either by installing them in an electrically heated coiled stainless-steel tube (Burger and Munro, 1986) or more simply using two GC ovens (Blomberg and Roeraade, 1987), the first one for the trap and the second one for the analytical column. Tuan *et al.* (1997) employed a commercially available capillary (5 m × 530 μm × 5 μm CP Sil-5 CB) to trap volatile organic compounds (VOCs) in air and to analyse them on-line, eliminating the need for a refocusing step by use of a new sample preconcentration technique. They exceeded the breakthrough volume in order to get a homogeneous dissolution of all volatiles along the trap (equilibrium absorption), thus providing the means for a homogenous desorption over time. As any part of the desorption flow is representative of the entire sample, this made possible the injection of only a 'slice' of the enriched sample plug into a portable micro high speed GC. This method could be very useful for field analysis.

To increase the thickness of the stationary phase film, Blomberg and Roeraade (1988) immobilised a thick prepolymer film formed on the column wall during dynamic coating. In this way they achieved coatings up to about 100 μm in thickness. In a further publication (Blomberg and Roeraade, 1990), they showed how to improve the solvent resistance of thick film traps by cross-bonding the polymer to the column wall (analysis of drug volatiles). Burger *et al.* (1991) even produced and used an open capillary trap coated with an ultra-thick film of 145 μm. However, even in their system, highly volatile compounds were not effectively retained.

The technique of dynamic coating requires considerable technical skill which is not always available. An ingenious way of preparing capillary traps with commercially available materials was found (Burger *et al.*, 1990): a silicon rubber tube was inserted into a 0.53-mm i.d. fused silica capillary by an innovative stretch and freeze method. The traps so obtained were suitable both for the determination of volatiles in headspace and in water samples (Burger and Le Roux, 1993).

Multi-channel traps. Increasing either capillary length or film thickness is a tedious process. Moreover it allows only low sampling flow rates (typically of the order of 10 mL/min) and normally requires a further refocusing step. To overcome these problems, Ortner and Rohwer (1996) designed a thick film silicone rubber trap within a novel multi-channel configuration. This device consists of several silicone rubber tubes in parallel fitted in a glass tube. They obtained very promising results for breakthrough volumes and thermal desorption characteristics for semivolatile components. A similar design has also been tested for the concentration of odorous compounds in water (Hassett and Rohwer, 1999). But even with this new configuration the maximum admissible flow rate for quantitative trapping is only about 15 mL/min. A large scale device has also been designed for air pollution studies (Krieger and Hites, 1992). It was made of a bundle of 120 DB-1 coated capillaries, achieving sampling flow rates up to 1.5 L/min in total.

Packed traps. Despite several clear advantages of open tubular traps (OTT) *vs.* classic adsorbents, they never gained widespread acceptance. This is due to their limited loading capacity and above all to their very low sampling rate. The main reason for developing open capillary traps rather than packed traps was to keep the flow compatibility between the trap and the GC column. Baltussen *et al.* (1997*a,b*) used a packed bed of PDMS as a sorption device and tested two methods for solving the flow incompatibility problem. In the first one, called split desorption, the flow exiting the trap was split allowing only a few mL/min to enter the column. This method resulted in a significant loss of sensivity. In the second method, called splitless desorption, the system contained two split points and a capillary cold trap placed between these split points. The analytes were first desorbed splitless onto the cold trap. Thereafter the analytes were transferred from the cryotrap onto the GC-column.
One of the main advantages of packed traps is their high sampling flow rate up to 2.5 L/min. In a further paper (Baltussen *et al.*, 1998), they compared the packed PDMS traps with four different adsorbents: Carbotrap 300, Tenax TA, Chromosorb 101, Lichrolut EN. For both polar and nonpolar analytes, the PDMS packed trap was better than or comparable with the tested adsorbents. In particular, PDMS showed better performances for analytes such as triethylamine, butanone, diacetyl, nicotine, and acetic acid.

Solvent trapping. Instead of being absorbed into a liquid coating, the volatiles can be dissolved (i.e. retained) in a solvent. The carrier gas loaded with the volatile components flows through a cooled U-tube filled with about 150 μL of an appropriate solvent. The solvent with the entrapped volatiles is then injected into the analytical device (Jurisk *et al.*, 1991).
Solvent trapping is difficult to automate and, as for solvent desorption, allows only the injection of an aliquot or requires a time-consuming solvent evaporation leading generally to large losses. Liquid trapping is rarely used.

Cold trapping
The stripped volatile compounds are trapped in a cold trap, mostly a capillary tube, cooled with either liquid nitrogen or solid carbon dioxide. To prevent clogging of the trap, water has to be removed very efficiently from the charged carrier gas (e.g. with a water condenser) before entering the cooling trap. Badings and De Jong (1985) tested the efficiency of different capillary traps using both mechanisms. They compared deactivated open capillary traps (pure condensation), open capillary traps coated with CP-sil 5 CB or Al_2O_3/KCl (sorption) and capillary traps packed with Tenax TA or Chromosorb 101 (adsorption). The results showed that the use of capillary traps combined with a liquid coating or an adsorbent considerably increased the efficiency of cold trapping. Cryogenic focusing is therefore recommended for analysis of samples containing highly volatile components (Kolb, 1999).
The three main advantages of cryogenic trapping vs. other trapping techniques are: (i) no artifact from thermal desorption, (ii) no carry over and (iii) no breakthrough problem. However, the additional equipment needed to handle the cryogen is quite expensive and very sensitive to water. Moreover, it takes quite some time to warm up the trap and the adjacent fittings as well as to cool them down again (Wampler, 1997).

Direct thermal desorption
In the direct thermal desorption (DTD) technique, a small amount of an homogeneous sample is placed directly in a thermal desorption unit. The desorbed volatiles are stripped into a cold trap placed in the injector. Description and various applications of this system to food products have been listed (Hartman *et al.*, 1991; Hartmann *et al.*, 1992; Manura and Hartman, 1992; Grimm *et al.*, 1997). DTD has been compared to static headspace and SPME for dried and fresh basil, pine needles and coffee (Pfannkoch, 2000): SPME with a PDMS coating provided 10–50 × higher sensivity than static headspace while DTD showed an even higher sensivity than SPME by a factor of 50–100. Though this method is strictly limited to samples with a low water content, fresh basil could be analysed by replacing the cold trap with a liner packed with Tenax TA cooled to 5 °C. Good results have been obtained for a blue cheese by mixing it with an hygroscopic salt (sodium sulphate) prior to desorption (Valero *et al.*, 1997).

Water interferences
One of the main problems of dynamic headspace is the adsorption of water on the trap. Water can be a major source of trouble if the sample contains it in high amounts (beverage, aqueous sample, foods). In the case of adsorption of the analytes onto a solid sorbent, a certain percentage of water will also be retained. The

water subsequently released during desorption may clog up the cold trap or the cryofocusing trap at the head of the column. Therefore efforts have been made to develop a sorbent with low water affinity. But even in the case of the hydrophobic Tenax TA or Carboxen 564, the trapping of water can cause trouble when the relative humidity of the sample is above 90% (Guillot *et al.*, 2000). Too much water entering the system can also damage the MS-detector (Hinshaw, 1990) and induce a modification of the spectrum, rendering identification difficult (Westendorf, 1992). Therefore it may be necessary to introduce one or a combination of the following solutions:

Dry purge: immediately before desorption, the solid trap can be flushed with an inert dry gas (helium) to remove part of the water. A part of the highly volatile compounds will inevitably be lost. The effects of a dry purge have been described for the volatile fraction of a cheese by Canac-Arteaga *et al.* (1999*a*). Dry purge is the most widely used method for water removal from solid sorbents.

Condensation: water can be condensed in a cold water trap (condenser) held at −10–−15 °C and located between the sparging vessel and the trap. This technique can be applied to solid trap (Canac-Arteaga *et al.*, 1999*b*) as well as to cold trap systems (Badings and De Jong, 1985; Wood *et al.*, 1994). Noji *et al.* (1987) investigated the efficiency of the cold water trap and its influence on overall recovery. They found that polar and high boiling non polar solutes were partly or completely lost due to a reflux effect, limiting this method to apolar low boiling analytes. Similar results were obtained during investigation of cheese volatiles (Canac-Arteaga *et al.*, 1999*b*). The insertion of a condenser improved the overall quality of the chromatographic signal, but caused major changes in the chromatographic profile of the cheese. Werkhoff and Bretschneider (1987) used a reflux condenser cooled to between +5 and +10 °C, limiting the loss of analytes but increasing water uptake from the trap.

Hygroscopic trap and drying of the sample: A cartridge packed with hygroscopic salts can be placed in front of the trap to absorb water (Ambrus and Thier, 1986; Tangerman, 1986). It was shown that of the three hygroscopic salts, sodium carbonate, magnesium sulphate and calcium chloride, only sodium carbonate seemed not to absorb significant amounts of the volatile components (Guillot *et al.*, 2000). Phosphorus pentoxide is also very effective for selective absorption of water (Valero *et al.*, 1997). (References to further drying agents are given in Helmig and Vierling, 1995; Kolb, 1999; Canac-Arteaga, 2001).
If the sample is not an aqueous solution, it is possible to mix it directly with some hygroscopic salt. Villaseñor *et al.* (2000) and Valero *et al.* (1997) dried cheese samples with Na_2SO_4. Canac-Arteaga *et al.* (2000) compared several hygroscopic salts for drying cheese samples. Calcium chloride and potassium carbonate lowered the relative humidity of the headspace of the cheese most effectively but also strongly modified the chromatographic profiles. Sodium chloride and sodium sulphate had a reduced drying effect but delivered chromatographic profiles close to the references. Some salts also caused a major pH modification, resulting in a release of different acidic and basic compounds such as amines and volatile fatty acids.

Permeation: water from the sample can diffuse through the wall of a drying tube while the analytes stay in the carrier stream. Nafion is the most widely used tubing for the purge-and-trap technique (Simmonds, 1984; Noij *et al.*, 1987; Mc Clenny *et al.*, 1989; Pankow, 1991) and for air samples (Borgerding and Wilkerson, 1996). This method is less attractive due to some selectivity of the Nafion membrane. It has been found that light, polar and oxygenated compounds are partially or completely removed from the stream (Burns *et al.*, 1982; Coohran and Henson, 1988; Pankow, 1991). Memory effects of Nafion driers have also been documented (Baker, 1974; Noij *et al.*, 1987).

Solid phase microextraction (SPME)

Solid phase microextraction makes a rapid sample preparation possible both in the laboratory and on-site where the system to be investigated is located. The first SPME devices were developed by Arthur and Pawliszyn (1990) as a preconcentration technique for the analysis of water pollutants. Within recent years, however, SPME has become a very popular tool and has extended its applications to numerous other fields. Among the 416 publications concerning SPME quoted under http://www.cm.utexas.edu/brodbelt/Brodsite/spme_refs.html (update 5/99), just under 40% were found to deal with environmentally relevant problems (**Fig. 1**). Food and botanical applications accounted for about 20%.
Many different implementations of SPME have been developed (Lord and Pawliszyn, 2000), most of them being limited to laboratory uses. This section will essentially deal with the well-known syringe-based SPME device supplied by Supelco.

SPME and SPDE configurations

SPME can be performed either in the direct extraction mode or in a headspace configuration. In the former mode, the coated fibre is directly immersed into the sample matrix (e.g. aqueous solution) where the analytes are extracted. An efficient agitation is required to counteract the low diffusion coefficients of analytes in liquid matrices and to facilitate a rapid extraction.
In the headspace mode, analytes first have to diffuse from the matrix into the air and then from the air into the fibre coating. This technique is necessary for analysing volatiles from highly complex matrices (as is mostly the case for food) that could damage the fibre. The extraction kinetics can be improved either by using very efficient agitation, by increasing the extraction temperature or by both together. Elevating sampling

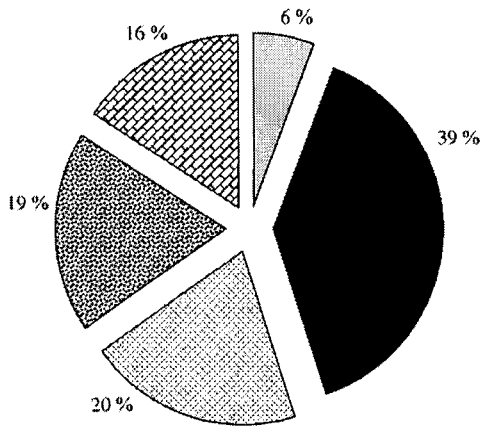

Fig. 1. Distribution of publications dealing with SPME; (□), General informative articles; (■), Environmental applications; (▨), Food and botanical applications; (▩), Clinical and forensic applications; (▧), Fundamental developments

temperature will however decrease the amount of extracted analytes in the fibre at equilibrium. To prevent this loss of sensivity, the coating can be cooled simultaneously with sample heating (Zhang and Pawliszyn, 1995; Eisert and Levsen, 1996). With an internally cooled SPME device, Zhang and Pawliszyn achieved a quantitative extraction of benzene, toluene, ethyl benzene and xylene from complex matrices such as sludge, waste water and soil.

For the simultaneous analysis of gases and less volatile analytes, the headspace SPME technique can be combined with static headspace sampling. A coated fused-silica fibre, mounted on a gas-tight syringe, is used for this purpose (Zhang and Pawliszyn, 1996). When the fibre is withdrawn into the syringe needle, a certain volume of gas is also introduced into the gas-tight syringe through the needle opening. With this technique the analysis of a whole range of compounds can be carried out with a single injection. The absorption phase can also be coated on the inside wall of the needle with the advantage of eliminating the mechanical damages due to the 'in and out' movement of the coated fibre in the needle. This configuration has newly been manufactured by Chromtech (Idstein, Germany) and is sold under the name SPDE (Solid Phase Dynamic Extraction) (Berg, 2000).

For the determination of very high boiling analytes from dirty matrices neither direct extraction nor headspace are suitable. The analyte concentration is too low in the headspace and the fibre can be damaged by high molecular weight compounds from the matrix. The indirect SPME extraction through a membrane offers a promising alternative. The fibre of a SPME device is placed inside a hollow cellulose membrane, allowing only analytes with a molecular weight of less than 1000 Da to diffuse through it (Zhang *et al.*, 1996). In this way, the fibre is protected but extraction will last much longer. For compounds with molecular weight lower than phenanthrene, the headspace approach is still more efficient. The development of membranes with different cut-off values should broaden the application field of membrane protected SPME sampling.

Fibre coatings

Six specific fibres with various film thicknesses and coating materials have been developed for a wide range of applications. Coatings can be classified into two groups: the pure liquid polymer coating such as polydimethylsiloxane (PDMS) or polyacrylate (PA) and the mixed film, containing liquid polymer and solid particles such as Carboxen-PDMS, Divinylbenzene (DVB)-PDMS, Carbowax-DVB and DVB-Carboxen-PDMS. The mixed films combine the absorption properties of the liquid polymer with the adsorption properties of porous particles.

Pure PDMS is strongly hydrophobic and was originally designed to extract pollutants from aqueous samples. Polyacrylate is currently the most polar coating available for SPME and is applied to the extraction of more polar components, such as fatty acids (Pan *et al.*, 1995) and reduced sulphur compounds (Shooter *et al.*, 1999).

Carboxen is a carbon molecular sieve containing macro-, meso- and micropores and is used in combination with PDMS. The pore size does not allow the bigger molecules to enter the micropores (where the interactions are the strongest), so that the combination of Carboxen and PDMS improves the extraction for small molecules. Shirey (2000) compared all six available fibres for the extraction of low-molecular-weight analytes. In many cases, the responses for the analytes extracted with the Carboxen-PDMS fibre were over 200 times greater. Other authors came to similar conclusions (Popp and Paschke, 1997; Azodanlou *et al.*, 1999). An alternative is given with the DVB-PDMS coating. The divinylbenzene solid polymer has larger pores than Carboxen and is thus better adapted for the extraction of bigger molecules such as aniline derivates (Müller *et al.*, 1997; Debruin *et al.*, 1998). The newest combination of DVB, Carboxen and PDMS now offers a full screening capacity. The first layer is made of PDMS/Carboxen and is covered with a second layer made of PDMS/DVB. The small molecules, having a higher diffusion coefficient, reach the inner layer faster where they are adsorbed onto the Carboxen. The heavier molecules are retained in the outer of DVB layer. Desorption is also facilitated with this configuration. However the DVB/Carboxen/PDMS fibres are difficult to produce and they are sometimes delivered with visible fissures in the coating. The last fibre available, Carbowax-DVB, is the most polar fibre of the second group. **Table 1** lists the recommendations of the manufacturer for the best choice of fibre.

The effect of fibre polarity has been demonstrated in several studies comparing fibres for the extraction of polar analytes such as phenol (Bartak and Cap, 1997), triazine herbicides (Ferrari *et al.*, 1998) and barbiturates (Hall and Brodbelt, 1997). However, the fibre polarity showed only a minimal effect on the extraction of low-molecular-weight molecules (Shirey, 2000). It seems that the porosity and the film thickness rather than the fibre polarity influence the extraction of small molecules.

To enhance the extraction efficiency of trace compounds in organic solvents, a new generation of SPME fibres is

Table 1 Recommended application fields for the different SPME fibres

Fibre	Application
PDMS 100 μm	Volatiles
30 μm	Nonpolar semi-volatiles
7 μm	Nonpolar high molecular weight compounds
PA	Polar semi-volatiles
PDMS/DVB	Volatiles, amines, nitroaromatic compounds
Carbowax/DVB	Alcohols and polar compounds
Carboxen /PDMS	Gases and low molecular weight compounds
DVB/Carboxen/PDMS	Volatile and semi-volatile flavours and odours

being developed. The polyacrylate coatings used so far absorb too much solvent for such analyses resulting in an overloaded chromatographic column. To limit solvent uptake, the fibre has been coated with a Nafion layer. This promising new type of fibre is still under development (Gorecki *et al.*, 1998; Haag, 1999).

Sampling conditions

The type of extraction (direct or headspace) has an appreciable influence on the results. As a general guideline, it can be said that ambient headspace and immersion techniques are best for extracting nonpolar analytes while heated headspace and immersion are the best techniques for extracting polar analytes. The type of extraction affects the results to a greater extent with a pure absorbent-type fibre than with adsorbent-type fibres such as the Carboxen-PDMS (Shirey, 2000).

The salting out effect also gives interesting results in increasing the amount of extracted analytes, both in headspace or in immersion mode. Four types of behaviour can be differentiated (Yang and Peppard, 1994): (a) extraction increases with increasing salt concentration, (b) extraction increases initially and then levels off at higher salt concentration, (c) extraction increases first and then decreases with increasing salt concentration, (d) extraction decreases with increasing salt concentration. The last case is fairly rare but shows the importance of carefully checking the salting out effect. As a general guideline, it can be said that the addition of salt greatly enhances the extraction rate of polar analytes such as isopropylamine, isopropanol (Shirey, 2000), carboxylic acids (Pan *et al.*, 1995; Harmon, 1997) and phenols (Buchholz and Pawliszyn, 1994). The effect is slightly weaker for less polar molecules such as benzene, toluene, ethylbenzene and xylene (Djozan and Assadi, 1997). Other applications of the salting out effect are quoted for acetaldehyde (Huynh and Vu-Duc, 1998), terpenoids (De la Calla Garcia *et al.*, 1998), aromatic amines (Müller *et al.*, 1997), and Maillard reaction products (Colemann, 1996). The addition of salt may not always be suitable for high molecular weight molecules as the salt may cause the analytes to adhere to the glass vials (Degorce-Dumas *et al.*, 1986; Yang and Peppard, 1994). The most commonly used salts are sodium chloride and sodium sulphate (Canac-Arteaga, 2001). The salting out effect is also commonly used with the purge-and-trap method. For example potassium carbonate was used in yoghurt (Urbach, 1987) and in milk samples (Boyd–Boland *et al.*, 1996) but the reproducibility was poor. Ammonium sulphate was added to yoghurt (Urbach, 1987).

The headspace volume should be kept as small as possible as the extraction yield decreases with increasing headspace volume (dilution effect), the amount of liquid phase being kept constant (Yang and Peppard, 1994).

Stirring liquid samples can also be useful for headspace sampling. The equilibration time for less volatile compounds (e.g. PAHs) is substantially reduced by stirring while it only has a minimal effect for highly volatile compounds (Matich, 1999).

If a compound with a high partition coefficient $K_{\text{fibre-air}}$ is present at high concentration, it may induce a competition effect, possibly skewing results. Short-time sampling can help to avoid this (Roberts *et al.*, 2000).

Microwave assisted extraction (MAE) can also be applied in combination with SPME. Solid samples are grounded and mixed with water (the best solvent for this purpose) before treatment with microwaves. An application with potato chips was proposed by Wang *et al.* (1997).

In field or in off-line sampling, it is very important to preserve extracted analytes on the fibre for longer time periods and to protect the coating from contamination. As organic analytes can dissolve in polymeric material, capping the needle with a polymeric septum is only effective for a short time (Chai and Pawliszyn, 1995). A more appropriate approach is based on metal to metal seals (Pawliszyn, 1997).

New generation of SPME implementations

The main limitation of SPME is the relatively small amount of sorbent available on the fibre, typically of the order of 0.5 μL (100 μm PDMS fibre). This means that in a 10 mL aqueous sample, an analyte with a $K_{\text{water-fibre}}$ value as high as 20,000 will be extracted with only 50% recovery. To circumvent this problem, Baltussen *et al.* (1999) developed a novel technique called Stir Bar Sorptive Extraction (SBSE). SBSE approaches the high enrichment factors of PDMS-packed beds but with the application range and the simplicity of use of SPME. Stir bars are commercially available under the name of 'Twister' (Gerstel, Müllheim a/d Ruhr, Germany). They consist of a glass-coated metal bar (1.0–4.0 cm in length) with a coating of 50–200 μL of PDMS. They are exposed to an aqueous sample and stirred for a predefined time. Desorption takes place in an automated thermal desorption unit followed by cryogenic refocusing at the

Table 2 Some applications to dairy products in a dynamic headspace mode

Application	Sort	Author	Preconcentration	Water management	Detector	Pattern recognition
Cheese	Parmigiano Reggiano Mahón, Fontina, Comté Beaufort, Appenzeller	Bosset and Gauch (1993)	Carbosieve SIII/Carbopack B60/80	Dry purge	GC-FID	No
	Mini-Babybel	Canac-Arteaga *et al.* (1999*a*)	Tenax trap	Dry purge	GC-MS	No
	Mini-Babybel	Canac-Arteaga *et al.* (2000*b*)	Tenax trap	Condenser	GC-MS	No
	Cheddar, Gouda, Proosdij, Maasdam, Gruyère, Parmesan	Engels et al. (1997)	Carbosieve SIII/Carbotrap	Not specified	GC-MS	No
	Ewe's milk cheeses	Larrayoz *et al.* (2000)	Carbosieve SIII/Carbopack B60/80	Dry purge	GC-MS	No
	Processed cheese	Mariaca *et al.* (1998)	Carbosieve SIII/Carbopack B60/80	Dry purge	GC-MS	No
	Blue cheese	Valero *et al.* (1997)	Tenax TA	P_2O_5	GC-MS	No
	French cheeses	Vernat Berdagué (1995)	Tenax TA	Not specified	MS	Type not specified
	Manchego (ewe's milk)	Villaseñor *et al.* (2000)	Tenax TA	Not specified	GC-MS	No
	Cheddar	Wood *et al.* (1994)	Cold trap	Condenser	GC-MS	No
	Cheddar and Swiss	Yang and Min (1994)	Tenax TA	Not specified	GC-FID	No
Milk		Badings *et al.* (1985)	Cold trap	Condenser	GC-MS	no
		Contarini et al. (1997)	Tenax TA	Not specified	GC-FID	PCA, LDA
		Marsili and Miller (1998)	Tenax trap	Dry purge	GC-MS	PCA
		Horimoto *et al.* (1997*a*)	Tenax TA	Dry purge	GC-FID	PCA, PCSA
		Horimoto *et al.* (1997*b*)	Tenax TA	Dry purge	GC-FID	ANN, PLS, PCR
		Vallejo-Cordoba (1994*a*)	Tenax TA	Not specified	GC-FID	PCA, PCR
		Vallejo-Cordoba (1994*b*)	Tenax TA	Not specified	GC-FID	LDA
		Valero *et al.* (1997)	Tenax TA	P_2O_5	GC-MS	No
Yoghurt		Ott *et al.* (1997)	Tenax TA	Not specified	GC-MS	No
Drinking yoghurt		Linssen *et al.* (1991)	Tenax TA	Condenser	GC-MS	No
Butter		Christensen and Hølmer (1996)	Tenax GR	Dry purge	GC-MS	No
Buttermilk		Heiler and Schiberle (1997)	Cold trap	Not specified	GC-O	No

PCA: Principle Component Analysis.
PCSA: Principle Component Similarity Analysis.
PLS: Partial Least Square.
LDA: Linear Discriminant Analysis.
ANN: Artificial Neural Network.
PCR: Principle Component Regression.

Table 3 Applications to dairy products using SPME or SBSE

Product	Sort	Author	Type of fibre	Detection	Pattern recognition
Cheese	Raclette, Gruyère Emmentaler	Burgraaf (2000)	PDMS, CW/DVB, PDMS/DVB, Carboxen/PDMS, PA	GC-FID	No
	Swiss, Cheddar and Romano Cheeses	Chin *et al.* (1996)	PDMS *vs.* PA	GC-MS	PCA
	Serra da Estrela (ewe's milk)	Dahl *et al.* (2000)	PA	GC-FID	PCA
	Cream cheese	Hoffman and Heiden (2000)	PDMS (SBSE)	GC-MS	No
	Camembert	Jaillais *et al.* (1999)	PDMS *vs.* PA	GC-MS	No
	Swiss cheeses	Jou and Harper (1998)	polyacrylate	GC-FID	ANN
	Cheddar and Colby cheese	Braggins *et al.* (1999)	PDMS *vs.* PDMS/DVB *vs.* PA	GC-FID	PCA
	Emmental	Schaller *et al.* (2000)	CW/DVB *vs.* Dynamic Headspace (Carbosieve SIII/Carbopack B60/80)	MS	PCA
	Cheddar	Wijesundera *et al.* (1998)	PA	GC-FID	No
Milk		Hoffman and Heiden (2000)	PDMS (SBSE)	GC-MS	No
		Marsili (1999*a*)	PDMS	MS	PCA
		Marsili (1999*b*)	Carboxen *vs.* Dynamic Headspace (Tenax)	GC-MS	No
		Lunden *et al.* (2001)	PDMS/DVB	GC-MS+FJD+O+NPD	
		Marsili (2000)	Carboxen	MS	PCA, PLS
Yoghurt	Strawberry	Hoffman and Heiden (2000)	PDMS (SBSE)	GC-MS	No
Butter		Shooter *et al.* (1999)	PA	GC-MS	No

Abbreviations: see **Table 2**.
GC-O: Gas chromatography-olfactometry; FJD: Flame ionisation detector; NPD: Nitrogen Phosphor detector.

head of the GC-column. SBSE has been used for the determination of volatiles from beverages (Hoffmann *et al.*, 2000*a*; Hoffman *et al.*, 2000*b*; Tredoux et al., 2000), wine (David, 2000; Hoffmann *et al.*, 2000*c*), and dairy products (Hoffmann and Heiden, 2000). As an extension of SBSE, Headspace Sorptive Extraction (HSSE) has been developed to extract analytes from the headspace with a higher loading capacity than SPME (Tienpont *et al.*, 2000*a*). HSSE-PDMS bars with 51.5 μL PDMS are commercially available from Gerstel GmbH. They consist of a glass rod of 5 cm length with an O.D. of 2 mm, the last cm being coated with PDMS. The bar is held in the sample headspace by a special device. The desorption also takes place in an automated thermal desorption unit followed by cryofocusing at the head of the column. HSSE has been used for determining volatiles from a commercial shampoo, coffee and banana (Tienpont *et al.*, 2000*a*) as well as from aromatic and medicinal plants (Bicchi *et al.*, 2000).
A paper comparing SPME, HSSE and SBSE for the enrichment of volatiles is in preparation (Tienpont *et al.*, 2000*b*).

Conclusion

Many of the preconcentration methods mentioned have already been used for analysing volatile compounds from food products. They all offer the possibility for on- or off-line sampling. **Tables 2** and **3** list several applications specific to dairy products carried out either in a dynamic headspace mode or using SPME. Sampling by dynamic headspace usually requires 1–2 h, which cannot be regarded as rapid. By using several traps in parallel, one being desorbed while the others are being loaded, it would be possible to considerably reduce the overall sampling time.
Sorption of analytes into a liquid polymer has found applications to foods only with the SPME mode. Such a method has already shown its superiority over the dynamic headspace mode with respect to repeatability, background and carry-over peaks (Marsili, 1999*b*; Schaller *et al.*, 2000). Therefore, SPME could become the method of choice for future investigations as it is rapid, not expensive and easy to use and to automate. The rapid deterioration of the coating requires however lots of care (use of calibration standards) for long term studies. The new SBSE and HSSE versions seem also to be very promising and less subject to deterioration. So far, no application with SPDE has been found in the literature as this is quite new.

Acknowledgements

The authors thank Mrs G. Urbach for her careful reviewing of the paper. This work was supported by a grant from the Swiss Federal Commission for Technology and Innovation (CTI, project 4614.1).

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CHAPTER 2

Long term study of volatile compounds from deep frozen canned processed cheeses proposed as control standards.

L. Pillonel, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Mitteilungen aus Lebensmitteluntersuchung und Hygiene, 93, 140-153 (2002)

Introduction

When using dynamic headspace gas chromatographic (DH-GC) analysis of volatile compounds in food, a very old problem is the lack of simple and stable materials usable for calibration in (semi-)quantitative analysis. The four main calibration methods used in GC have been described in a comprehensive paper by *Lord* and *Pawliszyn** (1).

The first is the so called standard addition. It is a widely used calibration method but it is extremely time consuming and laborious. To obtain a precision in the range of 2–5 %, the recommendation is to analyse three standard addition samples in triplicate. Further authors even propose the preparation of 7–8 standard addition samples to check the dynamic or linear range of the signal and to estimate the uncertainty of the measurements (2). Also the method becomes overly cumbersome if more than a few volatile compounds are to be quantified.

The second method is the spiking of samples with an internal standard. The major problem here is to get equilibrium of the internal standard in the matrix, specially if the standard is a highly volatile compound and the matrix a solid or viscous food sample. Furthermore, the behaviour of the internal standard during the various steps of the analysis is unpredictable and may be different from the analytes of interest, even if they are chemically very close.

* Their description focuses on SPME applications but the calibration methods remains valid for purge & trap

The third technique is the use of isotopically labelled standards. This is by far the most accurate method but is only usable with a mass sensitive detector (MS). Unfortunately these standards are very expensive and not always commercially available. Moreover the same problem of equilibrium occurs as with internal standards.

The fourth and final quantification method available is external calibration. Usually three different concentrations of standard solutions are measured in triplicate, leading to a calibration line. However, these solutions present several problems: if the standards are dissolved in a pure organic solvent, the latter will be overabundant, saturate the trap in DH-GC and mask the other peaks by overlapping; if the standards are dissolved in water (which is not always possible due to their lipophilic character), the risk of artifact formation due to the high amount of water vapour renders the use of standards problematic (3). Another way is to inject a known volume of standard in a flask or a bag and allow the system to equilibrate. A known volume of air-standard mix is then withdrawn with a syringe and injected into the GC-port. In the case of a purge&trap system, the introduction of the syringe content into the latter is not possible without a technical modification of the instrument. The last solution would be the addition of standards to an adequate matrix such as a mixture of triglycerides. Once again, the equilibrium between the added standard and the matrix presents a major problem. Furthermore, the matrix should in principle have a composition very close to the investigated samples to simulate the reactions and the interactions in the matrix and between the compounds themselves during the analysis. This makes it possible to take into account the alteration of the ad(ab)sorbent material used for preconcentration as well as possible displacement effects, breakthrough, etc. Such stable and homogeneous matrices have however never been related for volatile compounds analysis in cheeses. As no easy calibration method is available for a GC system with a preconcentration step, a simple control standard would be very useful in a first step.

The main objective of the current paper was to follow volatile compounds from four different deep frozen processed cheeses to check their ability to be used as native control standards. The measurements were carried out on both a Tekmar LSC 2000 and a Tekmar 3100 over approx. one year each. The stability of the FID signal for the volatile compounds previously identified with MS was studied, with the aim of selecting a few of them for cheese flavour research. Applications of such stable, homogeneous and reproducible standards are e.g.: comparison of different types of analytical equipment or quality control charts of gas chromatographs (combined with purge&trap or solid phase microextraction) and electronic noses. These materials have already been successfully used within a data transferability test between two electronic noses (4).

In addition to the main objective, it was possible to make some comparison between the two purge&trap concentrators used.

Experimental

Materials

Within the eleven processed cheese types already investigated for their volatile compounds (5), four varieties were chosen for their interesting chromatographic profiles. They were supplied by Tiger Käse AG (CH-3550 Langnau): ¼ fett, Emmentaler, Glarissa (with herbs) and Salami (with small salami pieces). They were all conditioned in gas-tight polymer coated aluminium cans (approximately 25 g). All cans of the same variety originated from the same production batch ensuring the needed homogeneity/reproducibility of the samples. All samples were stored at –20° C and left at +4° C overnight prior to sample preparation.

Sample preparation

Processed cheese samples were manually grated using a domestic rasp. Ten g of grated cheese were placed in the 25 ml non-fritted glass sparger of the Tekmar instrument. The bath was kept at 30° C, slightly below the melting point of the cheese. Table 1 summarises the plan of the analyses. Each repetition of an analysis was carried out using a fresh cheese can.

Table 1
Day and number of repetitions of the analyses

"2000" series	*Days*	*0*	*10*	*15*	*27*	*32*	*93*	*136*	*210*	*281*	*350*
	Repetitions (*n*=)	1	1	1	2	2	2	2	2	2	2
"3100" series	*Days*	*0*	*69*	*119*	*187*	*285*	*341*	*416*			
	Repetitions (*n*=)	6	2	2	2	2	2	2			

Dynamic headspace analyses

The Purge & Trap systems were successively the following: i) Tekmar LSC 2000 and ii) Tekmar 3100 sample concentrator (Tekmar, Cincinnati, OH, USA) over one year each, with a one-year break between these two periods. They included a slightly modified 25 ml non-fritted sparger (Schmidlin Co, part no. 14-2333-4SL, CH-6345 Neuheim), a trap (no. 8, containing a mixture of Carbosieve SIII (0.05 g) and Carbopack B60/80 (0.2 g)) as well as a cryofocusing unit. The modification allowed the glass sparger to be placed in a water bath for better temperature control (±0.1° C).

The Moisture Control Module (MCM) was removed from the 2000 series. The corresponding device on the 3100 series, the Moisture Control System (MCS), was set at a temperature of 150° C.

Operating conditions were as follows (values in brackets are specific for the "3100" series): prepurge time, 1 min; purge gas, nitrogen; purge flow, 30 ml/min;

purge pressure, 150 kPa; purge time, 10 min, dry purge time, 10 min; desorb preheat, 210° C (240° C); desorption at 220° C (240° C) for 4 min; cryofocus temperature, –125° C (–140° C); injection temperature program, within 1.5 min (1 min) from –125 to 200° C (225° C); bake, 5 min at 260° C; 6-port valve, 150° C; line, 150° C; transfer line from P&T to GC, 150° C; mount temperature, 60° C.

A Hewlett-Packard (HP) 5890 GC, Serie II was used. Separations were performed on a 30 m × 0.32 mm i.d. × 4 µm SPB1 sulphur column (Supelco). Helium was employed as carrier gas with an inlet pressure of 40 kPa (50 kPa). Following sample transfer, the oven temperature was maintained at 45° C for 13 min and then programmed at 5° C/min to 240° C which was held for 5 min.

Detection

Two detectors were mounted in parallel by splitting the flow at the end of the capillary column; one stream (0.79 ml/min at 45° C) led to a flame ionisation detector (FID), the other (0.86 ml/min at 45° C) to a mass-selective detector (MSD model HP 5972). The latter operated in the scan mode (TIC) from 26 to 250 amu at 1.1 scan/s, ionisation was by EI at 70 eV by autotuning.

The MSD was used for the identification of the volatile compounds. In addition, the identity was confirmed by comparison of retention times of authentic reference compounds. The FID signal was used for the semi-quantitative determination of the peak height. Only compounds with a peak height greater than the value of 360 arbitrary units (fixed threshold) have been considered for this study. This value corresponds approximately to a MSD signal-to-noise ratio of 5. Peaks suffering from peak tailing or having a high base line, a poor resolution or an asymmetric shape have been excluded. For these reasons, the whole range from compounds no. 20 to 26 in "Emmental" and from compounds no. 14 to 29 in "Glarissa" have been excluded. The presence of volatile fatty acids within these ranges is responsible for the poor resolution.

Results and discussion

The volatile compounds found in the four processed cheese types are listed in tables 2 and 3. Their chromatograms are shown in figure 1. Three criteria have been applied to the selection of the volatile compounds most appropriate as standards for GC: i) a relative standard deviation lower than 6%, ii) no more than one outlier over the period of the trial and iii) an average peak height of at least 1000 arbitrary units (corresponds approximately to the height of peak no. 12 from "Glarissa" in fig. 1). The volatile compounds fulfilling these requirements appear in italic and underlined characters in tables 2 and 3.

Table 2

Occurrence of the volatile compounds meeting the conditions of detection using the Tekmar LSC 2000 series. Italic and underlined characters indicate compounds with less than 6% RSD, a maximum of one outlier and an average peak height of at least 1000 arbitrary units

				Processed cheeses							
Functional Group	*Peak No.*	*Compound*	*Retention Index*[a]	*¼ fett*		*Emmentaler*		*Glarissa*		*Salami*	
				RSD	*Outliers*	*RSD*	*Outliers*	*RSD*	*Outliers*	*RSD*	*Outliers*
Alcohol	1	Ethanol	443	0.081	1	0.139	0	0.069	0	0.090	0
	3	Propan-2-ol	482	0.064	1	0.140	0	0.084	1	0.067	0
	5	Propan-1-ol	536	0.082	0	0.095	0	*0.050*	0	0.066	1
	10	Butan-2-ol	583			0.154	0	0.090	0	0.135	0
	12	2-Methylpropanol	616			0.149	0	0.220	0	0.149	0
	18	Pentan-2-ol	685							0.233	0
	21	3-Methylbutanol	720	0.115	0					0.155	0
	23	2-Methylbutanol	724							0.172	0
	31	2-Furanmethanol	830					0.312	0		
	34	Heptan-2-ol	884			0.249	0				
Ketone	2	Propan-2-one	466	0.097	0	0.066	0	0.077	0	*0.058*	0
	7	Butane-2,3-dione	557	0.096	1	0.142	0	0.086	0	0.083	1
	9	Butan-2-one	568	0.089	0	*0.058*	0	*0.049*	0	*0.051*	0
	15	Pentan-2-one	669	0.072	0	*0.049*	0			*0.043*	0
	16	Pentane-2,3-dione	674	0.194	0					0.128	0
	22	4-Methylpentan-2-one	723	0.080	0						
	26	Hexan-2-one	770			0.072	0				
	32	Heptan-2-one	871	0.061	0	*0.055*	0	*0.042*	0	*0.035*	0
	47	Nonan-2-one	1075	0.111	0	0.076	0	0.070	0	0.071	0
Aldehyde	4	2-Methylpropanal	531	0.257	0	0.203	0	0.239	0	0.176	0
	6	2-Butenal[†]	542	0.164	0	0.134	0			0.118	1
	8	Butanal	563					0.145	0		
	13	3-Methylbutanal	638	0.079	0	0.100	0	0.066	0	0.108	0
	14	2-Methylbutanal	648	0.066	1	*0.057*	0			0.081	0
	17	Pentanal	677	0.131	1					0.072	0
	27	Hexanal	779							0.115	0

Functional Group	Peak No.	Compound	Retention Index[a]	Processed cheeses: ¼ fett RSD	¼ fett Outliers	Emmentaler RSD	Emmentaler Outliers	Glarissa RSD	Glarissa Outliers	Salami RSD	Salami Outliers
Ester	11	Acetic acid ethyl ester	598	0.199	0	0.082	0	0.201	0	0.063	0
	19	Propanoic acid ethyl ester	695			*0.050*	0			*0.056*	0
	28	Butanoic acid ethyl ester	784			*0.055*	0	0.267	1	0.052	0
	29	Acetic acid butyl ester	795					*0.045*	0		
	33	Butanoic acid propyl ester	880					*0.044*	0		
	35	Propanoic acid butyl ester	888					0.067	0		
	38	Butanoic acid butyl ester	976					0.070	0		
Terpene	36	Alpha-thujene	934							0.106	2
	37	Alpha-pinene	946							0.106	1
	39	Sabinene	980							0.149	1
	40	Beta-myrcene	985							0.068	1
	41	Beta-pinene	990							0.085	1
	43	Alpha-phellandrene	1011							0.071	1
	44	Delta-3-carene	1021							*0.059*	1
	45	Limonene	1037							*0.050*	1
	46	Gamma-terpinene	1062							0.129	1
Other	20	Heptane	700			0.092	0				
	24	Dimethyldisulfide	732	*0.049*	0						
	25	Toluene	761	0.321	0	0.161	0			1.004	0
	30	Octane	800			*0.060*	0	*0.041*	0	0.111	0
	42	2,2,4,6,6-pentamethylheptane	1006							0.067	0
Number of compounds considered					20		23		21		36
Number of compounds usable as control standards					1		7		6		7

†=tentatively identified by MS (86%)

[a]=SPB1 chromatographic column

Table 3

Occurrence of the volatile compounds meeting the conditions of detection using the Tekmar 3100 series. Italic and underlined characters indicate compounds with less than 6% RSD, a maximum of one outlier and an average peak height of at least 1000 arbitrary units

						Processed cheeses					
Functional Group	*Peak No.*	*Compound*	*Retention Index*[a]	*¼ fett*		*Emmentaler*		*Glarissa*		*Salami*	
				RSD	*Outliers*	*RSD*	*Outliers*	*RSD*	*Outliers*	*RSD*	*Outliers*
Alcohol	1	Ethanol	443	0.226	0	0.223	0	0.138	0	0.360	0
	3	Propan-2-ol	482	0.199	0	0.235	0	0.161	0	0.112	0
	5	Propan-1-ol	536	0.223	0	0.182	0	0.127	0	0.157	0
	10	Butan-2-ol	583			0.177	0	0.130	0	0.128	0
	12	2-Methylpropanol	616			0.125	2	0.545	0	0.143	0
	18	Pentan-2-ol	685							0.168	0
	21	3-Methylbutanol	720	0.152	0					0.111	0
	23	2-Methylbutanol	724							0.108	0
	31	2-Furanmethanol	830					0.764	0		
	34	Heptan-2-ol	884			0.131	0				
Ketone	2	Propan-2-one	466	0.114	1	0.075	0	0.061	2	*0.042*	0
	7	Butane-2,3-dione	557	0.176	0	0.263	0	0.260	0	0.139	0
	9	Butan-2-one	568	0.088	1	0.067	0	*0.047*	0	0.064	0
	15	Pentan-2-one	669	0.065	1	*0.059*	0			*0.058*	0
	16	Pentane-2,3-dione	674	n.d.	–					0.157	1
	22	4-Methylpentan-2-one	723	0.073	1						
	26	Hexan-2-one	770			0.078	0				
	32	Heptan-2-one	871	*0.046*	1	0.069	0	0.038	2	*0.056*	0
	47	Nonan-2-one	1075	n.d.	–	0.098	0	0.076	2	0.103	0
Aldehyde	4	2-Methylpropanal	531	0.509	0	0.355	0	0.412	2	0.253	0
	6	2-Butenal[†]	542	n.d.	–	0.156	2			n.d.	–
	8	Butanal	563					0.458	0		
	13	3-Methylbutanal	638	*0.050*	1	*0.051*	0	0.056	2	*0.038*	0
	14	2-Methylbutanal	648	0.095	1	*0.057*	0			*0.034*	0
	17	Pentanal	677	n.d.	–					0.080	0
	27	Hexanal	779							0.109	0

Functional Group	Peak No.	Compound	Retention Index[a]	Processed cheeses: ¼ fett RSD	¼ fett Outliers	Emmentaler RSD	Emmentaler Outliers	Glarissa RSD	Glarissa Outliers	Salami RSD	Salami Outliers
Ester	11	Acetic acid ethyl ester	598	0.242	1	0.066	1	0.308	0	0.062	1
	19	Propanoic acid ethyl ester	695			*0.056*	0			0.052	0
	28	Butanoic acid ethyl ester	784			0.065	0	n.d.	–	0.079	0
	29	Acetic acid butyl ester	795					n.d.	–		
	33	Butanoic acid propyl ester	880					0.039	2		
	35	Propanoic acid butyl ester	888					0.068	0		
	38	Butanoic acid butyl ester	976					0.076	2		
Terpene	36	Alpha-thujene	934							0.196	1
	37	Alpha-pinene	946							0.195	0
	39	Sabinene	980							0.439	0
	40	Beta-myrcene	985							n.d.	–
	41	Beta-pinene	990							0.184	1
	43	Alpha-phellandrene	1011							n. d.	–
	44	Delta-3-carene	1021							0.064	1
	45	Limonene	1037							0.076	1
	46	Gamma-terpinene	1062							0.344	0
Other	20	Heptane	700			0.086	0				
	24	Dimethyldisulfide	732	*0.039*	1						
	25	Toluene	761	0.366	0	0.127	0			0.141	3
	30	Octane	800			*0.059*	0	0.032	2	n.d.	–
	42	2,2,4,6,6-pentamethylheptane	1006							n.d.	–
Number of compounds considered					20		23		21		36
Number of compounds usable as control standards					3		5		1		5

†=tentatively identified by MS (86%)

[a]=SPB1 chromatographic column

n.d.=below the detection limits of Tekmar 3100 but detected using LSC 2000

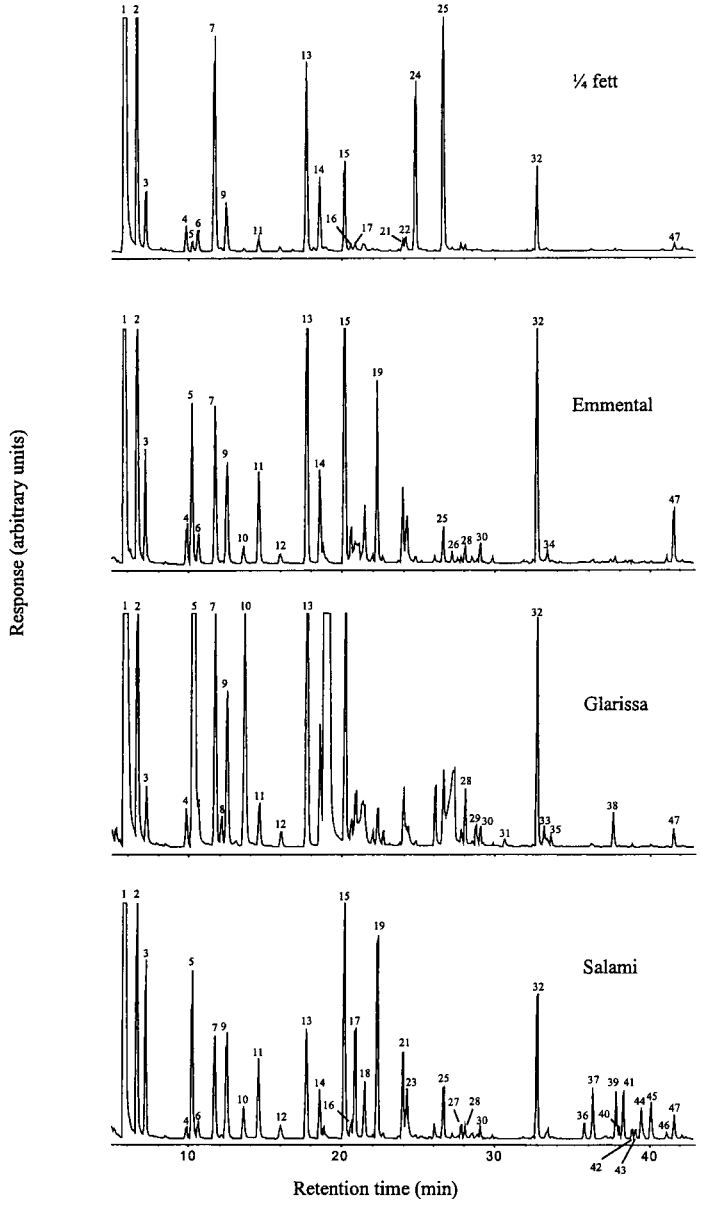

Figure 1 **Typical GC/FID chromatogram of volatile compounds found in the four cheese types.** Caption: see corresponding compounds listed in table 2

Stable compounds for the Tekmar LSC 2000 series

A single compound met the conditions in the "¼ fett" type cheese, seven compounds in "Emmentaler", six in "Glarissa" and seven in "Salami" (table 2). All these compounds would in practice be stable enough to be used as control standards.

The most interesting processed cheese type is Salami. The stability of the selected compounds is illustrated in figure 2. "Salami" provided stable compounds in three different chemical groups which were spread over the whole chromatogram (fig. 1 peaks no. 2, 9, 15, 19, 32, 44, 45). Two of them, Δ-3-carene and limonene, were terpenes from the spices added. This could be of particular interest when the purge & trap/GC-FID system has to be controlled for the analysis of cheese samples containing terpenes.

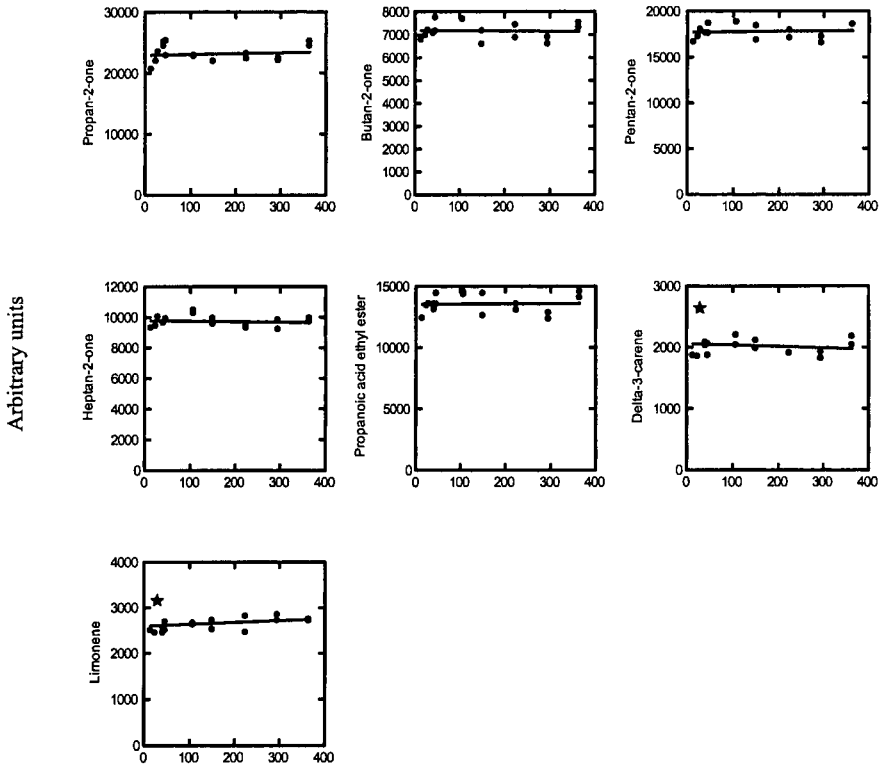

Figure 2 **Stability of the selected compounds in "Salami" for the Tekmar LSC 2000 instrument**

"Emmentaler" and "Glarissa" both provided stable compounds in four different chemical groups. "Emmentaler" even possessed a stable compound within the aldehyde group and "Glarissa" within the alcohol group; "¼ fett" may be, considered when dimethylsulfide is to be estimated in an unknown sample.

Stable compounds for the Tekmar 3100 series

Three compounds met the conditions mentioned above in the "¼ fett" type cheese, five in "Emmentaler", one in "Glarissa" and five in "Salami" (table 3). Except for the presence of three ketones, "Salami" is no longer of special interest. The most appropriate cheese here would be "Emmentaler", with stable compounds in four different chemical groups. The stability of the selected compounds is illustrated in figure 3. Though having three stable compounds, "¼ fett" is still only of interest for the presence of dimethylsulfide. "Glarissa" is no longer suitable for this purpose.

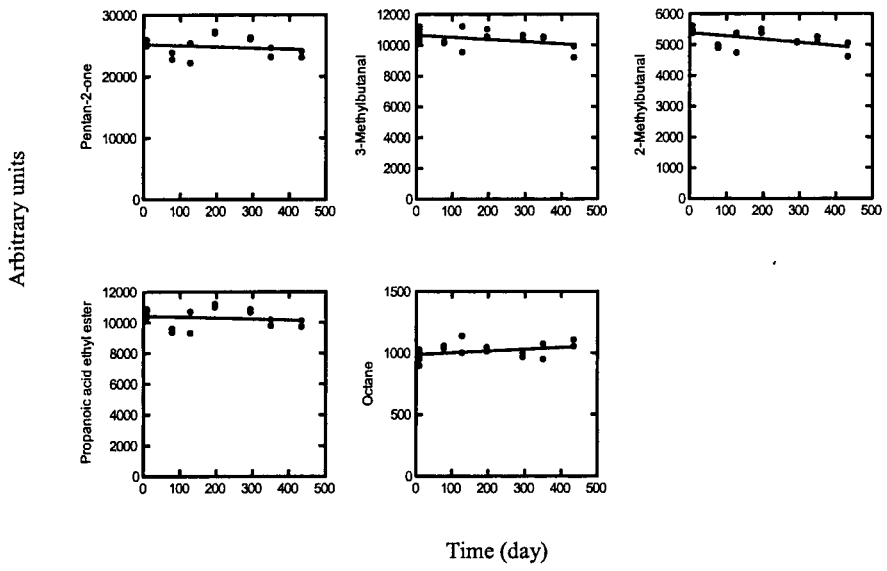

Figure 3 **Stability of the selected compounds in "Emmental" for the Tekmar 3100 instrument**

Differences between Tekmar LSC 2000 and Tekmar 3100

There were two general trends which differentiated the two series of analysers. The system with the concentrator Tekmar 3100 showed a poorer repeatability (higher RSD) and a poorer sensivity (some compounds found with the other system

are below the detection limit). This may be due to aging of the column, deterioration of the cheese samples or also to the concentrator itself.

There are three main modifications of design which differentiate the two concentrators compared, which could explain the differences in sensivity and reproducibility observed. Firstly, the trap of the 3100 series works differently from that of the older version. In the 3100 series there is a back pressure on the trap, which increases the total pressure in the cartridge and shifts the partitioning of the volatile analytes from the gas phase to the adsorbent. Secondly, the Moisture Control Module (MCM) of the 2000 series was removed and the one from the 3100 series was only kept as a "by pass" at the highest possible temperature, namely 150°C. Thirdly, the glass line is longer in the "3100", increasing the risk of condensation for high boiling compounds.

Conclusion

Several volatile components from processed cheeses packaged in gas-tight cans stored deep frozen (e.g. at –20° C) were stable enough, at least over three years, to be used as native control standards for gas chromatography with a preconcentration step. Such standards are therefore valuable e.g. for control charts, comparison of different types of analytical equipment and data transferability tests between electronic noses. The single necessary condition is obviously that these products originate from the same production batch to warrant the homogeneity of the filling material.

With a relative standard deviation of less than 6 %, no more than one outlier and an average peak height of at least 1000 arbitrary units, the following compounds may be recommended as native control standards:

- propan-2-one (RI=466), butan-2-one (RI=568), pentan-2-one (RI=669), propanoic acid ethyl ester (RI=695), heptan-2-one (RI=871), Δ-3-carene (RI= 1021) and limonene (RI=1037) from "Salami" processed cheese using Tekmar LSC 2000;
- 3-methylbutanal (RI=638), 2-methylbutanal (RI=648), pentan-2-one (RI= 669), propanoic acid ethyl ester (RI=695) and octane (RI=800) from "Emmentaler" processed cheese using Tekmar 3100.

There are significant differences between the performances of the Tekmar LSC 2000 and 3100 series. They are however difficult to interpret because the chromatographic conditions were not strictly identical.

Acknowledgement

The authors thank *Roland Gauch* for his technical assistance, *Thomas Berger* as well as *Gerda Urbach* (Australia) for their valuable comments and reviewing of the manuscript and the Tiger Käse AG (Langnau) for supplying the processed cheese samples used. This work was supported by a grant from the Swiss Federal "Commission for Technology and Innovation" (CTI, project 4614.1).

Summary

For the analysis of food volatiles using analytical techniques where no simple calibration method exists, standards are necessary for control charts, comparison of different types of equipment and stability control of the latter. For this purpose, volatile components from four industrial processed cheese types (¼ fat, Emmental, Glarissa and Salami), stored in gas-tight aluminium cans at –20° C, were investigated by dynamic headspace gas chromatography using two concentrators, Tekmar LSC 2000 and Tekmar 3100, over one year each, with a one-year break between the two periods. On the basis of their relative standard deviation (<6 %), repeatability (≤1 outlier) and signal intensity (>1000 arbitrary units), seven compounds in the "Salami"-type for the Tekmar LSC 2000 and five in the "Emmentaler"-type for the Tekmar 3100 respectively were selected as potential control standards with very different retention times. Moreover some differences of performance were found between the two purge & trap concentrators used.

Zusammenfassung

Bei der Bestimmung flüchtiger Verbindungen in Lebensmitteln, bei der keine einfachen Kalibrierungsmethoden existieren, sind Standards für Regelkarten, für den Vergleich verschiedener Analysengeräte und für ihre Stabilitätskontrolle notwendig. Deshalb wurden flüchtige Verbindungen von vier industriellen, in gasdichten Aludosen bei –20° C gelagerten Schmelzkäsesorten (¼ fett, Emmentaler, Glarissa und Salami) im Laufe eines Jahres mit Hilfe einer dynamischen Headspace-Gaschromatographie mit dem Aufkonzentrierungsgerät Tekmar LSC 2000 untersucht. Nach einem Jahr Unterbruch wurden diese Untersuchungen mit dem Tekmar 3100 wiederholt. Auf der Basis ihrer relativen Standardabweichung (<6 %), Wiederholbarkeit (≤1 Ausreisser) und Signalintensität (>1000 willkürliche Einheiten) wurden sieben Verbindungen in der Sorte «Salami» mit dem Tekmar 2000, respektive fünf in der Sorte «Emmentaler» mit dem Tekmar 3100, als potentielle Standards mit sehr unterschiedlichen Retentionszeiten ausgewählt. Einige Unterschiede in der Leistung der beiden verwendeten Purge & Trap-Apparaturen wurden dabei festgestellt.

Résumé

Pour l'analyse des composés volatils d'aliments où l'on ne dispose d'aucune méthode simple de calibrage, des standards sont nécessaires pour des applications telles que cartes de contrôle, comparaisons de différents types d'analyseurs et contrôles de leur stabilité. A cette fin, les composés volatils de quatre sortes de fromages fondus industriels (¼ gras, Emmental, Glarissa et Salami), stockés dans des boîtes étanches en aluminium à –20° C, ont été analysés par GC avec une préconcentration par espace de tête dynamique. Les analyses ont été effectuées consécutivement avec deux types d'appareils (Tekmar LSC 2000 & Tekmar 3100), durant une année chacun, avec une interruption d'une année entre les deux périodes d'essai. Sur

la base de leur déviation standard relative (<6%), de leur répétabilité (≤1 valeur aberrante) et l'intensité de leur signal (>1000 unités arbitraires), sept composés dans le type «Salami» pour le Tekmar LSC 2000 et cinq dans le type «Emmentaler» pour le Tekmar 3100 respectivement ont été sélectionnés comme standards de contrôle potentiels avec des temps de rétention très différents. Les deux appareils purge&trap utilisés présentent quelques différences de performance.

Key words
Volatile compound, Gas chromatography, Processed cheese, Control standard

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Corresponding author: Dr. Jacques-Olivier Bosset, Chemistry & Physic unit,
Swiss Federal Dairy Research Station, CH-3003 Berne,
E-mail: jacques-olivier.bosset@fam.admin.ch

CHAPTER 3

Data transferability between two MS-based electronic noses using processed cheeses and evaporated milk as reference materials.

L. Pillonel, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

European Food Research and Technology, 214, 160-162 (2002)

Abstract Electronic noses produce data which are difficult to transfer between identical instruments because of differences in the characteristics of "identical" instruments and because of sensor drift. The transportability of data is, however, essential for the setting up of databanks. Results of the present investigation, using processed cheeses and evaporated milk as reference materials, show that data transfer between two electronic noses based on mass spectrometry is possible and simple.

Keywords Processed cheese · Evaporated milk · Electronic nose · Mass spectrometry

Introduction

Electronic noses (ENs) have been developed to mimic the human sense of smell using an array of chemical sensors able to detect volatile compounds, and have been used in many sectors of the food industry [1]. There are four types of commercially available chemical sensors: the quartz microbalance (QMB), the conducting polymer, the metal oxide sensor (MOS) and the field effect transistor MOS. Apart from those traditional ENs, a further technology based on mass spectrometry (MS) is emerging. In this type of instrument, the mass selective detector acts as sensor array, each mass-to-charge ratio representing a sensor type. MS-based ENs are commercially available from a few manufacturers (chronologically SMartnose, Epagnier-Marin, Switzerland; Alpha-Mos, Toulouse, France; Agilent, USA).

To be effective, ENs need databases containing a set of measurements describing a reference product and other sets related to geographic origins, off-odour, adulteration, etc. By comparing the fingerprint of an unknown sample with fingerprints of the database, it is then possible to classify it into a defined group. To gain widespread acceptance, such databases must have international validity. As pointed out by Balaban et al. [2], this requires two conditions: (1) a database obtained from one instrument must be usable with other similar instruments, and (2) data collected from different laboratories with similar instruments must be identical so that a unique database can be built up.

Furthermore, ENs based on chemical sensors often suffer from sensor drift, so that calibration is made very difficult [3, 4] and the database becomes useless. This problem is hardly mentioned in the literature: Bazzo et al. [5] confirmed the possibility of transfer between ENs working with MOS sensors; without, however, mentioning the method used. Three different mathematical functions and two neural network configurations have been tested for transformation of data from one QMB to another [2]. When using the calibration data from QMB 1 and the transformed unknown milk samples from QMB 2, a correct classification rate of only 7/12 with the matrix based mathematical function as well as with the neural networks was found. Such a transferability rate is absolutely useless for any industrial application.

The present investigation aims to test the ability of MS-based ENs to overcome this problem. To compare two ENs, at least one reference material is needed. Instead of using standard solutions, it would be of great interest to have available a reference material with matrices similar to the test samples, e.g. cheese in our case. A study on four processed cheeses and one evaporated milk which could be used as reference materials for a preconcentration step followed by gas chromatography or EN is in progress. These materials have been tested within the current study on two MS-based ENs to check their suitability as reference materials and to investigate the transferability of data between two instruments.

L. Pillonel · J.O. Bosset (✉)
Federal Dairy Research Institute (FAM), Liebefeld, 3003-Bern, Switzerland
e-mail: jacques-olivier.bosset@fam.admin.ch
Tel.: +41 31 323 81 67, Fax: +41 31 323 82 27

R. Tabacchi
University of Neuchâtel, 2007-Neuchâtel, Switzerland

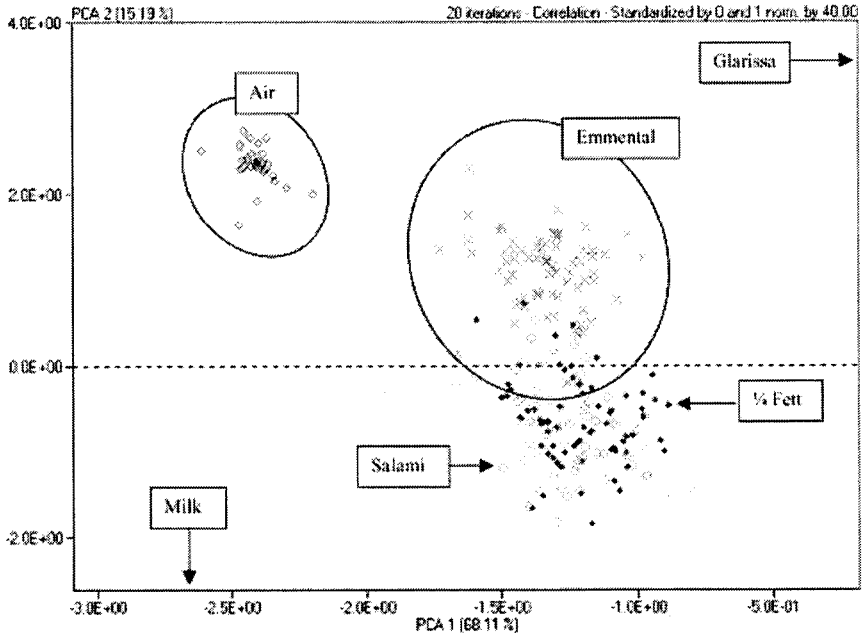

Fig. 1 Discrimination with a SMartnose between four different processed cheeses (1/4 Fett, Emmental, Glarissa, Salami), one evaporated milk and ambient air using a principal component analysis (*PCA*)

Materials and methods

Reference materials. Four commercial processed cheese varieties were supplied by Tiger Käse (CH-3550 Langnau): "1/4 fett", "Emmental", "Glarissa"(with herbs) and "Salami" (with small salami pieces and spices). They were all conditioned in gas-tight polymer coated aluminium cans (approximately 25 g). All cans of the same variety originated from the same production batch ensuring the required homogeneity and reproducibility for the samples.

Full cream unsweetened evaporated milk was supplied by Hochwald, Nahrungsmittelwerke, Thalfang, Germany. The milk all originated from the same production batch and was conditioned in tin cans.

All samples were stored at –20 °C and brought to room temperature prior to sample preparation.

Sample preparation. Processed cheese samples were cut into eight uniform pieces of approximately 3 g and filled into 10-ml vials adapted for the Combi Pal autosampler. Samples of evaporated milk (3 g) were placed in 10-ml vials with a Pasteur pipette. All vials were closed with a butyl/PTFE septum and a cap.

Analytical instruments and conditions. Two SMartnose (LDZ, CH-2074 Marin), referred to for simplicity as SN1 and SN2, were equipped with a Combi PAL autosampler (CTC Analytics, CH-4222 Zwingen). The main operating conditions were as follow: incubation temp, 60 °C; incubation time, 20 min; injection volume, 2.5 ml; syringe temp, 110 °C; injector temp, 130 °C; purge gas, nitrogen; purge flow, 200 ml/min; syringe purge time, 5 min; mass spectrometer scan speed, 0.5 s/mass; mass range, 10–140 amu; SEM voltage, 1030–1080 V (set to obtain a constant value for m/z=40 when air is injected). The total acquisition time was set to 170 s so that three cycles were measured for each injection, the first one being used to equilibate the measuring system.

SN1 and SN2 were located in two different laboratories, SN2 being an older version of SN1. Under these conditions, the robustness of the transferability test described below was ensured.

Samples analysis. The four cheeses and the evaporated milk were analysed seven times (called series 1) using SN1 over a period of 2 months, and twice (called series 2) using SN2 over 1 week. For each series, eight true replications of each sample were carried out. For air analyses, only five replications were performed. All samples were analysed in a randomised order. At the beginning of a series, eight test cheese samples were analysed (but not considered) to allow the system to equilibrate.

Data treatment. All the following data set transformations were carried out using the software supplied with the SMartnose. First, the mean value of the second and third cycle was calculated. Then the processed data set was normalised using the atomic ion of argon (m/z=40) from air. This mass-to-charge ratio is subject to practically no contamination from other compounds and the concentration of this gas in the headspace can be considered as constant. Such a normalisation makes it possible to correct the drift both within a single series of measurements and between different series. Then a principal component analysis (PCA) and a discriminant function analysis (DFA) were calculated. The data obtained with SN1 on seven different dates were thereafter used as the training set. The data obtained for air and for evaporated milk were then used as references for standardising the other values. This means that the axes of the PCA and DFA from each series of measurements were set in such a way that evaporated milk and air samples from each series overlapped. The data obtained using SN2 on two different dates were then imported as unknowns into the training set of SN1, undergoing the same standardisation procedure.

Results and discussions

Prior to performing a PCA, it was necessary to select the most discriminating variables (m/z). As evaporated milk, air and Glarissa processed cheese are highly differentiated, only 1/4 Fett, Emmental and Salami processed cheeses were considered for calculating the discriminating power of the mass-to-charge ratios. Therefore the following m/z values were selected from the training set: 58, 56, 54, 60, 41, 44, 64, and 47 (in order of decreasing discriminating power).

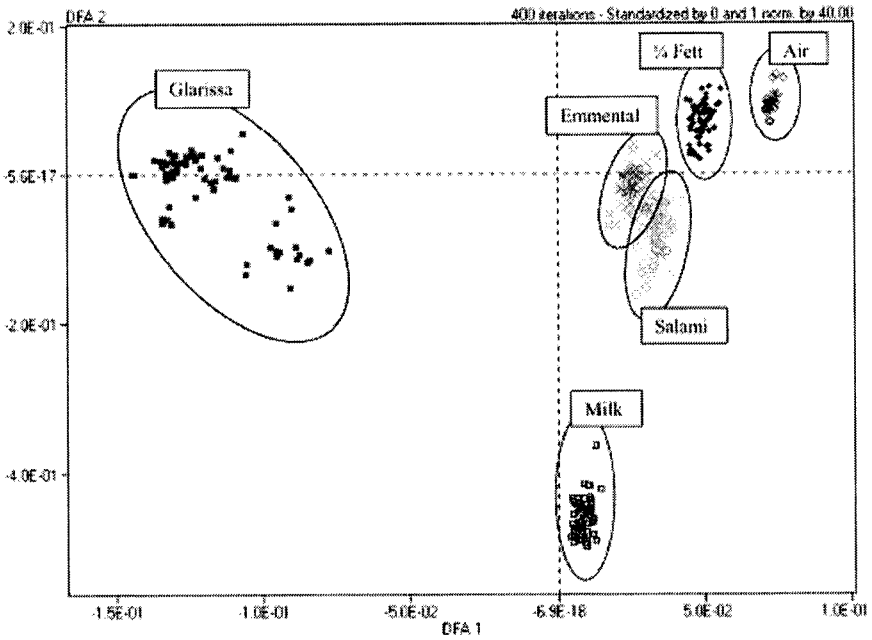

Fig. 2 Discrimination with a SMartnose between four different processed cheeses (1/4 Fett, Emmental, Glarissa, Salami), one evaporated milk and ambient air using a discriminant function analysis (DFA)

Figure 1 shows the results of a PCA on the seven series of SN1, with the two series of SN2 being added as unknowns. In the training set, 97.3% of the samples are correctly recognised whereas 96.9% of the unknowns are located in the correct group. The fact that failures occur within the training set proves that the discrimination is not trivial and increases the robustness of this test.

The results of a DFA for the same parameters are shown in Fig. 2. In the training set, 99.1% of correct placement is achieved, whereas 100% of the unknowns are located correctly (Fig. 1, Fig. 2).

In conclusion, the transfer of data between two SMartnoses does not require any complicated operations and is very user friendly. We showed that data imported from another instrument could easily be transferred to the training set of the original instrument and fit into the discrimination profile. In this study, we used evaporated milk and air as references for standardisation. Except for Glarissa, which shows a poorer repeatability in the PCA, all other cheeses (1/4 Fett, Emmental, Salami) and evaporated milk can be used as reference materials for standardisation.

Acknowledgements The authors thank Mrs G. Urbach for her careful reviewing of the paper and M.T. Zesiger for his technical and scientific support. This work was supported by a grant from the Swiss Federal Commission for Technology and Innovation (CTI, project 4614.1).

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CHAPTER 4

Comparison of efficiency and stability of two preconcentration techniques (SPME and INDEx) coupled to an MS-based "electronic nose"

L. Pillonel, D. Altieri, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Mitteilungen aus Lebensmitteluntersuchung und Hygiene, 95, 85-98 (2004)

Introduction

Electronic noses (ENs) may be very useful instruments for rapid analyses of volatile organic compounds (VOCs). They use an array of sensors which react differently to given volatile compounds, delivering a fingerprint which can be analysed by multivariate statistical analysis. MS-based ENs consider each m/z signal as one "sensor" instead of the chemical sensors encountered in the traditional ENs. The advantages of the former are the high number of "sensors" available, their robustness, selectivity and reproducibility (1). Furthermore, it is possible to explain some of the differences found in the MS fingerprint in terms of chemical compounds in particular when the corresponding GC-MS data are available (2).

However, to gain widespread acceptance, ENs need databases with international validity (3). It means that repeatability and reproducibility of the measurements must be ensured. Both factors were investigated by *Pillonel et al.* (4) using various processed cheese samples as standard materials. These guarantee excellent long term stability when stored deep frozen in small canned portions from a single production batch. A database was actually produced using one instrument and measurements carried out using a second instrument were successfully imported and classified in this database. An indirect correction for signal drifts was carried out by standardising the signals with two control standards (4). The analyses were however carried out in the static headspace mode and, though the three processed cheese types analysed had quite different volatile profiles, the separation into three groups was

only just achieved. This weakness, which strongly limits the use of MS-based ENs for compounds with a lower volatility, can be drastically improved by preconcentrating the headspace prior to the analysis.

A comparison between SPME, Purge & Trap and static headspace techniques for classifying Swiss Emmentaler at four different ripening times has already been carried out by *Schaller et al.* (5). They established that for small molecular masses (i.e. up to m/z=45) the signal intensity was comparable between the three techniques, whereas for the medium and higher molecular masses, the static headspace exhibited very low responses in comparison to the other two techniques. But while static headspace and SPME showed a good repeatability, the variation was significantly higher using Purge & Trap. Further drawbacks of the latter system are the size of the instrument and the numerous manual operations required even with a new system for automated Purge&Trap-(GC)-MS analysis as was recently described (2).

Two commercial systems which can easily be coupled to an EN are available: solid phase micro-extraction (SPME) and inside needle dynamic extraction (INDEx), a special type of solid phase dynamic extraction (SPDE). Both techniques can be automated using an auto-sampler. SPME has already found many applications in analysis of food volatiles whereas INDEx/SPDE is a newly developed device. So far few investigations have been carried out using the latter. *Lachenmeier et al.* (6) used SPDE coupled with gas chromatography/tandem mass spectrometry (SPDE/GC-MS/MS) to determine drugs of abuse in hair samples. *Musshoff et al.* (7) used SPDE-GC-MS for the determination of amphetamines and synthetic designer drugs in identical samples. *Lipinski* (8) applied the same technique to analyse pesticides in water. *Ampuero at al.* (9) compared the use of INDEx, SPME and static headspace for the classification of monofloral honeys.

Compared to SPME, INDEx/SPDE often shows a more effective and faster extraction of VOCs due to the advantage of a mechanically robust metallic steel needle instead of a fragile polymer fibre. The INDEx/SPDE device consists of a hollow needle internally coated (SPDE) or packed (INDEx) with an absorption material. The headspace may be aspirated several times consecutively through the syringe needle by moving the plunger up and down.

The objective of this paper was to compare the performances of SPME and INDEx applied to a MS-based EN and to check their stability by carrying out analyses with different syringes of the same type using canned processed cheese stored deep frozen which has already been shown to be a stable and well defined control material (10). Furthermore, the correlation between the highly discriminating masses selected from the EN and differences in compound concentrations analysed using GC-MS were investigated for SPME.

Materials and methods

Sample selection

The processed cheeses (¼-Fett, Emmental and Salami) and the unsweetened evaporated milk were conditioned in gas tight metallic cans and stored at approx. –18 °C. A detailed description can be found in (10).

Mass Spectrometry-based Electronic Nose

The instrument used was a SMart Nose (LDZ, CH-2074 Marin) equipped with a Combi Pal autosampler (CTC Analytics, CH-4222 Zwingen) controlled by the software CTC Cycle Composer.

SPME and INDEx

Of each sample 3 g were filled into 10 ml vials closed with a silicon septum and a cap. They were then incubated with agitation for 10 min at 60 °C for headspace equilibration.

The SPME preconcentration was carried out using a 1 cm Divinylbenzene/Carboxene/Polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Bellefonte, PA) left in the headspace of the vials for 30 min at 60 °C. The overall analysis time (i.e., equilibration, absorption, desorption, data acquisition) was 44 min per sample.

The INDEx extraction was carried out using a modified 2.5 ml Hamilton syringe (LDZ, CH-2047 Marin), whose needle was packed with Divinylbenzene, active charcoal and polydimethylsiloxane (diameter <0.3 mm) with a mass ratio 1:1:1. The manufacture is still under development, possibly leading to poor reproducibility from batch to batch. For this extraction, ten push and pull movements of the plunger (2 ml each) were carried out. The syringe temperature was set at 130 °C. To eliminate the possibility of water condensing on the wall of the needle and the packing material, the syringe was purged with dry nitrogen for 30 s prior to injection. Again ten push and pull movements of the plunger were executed in the injector port to help desorption. The overall analysis time was 21 min per sample.

Data acquisition and treatment using the SMart Nose

Injector temperature (200 °C) and acquisition parameters were identical for both SPME and SPDE modus. The quadrupole mass spectrometer was used in the EI ionisation mode (70 eV). The mass range was 10–130 amu and the mass spectrometer scan speed at 0.5 s/mass. The fibre, or the needle, was conditioned in the injector flushed with 200 ml/min nitrogen.

The total ion current (TIC) profile gave a global MS fingerprint of each sample headspace. All the data sets recorded were processed by the software supplied with the SMart Nose. The mass intensities were normalized with m/z 100 (a fragment of the background to correct the drift both in a single series of measurements and between series). The data obtained for air and evaporated milk were used as refer-

ences for standardising the other values (4). This means that the axes of the PCA and DFA from each series of measurements were set in such a way, that evaporated milk and air samples from each series overlapped in an optimal way. Using the SMart Nose, the three cheese types and the evaporated milk were analysed in three series using a new SPME fibre for each series and in a further series using a fibre used previously for 80 injections. For INDEx, only two series were analysed because only two INDEx syringes were available (still in the stage of development).

A principal component analysis (PCA) treatment was performed on the selected most discriminating masses. In both analyses (SPME and INDEx), the first series of data was used as training set and the remaining series as validation set. Eight true replications were carried out for each series. For air analysis (as second reference), only four replications were performed. All samples were analysed in randomised order.

Gas chromatography

To study the correlations between EN data and the chemical compounds present in different concentrations, a normal GC-MSD analysis was also carried out using SPME as preconcentration system. The three cheese types and the evaporated milk were analysed without replicate.

Extraction conditions and SPME fibre were the same as those used for the SMart Nose. Analyses were carried out on a Hewlett-Packard (HP) 5890, Series II gas chromatography system equipped with a narrow bore liner and a mass-sensitive detector (MSD) HP 5971. The temperature for the splitless injection was 260 °C. The volatiles were separated on a SPB-1 (Supelco) capillary column (60 m × 0.32 mm i.d., film thickness, 4 µm). Helium was used as carrier gas with a constant inlet pressure of 50 kPa. The temperature program was the following: initial temperature 35 °C for 3 min, heating rate, 5 °C/min to 260 °C and then 12 min at 260 °C.

MS detection was performed on a quadrupole mass spectrometer (model HP 5972) operating in full scan EI ionisation mode (70 EV). The scan range was 19–250 amu and the scan speed was 2.9 scans/s.

Data acquisition and processing were performed with the Hewlett Packard HP-Chem Station data software. The volatiles were identified using NIST MS library and further confirmed by comparison of retention indices of authentic reference compounds (10).

Statistical interpretation

Descriptive statistics and pairwise comparisons of mean values with Fisher's LSD test ($P \leq 0.001$) were performed with Systat for Windows version 9.0 (SPSS Inc., Chicago, IL).

Results and discussion

Discrimination using SPME

Three series of analyses were carried out using three new fibres of the same type (DVB/CAR/PDMS) to test their reproducibility and, a further series was repeated with one of the three fibres which had been used for 80 injections to test its robustness.

Data from the first series were used as training set to select the most discriminant variables (m/z). The three processed cheeses were considered for the elaboration of the PCA model (air and unsweetened evaporated milk were only used for the standardization). The following masses were chosen (in order of decreasing discriminant power): 74, 54, 58, 93, 94, 119. The data obtained by the remaining series were imported as unknowns into the PCA (validation set).

Figure 1 shows the plot of the PCA scores with the four series. All samples were correctly grouped. SPME therefore seemed to be a repeatable and reproducible preconcentration technique for this application. The samples analysed with the 80-times used fibre (empty marks) were also classified correctly, though the groups clearly laid closer together. This decrease in efficiency after a certain number of injections is a known weakness of SPME (ageing of the fibre).

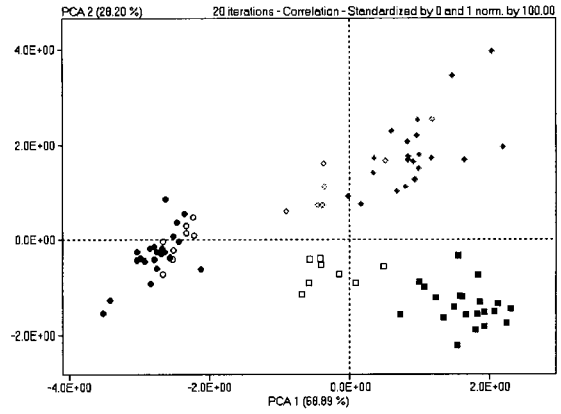

Figure 1 **Discrimination between three different processed cheeses (Emmental, ¼-Fett, Salami) using a new SPME fibre coupled to a MS-based electronic nose**
Score plots of the principal component analysis carried out using the masses m/z 74, 54, 58, 93, 94, 119.
(■) Emmental, (●) ¼-Fett, (◆) Salami, empty marks indicate the results obtained by an aged fibre.

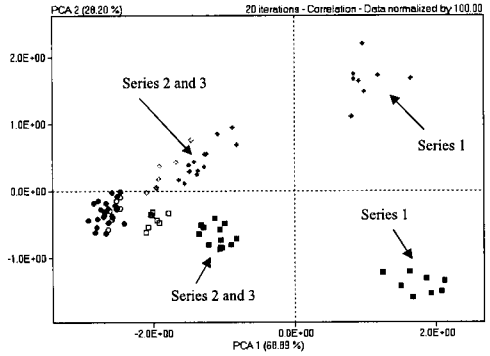

Figure 2 **Same PCA as in figure 1 but without applying the standardisation against air and unsweetened evaporated milk**
(■) Emmental, (●) ¼-Fett, (◆) Salami

To illustrate the magnitude and the efficiency of the standardisation, a PCA of the results was carried out omitting this correction (figure 2). The points of the first series were far away from the other series, probably due to the mass spectrometer being tuned differently. This problem may be corrected by the standardisation, which is only a translation of the groups to allow them to lie one above the other.

Discrimination using INDEx

Two different INDEx syringes of the same type were used to test their "reproducibility". The first syringe (first series) had already been used for more than 500 injections prior to the current work being carried out. The second syringe was new. The selected variables (m/z) were the following: 57, 94, 93, 74, 91, 48 (in order of decreasing discriminant power).

Figure 3 shows the PCA obtained using the first series as training set and the second series added as unknown. Once again 100 % of the samples were placed in the correct group.

Comparison of static headspace/SPME/INDEx

The PCA results obtained using both preconcentration techniques mentioned above (figures 1 and 3) as well as the results obtained using a static headspace (SHS) extraction (figure 4) were compared with one another.

For the latter technique, the data previously obtained by *Pillonel et al.* (4) were used to calculate the PCA omitting the Glarissa cheese data. The selected variables (m/z) were the following: 45, 64, 55, 58 (in order of decreasing discriminant power). In all three PCA, samples were placed 100 % correctly.

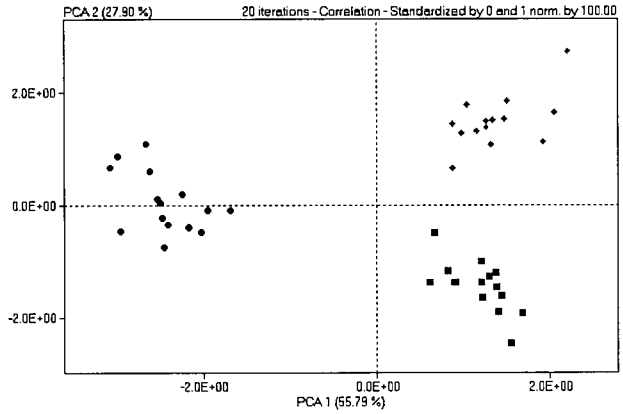

Figure 3 **Discrimination between three different processed cheeses (Emmental, ¼-Fett, Salami) using INDEx coupled to a MS-based electronic nose**
Score plots of the principal component analysis carried out using the masses m/z 57, 94, 93, 74, 91, 48.
(■) Emmental, (●) ¼-Fett, (♦) Salami

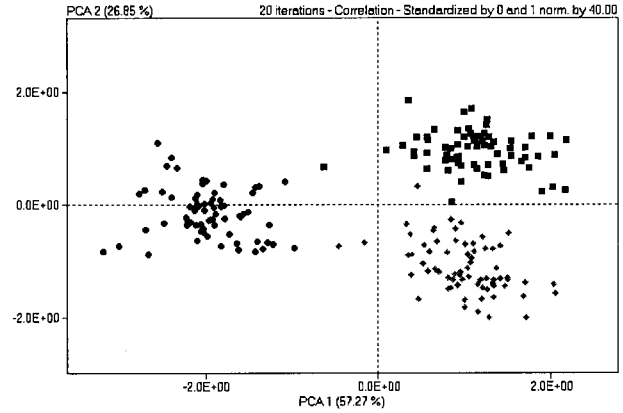

Figure 4 **Discrimination between three different processed cheeses (Emmental, ¼-Fett, Salami) using static headspace extraction coupled to a MS-based electronic nose**
Score plots of the principal component analysis carried out on the masses m/z 45, 64, 55, 58.
(■) Emmental, (●) ¼-Fett, (♦) Salami

Table 1
Average Euclidean distance between the points of a group and the centroid of another group for each series

	E-Q		E-S		Q-E		Q-S		S-E		S-Q	
	Mean	*Std*	*Mean*	*Std*	*Mean*	*Std*	*Mean*	*Std*	*Mean*	*Std*	*Mean*	*Std*
SPME												
Series 1[1]	4.68^{AB}	0.29	3.20^{B}	0.15	4.66^{AB}	0.09	4.26^{A}	0.11	3.17^{BC}	0.27	4.27^{AB}	0.30
Series 2[2]	4.63^{AB}	0.46	3.18^{B}	0.56	5.04^{A}	0.21	4.73^{A}	0.69	4.35^{A}	0.74	4.97^{A}	0.98
Series 3[2]	4.52^{AB}	0.53	3.32^{B}	0.45	4.37^{ABC}	0.38	4.29^{A}	0.39	3.19^{BC}	0.29	4.05^{ABC}	0.75
Series 4[3]	3.06^{C}	0.23	3.15^{B}	0.45	4.67^{AB}	0.10	4.15^{A}	0.32	3.66^{B}	0.29	3.42^{C}	0.77
INDEx												
Series 1[1]	4.22^{B}	0.34	3.04^{B}	0.32	4.26^{BC}	0.40	4.46^{A}	0.30	3.09^{BC}	0.53	4.46^{AB}	0.47
Series 2[2]	4.84^{A}	0.52	3.94^{A}	0.42	4.11^{BC}	0.30	4.61^{A}	0.38	3.18^{BC}	0.37	4.35^{AB}	0.22
SHS												
Series 1[1]	2.86^{C}	0.21	1.96^{C}	0.29	3.30^{D}	0.33	3.23^{B}	0.34	2.57^{CD}	0.26	3.27^{C}	0.25
Series 2[4]	3.37^{C}	0.38	2.18^{C}	0.20	3.32^{D}	0.51	3.38^{B}	0.54	2.31^{D}	0.46	3.31^{C}	0.71

E=Emmental, S=Salami, Q=¼-Fett

E-Q means the distance between the samples of E and the centroid of Q

Std=standard deviation

[1]These series were used to build the PCA model.

[2]These series correspond to a new SPME fibre or INDEx needle and were included as unknowns in the PCA.

[3]This series was obtained with the SPME fibre from series 1 but after 80 injections and was included as unknown in the PCA.

[4]Analysis under same conditions as series 1 but included as unknown in the PCA.

Series: A>B>C>D (=significantly different contents; $P \leq 0.001$) or AB=A and B overlap when using a univariate discriminant analysis

To compare the performances of the sampling modes, the distances between the groups were calculated using the SMart Nose software. As only the first series of each sampling mode was considered for building the PCA model, the centroid referred to the first series as well. Table 1 shows the average Euclidean distance between each point of a group and the centroid of the other two groups for each series separately. For the static headspace, the distances were virtually always the smallest. It showed the importance of less volatile compounds for differentiating cheese varieties. The distances between the groups were similar for both SPME and INDEx. Only the used SPME fibre showed a significantly poorer ability to discriminate the cheese varieties. This highlights the major advantage of preconcentration systems such as INDEx where the ad(ab)sorbents are packed into the needle and not exposed to mechanical stress thus limiting any ageing effects.

Table 2 shows the average Euclidean distance between each sample and the centroid of its own group (the centroid still referring to series 1 only) for all cheese types together. The eight replicates measured in series 1 shows the repeatability of the sampling whereas the replicates from the other series are more likely to be representative of the reproducibility due to changing the fibre or the syringe. For the first series, the lowest scattering was achieved using SPME. However the mean distance to the centroid in the case of SPME was not significantly different from the distance in the cases of SHS or INDEx. This means that all three sampling modes are equally repeatable.

Table 2
Average Euclidean distance between each sample of a group and the centroid of its own group for all cheese varieties together

	Mean	*Std deviation*
SPME		
Series 1[1]	0.44^{D}	0.26
Series 2[2]	1.33^{B}	0.63
Series 3[2]	1.10^{BC}	0.49
Series 4[3]	2.15^{A}	0.65
INDEx		
Series 1[1]	0.75^{CD}	0.35
Series 2[2]	1.83^{A}	0.34
SHS		
Series 1[1]	0.58^{CD}	0.30
Series 2[4]	1.29^{BC}	0.56

[1-4] see Table 1
Series: A>B>C>D (=significantly different contents; P≤0.001) or
AB=A and B overlap when using a univariate discriminant analysis

The scores obtained with the two other SPME fibres (series 2 and 3) were further away from the centroid. However they were still in the same range as that obtained using SHS (series 2), indicating a good reproducibility of the fibres. A large change

occurred with the aged fibre (series 4). The distance from its own centroid increased markedly. This was also translated into a closer grouping of the three cheese varieties.

The scores obtained using the second INDEx syringe were also significantly different from the ones from the first syringe. They were in the same range as that obtained using the aged SPME. However with the new INDEx, the distances between the groups were mostly increased (table 1). This may indicate some ageing effect of the older syringe. The liquid polymer particles filling the needle fuse together with time, decreasing the exchange surface with the headspace. The performances of the older INDEx after more than 500 injections were however still significantly better than those of the SPME fibre after 80 injections.

Interpretation of the discrimination using SPME-GC-MS

One major advantage of MS-based EN over the conventional EN is the possibility of interpreting the results by correlating them with compounds present in the sample.

Using SPME as preconcentration system for the SMart Nose analyses, the following ion mass values (m/z) were selected for their high discriminant power: 74, 54, 58, 93, 94, 119 (in decreasing order of discriminant power). Figure 1 shows the score plot of the PCA and figure 5 the corresponding loading plot. Ion masses 54, 58 and 74 were clearly characteristic of Emmental, 119 and 93 of Salami and 94 of ¼-Fett processed cheese.

To trace the chemical compounds responsible for the separation in the PCA, the selected ion masses were extracted from the chromatogram obtained using SPME-GC-MS. Peaks whose area was less than 4 % of the total peak area were not taken

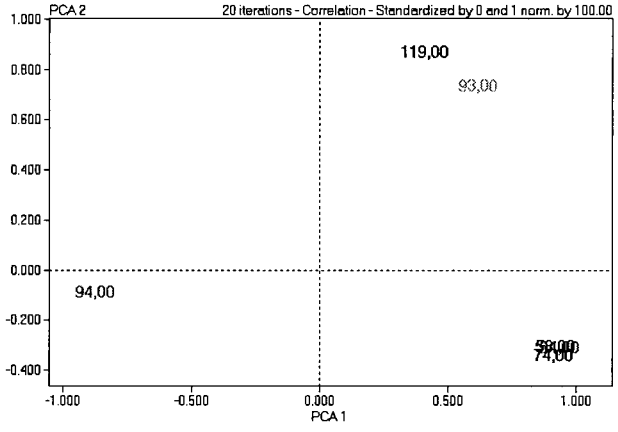

Figure 5 **Loading plots of the same PCA as in figure 1**
The masses 74, 58 and 54 are overlaid.

Table 3
Masses selected using the SPME-SMart Nose and their corresponding compounds from a SPME-GC-MS analysis
For each ion mass, the highest total ion mass of the three cheese varieties was taken as 100%.

Sample	*E*	*Q*	*S*	*E*	*Q*	*S*	*E*	*Q*	*S*	*E*	*Q*	*S*	*E*	*Q*	*S*	*E*	*Q*	*S*
Ion mass (m/z)		*54*			*58*			*74*			*93*			*94*			*119*	
Compounds																		
Acetic acid							11.0	7.0	6.6									
Propanoic acid	18.5																	
Butanoic acid							24.4	15.2	11.6									
Hexanoic acid	22.3		15.7				50.9	13.3	33.3									
Octanoic acid	18.0	6.4					13.7	4.2	12.6									
Decanoic acid	12.4		17.0															
2-Heptanone				33.1	11.0	20.8												
2-Nonanone	12.4		6.6	58.5	5.9	36.3												
2-Undecanone				8.4		6.3												
8-Nonen-2-one	7.8		24.4															
Nonanal		6.7	12.4															
Pirazine tetramethyl†	8.6		24.4															
Terpenes*												100.0						100.0
Phenol														88.2				
Disulfide dimethyl														11.8				
Total	100.0	13.1	76.1	100.0	16.9	63.4	100.0	39.7	64.2	0.0	0.0	100.0	0.0	100.0	0.0	0.0	0.0	100.0

* Terpenes: α-Thujene, α-Pinene, Sabinene, β-pinene, α-Phellandrene, γ-3-Carene, Limonene, β-Phellandrene, Caryophyllene
† Tentatively identified with MassLib

into consideration. The selected masses and their corresponding compounds are listed in table 3. For each ion mass, the highest total ion mass of the three cheese types was taken as 100%.

The results obtained from the SMart Nose were all confirmed by the GC-MS technique. The masses 54, 58 and 74 achieved the maximal values with processed Emmental cheese. The major compounds responsible were identified as carboxylic acids (m/z=54, 78) and ketones (m/z=58). The intensities of ion masses 93 and 119 were much higher in Salami due to the presence of a series of terpenes, probably originating from spices added in this processed cheese type. ¼-Fett processed cheese contained much more phenol which caused the significantly higher signal for m/z 94.

Further masses possessed high discriminant power but the information they contained were often redundant with information already obtained. For instance, the mass 48 was very specific for sulphur compounds contained only in ¼-Fett (methanethiol and disulfide dimethyl). The discrimination was however not improved by integrating it into the PCA.

Conclusion

The performances and stability of two different sampling modes, i.e. SPME and INDEx, for use with a MS-based electronic nose of type SMart Nose were compared. The discrimination between three processed cheeses analysed (Emmental, Salami and ¼-Fett) was clearly enhanced compared to the static headspace sampling mode. The most discriminating and therefore selected ion masses (m/z) for the PCA were as follows (in order of decreasing discriminant power): 45, 64, 55, 58 for SHS, 74, 54, 58, 93, 94, 119 for SPME and 57, 94, 93, 74, 91, 48 for INDEx. The improved discrimination after preconcentration was explained by the higher concentration of medium and low volatile compounds in the injection port.

The repeatability of the measurements was comparable for all sampling modes. The reproducibility between different SPME syringes was good except for the aged one (>80 injections) which showed a reduced retention power. The signal was therefore much lower than for the new SPME syringes. In the case of INDEx, the reproducibility was in the same range as for the aged SPME. The old INDEx was however used for more than 500 injections and still could compete with new SPME fibres for the discrimination. INDEx offers therefore an interesting alternative to the fragile and expensive SPME fibres, also with regard to the time of analysis per sample which was 21 min for INDEx compared to 44 min for SPME for similar performances.

Finally it could be shown that the discrimination obtained using an MS-based EN can be explained by differences in the concentrations of various compounds.

Acknowledgments

This study was supported by the Commission for Technology and Innovation (CTI, project no. 4614.1). The authors thank Mrs G. Urbach for her careful reviewing of the paper as well as T. Zesiger for his technical and scientific support.

Summary

Preconcentration of medium boiling organic compounds is generally useful to improve the efficiency of mass spectrometry-based electronic noses. Two different techniques, i.e. solid phase micro-extraction (SPME) and inside needle dynamic extraction (INDEx), a particular type of solid phase dynamic extraction (SPDE), were compared discriminating three processed cheeses used as standard materials. The reliability of these techniques was also investigated by repeating analyses using different SPME fibres and INDEx syringes of the same type. Both preconcentration techniques improved the efficiency of the MS-based electronic nose. The discrimination between the processed cheeses was complete, even when the syringe was exchanged. A significant loss of efficiency was observed for SPME which had previously been used for 80 injections (ageing of the fibre). The volatile organic compounds making possible the discrimination between the processed cheeses considered were identified by a parallel SPME-GC-MS analysis, tracing the characteristic fragments corresponding to the ion masses selected for the electronic nose.

Zusammenfassung

Vorkonzentration von mittelsiedenden organischen Verbindungen ist nützlich, um die Effizienz von auf Massenspektrometern basierenden elektronischen Nasen zu verbessern. Zwei verschiedene Techniken, Festphasenmikroextraktion (SPME) und die sogenannte «inside needle dynamic extraction» (INDEx), eine spezielle Art der dynamischen Festphasenextraktion (SPDE), wurden zur Diskriminierung zwischen drei Schmelzkäseproben als Standardmaterialen verglichen. Um ihre Zuverlässigkeit zu prüfen, wurden verschiedene SPME Fasern und INDEx Spritzen vom gleichen Typ ausprobiert. Beide Vorkonzentrationsmethoden verbesserten die Leistungsfähigkeit der elektronischen Nase. Die Diskriminierung der Schmelzkäseproben gelang vollständig, auch wenn die Spritze ausgetauscht wurde. Eine signifikante Abnahme der Leistungsfähigkeit der vorher 80-mal verwendeten SPME Faser wurde beobachtet. Die flüchtigen organischen Verbindungen, die die Diskriminierung zwischen den untersuchten Schmelzkäseproben ermöglichten, wurden mit Hilfe einer SPME-GC-MS Analyse identifiziert. Dabei wurden nur die charakteristischen Massenfragmente herangezogen, welche den ausgewählten Massen der elektronischen Nase entsprachen.

Résumé

La préconcentration des composés organiques volatils à moyen point d'ébullition est souvent utile pour améliorer les performances des nez électroniques basés sur la spectrométrie de masse. Deux différentes techniques, à savoir la micro-extraction sur phase solide (SPME) et celle dite «inside needle dynamic extraction» (INDEx), une forme d'extraction dynamique sur phase solide (SPDE), ont été comparées pour la discrimination de trois sortes de fromages fondus utilisés comme matériels standards. La fiabilité de ces techniques a été testée à l'aide d'analyses

répétées utilisant des seringues différentes mais de même type. Les deux techniques ont permis d'améliorer les performances du nez électronique. La discrimination des fromages fondus était complète, même après avoir changé de seringue. Cependant la seringue SPME déjà utilisée pour 80 injections antérieures a montré d'importants signes de vieillissement. Les composés organiques volatils qui permettent la discrimination des fromages fondus considérés ont été identifiés à l'aide d'une analyse SPME-GC-MS parallèle en traçant les fragments caractéristiques correspondants aux masses sélectionnée pour le nez électronique.

Key words

Electronic nose, processed cheese, mass spectrometry, SPME, SPDE, INDEx, preconcentration, volatile organic compounds (VOC)

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Address of correspondent: Dr. Jacques Olivier Bosset, 8 ch. des Prés, CH-1530 Payerne

CHAPTER 5

Analytical methods for the determination of the geographic origin of Emmental cheeses. Main framework of the project; chemical, biochemical, microbiological, colour and sensory analyses.

L. Pillonel, R. Badertscher, U. Bütikofer, M. Casey, M. Dalla Torre, P. Lavanchy, J. Meyer, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

European Food Research and Technology, 215, 260-267 (2002)

Abstract Emmentaler is a hard cheese produced and consumed in many countries. The authenticity of this cheese variety is becoming a matter of national importance with the imminent opening of the cheese market in Switzerland and the introduction of the PDO label for the Emmentaler Switzerland™. This paper is the first in a series reporting the search for parameters that make possible a geographic discrimination of Emmentalers. Twenty Emmentaler cheese samples from six regions in Europe (one from Switzerland, two from France, one from Austria, one from Germany and one from Finland) were considered. In this paper, chemical, biochemical, microbiological, colour and sensory parameters were investigated leading to the following preliminary results. The presence of *Lb. helveticus* seemed to be a good indicator of an origin other than Swiss. The concentration of enterococci, facultative and obligate heterofermentative lactobacilli and salt tolerant bacteria was significantly lower in Emmentaler Switzerland™ than in the foreign cheeses. Chemical parameters such as fat content and pH value as well as biochemical parameters such as L- and D-lactate and pyruvate allowed us to partially discriminate between the regions when these data were combined by principal component analysis. The colour of the body also showed significant differences.

Keywords Emmentaler · Swiss cheese · Authenticity · Geographic origin · Traceability · PDO

L. Pillonel · R. Badertscher · U. Bütikofer · M. Casey
M. Dalla Torre · P. Lavanchy · J. Meyer · J.O. Bosset (✉)
Federal Dairy Research Station (FAM), Liebefeld,
3003 Berne, Switzerland
e-mail: jacques-olivier.bosset@fam.admin.ch
Tel.: +41-31-3238167, Fax: +41-31-3238227

R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel, Switzerland

Introduction

Determination of food authenticity and geographic origin is a crucial issue in food quality control and safety. The 5th Food Authenticity and Security International Symposium, June 9–11, 1999 in La Baule (F) [1] highlighted the fact that food misrepresentations and adulterations are distinguished both by their abundance and their variety. Well-known adulterated products are wine [2] and olive oil [3, 4] for which several methods of authentication have already been developed. But adulterations are also known for dairy products. The geographic origin of butter [5] and milk [6], the addition of cow's milk to ewe's or goat's milk or other dairy products [7] and the use of added whey solids or processed cheeses in grated cheese [8] have already been investigated. A less common but potential type of misrepresentation may concern the geographic origin of cheese.

The originality of a cheese depends on several factors such as milk and cheesemaking procedures (incl. microbiology and technology), which are both dependent on the geographic origin. The climate, geology, forage and breed itself influence the milk quality while local, regional or national traditions influence the cheesemaking. The determination of origin is a key component of Protected Designation of Origin (PDO) products and, where industrial copies exist, of goods manufactured by a traditional method.

The imminent opening of the Swiss cheese market will unquestionably allow massive quantities of foreign cheeses to be imported. Moreover, Emment(h)al(er) cheese, often called "Swiss cheese," is the example par excellence of a cheese variety that is widespread and very popular in many countries. However, less than 10% of the European production is manufactured in Switzerland (Table 1) [9]. Bordering countries such as France and Germany together produce more than half of the European manufacture and at lower cost. Therefore the problem of the analytical authentication of Emmentaler cheese will be a major challenge in the near or more dis-

Table 1 Production level (1997) of Emmentaler cheese in several countries

Country	Production (t/y)
France	275 000
Netherlands	89 400
Germany	88 300
Switzerland	45 000
Sweden	28 400
Finland	26 400
Austria	12 800
Denmark	6 600
Ireland	5 000

tant future both for people responsible for suppression of misrepresentations as well as producers of Emmentaler. This consideration is not only true for Switzerland but also for every region in Europe producing a cheese type in a traditional and more expensive way than in large industrial plants.

Many investigations have been carried out to characterise the Emmentaler Switzerland™ [10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21], the German [22, 23, 24, 25] or the French Emmentaler [26, 27], but few are dedicated to the geographic origin of a cheese sample. Rohm [28] carried out a regional classification of Emmentaler cheese in western Austria based on some chemical parameters. Bosset et al. [29] and Collomb et al. [30] found significant differences in the amount of terpenes and conjugated linoleic acids in Gruyère cheese between a lowland and an highland production zone. Manca et al [31] could distinguish between Pecorino Sardo, Siciliano and Pugliese using stable isotope ratios and free amino acids ratios. Grappin et al. [32] could correctly discriminate the origin of 20 Comté cheeses made in five different cheese plants according to some physico-chemical variables, microbial counts and sensory characteristics.

Sensory analyses mainly focus on the characterisation of a cheese type or on the description of the evolution during ripening. However, such analyses have been applied to differentiate Parmigiano-Reggiano [33, 34] and Grana Padano [35] from their imitations. Muir et al. [36] showed differences in flavour and texture between raw milk farmhouse Cheddar and Cheddar from a factory made from pasteurised milk. However no differences were found between the different countries of production. The French Emmentaler "Grand-Cru" was characterised by a more intense flavour including the terms fruity, salted, pungent and sweet and by a weaker rancid odour than from the other French Emmentaler [27]. Monnet et al. [37] positively correlated the type of soil (edaphic parameters) with the sensory quality of 20 Comté cheeses from the French Jura. The influence of altitude on Abondance cheese flavour was investigated by Bugaud et al. [38]. Furthermore, no correlation seems yet to have been established between the origin of a cheese and its microbial flora.

Therefore, the present study aims to evaluate the potential of a large variety of analytical methods for determining the geographic origin of an Emmentaler cheese. This first paper in a series deals with chemical, biochemical, microbiological, sensory and colour analyses. The next publications will deal with further analytical techniques: compounds resulting from the proteolysis and rheology; infrared spectroscopy; detailed composition of fat (free fatty acids, fatty acid composition and triglycerides); volatile compounds using GC/MS and electronic nose; as well as the inorganic composition of the cheeses (major and trace elements, radioactivity, and stable isotope ratios). At the end, all the above mentioned methods will be compared and the best set of methods will be selected for a follow-up of the project, which also takes into account their cost, rapidity and robustness. Using these methods, a larger number of samples will then be investigated in the future in order i) to obtain a cheese fingerprint from each region of production, ii) to allow the cheeses to be easily classified according to their origin and iii) to build up a corresponding database.

Materials and methods

Origin and selection of the cheese samples

Table 2 summarises the geographic origin, the date of manufacture and the ripening period of the 20 Emmentaler cheeses selected from six regions (Fig. 1). Three samples were chosen from each region (except for Switzerland with six and Finland with two samples). Three regions were adjacent to Switzerland (Savoie (F), Allgäu (D), Vorarlberg (A)). The geographic and climatic proximity of these regions, as well as the similar technologies used, allows

Table 2 Origin and ripening time of the 20 cheese samples investigated

Samples	Region (country)	Date of fabrication	Ripening time (month)
AL 1–3	Allgäu (D)	20.12.2000	4
BR 1–3	Bretagne (F)	16.02.2001	2.5
CH 1–6	Switzerland (CH)	26.12.2000	4
FI 1–2	Middle Finland (FI)	07.02.2001	3
SA 1–3	Savoie (F)	08.02.2001	3
VO 1–3	Vorarlberg (A)	02.02.2001	3

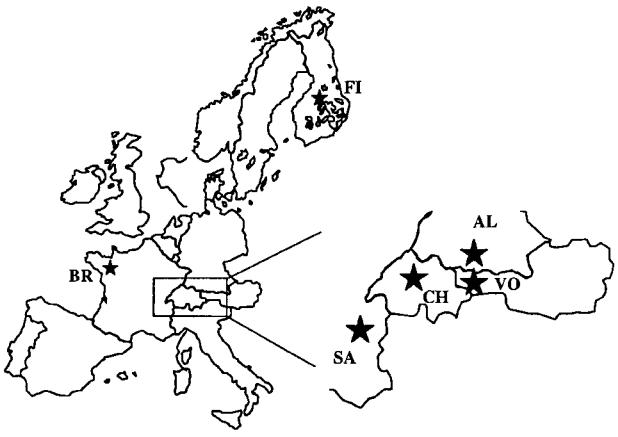

Fig. 1 Sampling areas

one to test the robustness of the methods (intra-variability within a region vs. inter-variability between regions). The two remaining regions (Bretagne (F) and the central region of Finland) are quite distant from Switzerland. Taken together, these six regions account for approx. 77% of European Emmentaler production. The samples were supplied directly by the cheese factories (Austria, Finland, Switzerland) or were collected with the help of a national dairy research centre (France and Germany). The samples (approx. 8 kg) were supplied as sectors or slices of a whole block. The first 2 cm from the rind were discarded. For analyses, where grated cheese was required, portions were cut across the whole block (height) to take into account concentration gradients. The cheeses investigated were chosen at ages between 2.5–4 months of ripening, which corresponds to that sold in stores. For most regions, this corresponds to the average ripening time. In Switzerland, a 4-month old Emmentaler is considered very young. Indeed, an important part of the market is made up of Emmentaler over 8 months old. We limited, however, our investigation to young Emmentaler Switzerland™ and the corresponding foreign samples since the risk of confusion is highest with young cheeses.

Chemical and biochemical analyses

The samples were kept frozen until the following analyses were performed: water gravimetric [39], fat according to Gerber van Gulik [40], total nitrogen according to Kjeldahl [41], sodium chloride potentiometric with a silver electrode [42], L- and D-lactate, citrate, succinate and pyruvate with an enzymatic test kit after extraction [43], volatile organic acids [44], alkaline phosphatase photometric with *p*-nitrophenylphosphate as substrate [45], vitamins A and E using HPLC [46]. The pH-value was determined at room temperature using a penetrometric electrode (Mettler-Toledo, no. 104063123).

Microbial analyses

Fresh samples were analysed except for *Lb. helveticus*. For the latter, samples were frozen. The following microorganisms were determined: enterococci (ECOC) [47], propionic acid bacteria (PAB) [48], facultative heterofermentative lactobacilli (FHL) [49], obligate heterofermentative lactobacilli (OHL) [49], salt tolerant bacteria (STB) [50]. The occurrence of *Lb. helveticus* was investigated by polymerase chain reaction (PCR) according to a method not yet published.

Sensory analyses

Fresh samples were analysed according to a protocol described in [51]. The samples were evaluated by the accredited sensory panel of the FAM in four series: Three series with two samples from each region (except Finland) and one series with the two Finnish Emmentalers.

Colour analyses

After storing the cheese samples at 15±0.5 °C overnight and preparing cheese slices (height >30 mm), tristimulus reflectance measurements (each one made twice) were carried out with a Hunterlab D 25-D-2 Optical Head in the system according to Hunter; L=brightness (L=0: black; L=100: white); a=green-red component (a<0: green; a>0: red); b=blue-yellow component (b<0: blue; b>0: yellow). The method has been described by Bosset et al. [52]

Statistical analyses

The decimal logarithm of the microbiological counts was used for the calculations. The averages and standard deviations were calculated for each parameters. Descriptive statistics, analysis of variance (ANOVA), pairwise comparisons of mean values with Fisher's LSD test, principal component analysis (PCA) and correlation test were performed with Systat for Windows version 9.0 (SPSS Inc., Chicago, IL). The correlation coefficients were always calculated using the individual values of each region.

Results and discussions

Chemical composition

The chemical compositions of the 20 Emmentaler samples from the six regions are listed in Tables 3 and 4. "Vorarlberg" showed a significantly higher fat content than all others except for "Allgäu." "Bretagne" had the lowest fat content. "Allgäu" contained significantly more sodium chloride than "Switzerland," "Savoie" and "Vorarlberg." "Savoie" showed the highest p*H*-value, "Bretagne" the lowest. No differences were found in the vitamin content.

The results of alkaline phosphatase (AP) were very surprising. This enzyme is denatured by pasteurisation and therefore used as indicator of milk heat treatment. Though the Swiss factories produce Emmentaler from raw milk, it showed the lowest AP values. On the contrary, in Finland, where thermisation is applied and the curd is heated at the highest temperature of all regions (54 °C), the samples nevertheless showed a high AP activity. The broad activity range of this enzyme may be explained as follows: i) the native milk may contain different AP concentrations (up to 30% relative standard deviation), ii) the duration of scalding, the temperature at which the coagulum is transferred into the moulds and the size of the block also influence the degree of AP denaturation, iii) some micro-organisms are known to produce significant amount of AP [53] and iv) the presence of a strong gradient within the block. From the zone under the rind to the core of the block, the activity of the enzyme can drop off by several orders of magnitude [54]. This gradient is due to the slower cooling inside the cheese. Moreover, the reliability of the measurements decreases strongly with the age of the cheese. Finally the value of these results must be put into perspective because the position of the samples within the block were not rigorously the same. Therefore, the alkaline phosphatase activity can be an appropriate method for the characterisation of heat treatment only under the strict condition that the sample to be investigated is located just below the rind.

"Finland" no longer contained L- and D-lactate nor pyruvate. This fact indicates a very high activity of propionic acid bacteria. "Allgäu," "Bretagne" and "Vorarlberg" had the highest lactate concentrations, which indicates a lower propionic fermentation. The concentrations of propionic acid and L+D-lactate were highly negatively correlated (r=–0.94). "Bretagne" showed a high average citrate concentration due to one sample (BR3) containing 7.7 mmol/kg. Citrate is normally catabolised to formate by facultative heterofermentative bacteria (FHL). How-

Table 3 Gross chemical composition, pH-value, alkaline phosphatase and vitamins in the 20 Emmentaler cheese samples investigated

Region (n=)	ANOVA	AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s
Total nitrogen (g/kg)	*	**44.1**^{AB}	1.8	**45.0**^{AB}	0.72	**44.4**^{AB}	0.85	**45.5**^{AB}	1.3	**46.2**^{A}	0.77	**42.9**^{B}	1.6
Fat (g/kg)	***	**322**^{AB}	15	**300**^{C}	7.9	**318**^{B}	7.4	**307**^{BC}	2.5	**306**^{BC}	7.7	**342**^{A}	6.3
Fat in dry matter (g/kg)	**	**494**^{AB}	19	**473**^{B}	3.6	**494**^{B}	9.5	**483**^{B}	4.1	**474**^{B}	11	**520**^{A}	13
Water (g/kg)	*	**346**^{AB}	9.2	**366**^{AB}	12	**355**^{AB}	6.8	**363**^{AB}	11	**354**^{AB}	4.9	**341**^{B}	9.2
Water in fat-free matter (g/kg)	ns	**511**	11	**523**	11	**521**	8.4	**525**	14	**511**	7.1	**519**	16
Sodium chloride (g/kg)	**	**6.43**^{A}	0.83	**4.37**^{AB}	1.58	**3.43**^{B}	0.41	**5.60**^{AB}	1.13	**3.60**^{B}	1.04	**3.57**^{B}	0.98
pH-value	***	**5.73**^{AB}	0.05	**5.57**^{C}	0.05	**5.63**^{BC}	0.06	**5.73**^{AB}	0.02	**5.79**^{A}	0.08	**5.65**^{BC}	0.03
Alkaline phosphatase (IU)	ns	**1700**	1834	**226**	176	**29**	47	**1500**	675	**945**	660	**211**	216
Vitamin A (IE/kg)	ns	**6179**	1274	**6936**	1048	**6950**	1391	**6318**	181	**5992**	607	**5534**	719
Vitamin E (mg/kg)	ns	**4.00**	0.39	**3.32**	0.49	**4.13**	0.60	**4.01**	0.34	**3.81**	0.62	**4.48**	0.75

x=mean value; s_x=standard deviation; ANOVA: ns=not significant, *p=0.05, **p=0.01, ***p=0.001
Production sites: A>B>C>D (=significantly different contents p=0.01) or AB=A and B overlap by using an univariate discriminant analysis
AL=Allgäu, BR=Bretagne, CH=Switzerland, FI=Finland, SA=Savoie, VO=Vorarlberg
n=number of samples

Table 4 Organic acids content of the 20 Emmentaler cheese samples investigated

Region (n=) (mmol/kg)	ANOVA	AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s
L-Lactate	***	**45**^{A}	13	**47**^{A}	15	**23.8**^{B}	4.4	**0.00**^{C}	0.00	**27.0**^{AB}	9.5	**45.7**^{A}	4.5
D-Lactate	***	**59**^{A}	29	**48.7**^{AB}	7.6	**21.8**^{CD}	6.2	**0.00**^{D}	0.00	**24.3**^{BCD}	9.9	**35.0**^{ABC}	2.7
L/D ratio (no unit)	ns	**0.87**	0.31	**0.96**	0.19	**1.13**	0.21	–	–	**1.13**	0.10	**1.31**	0.18
Citrate	ns	**0.10**	0.10	**2.6**	4.4	**0.22**	0.48	**0.05**	0.07	**0.13**	0.06	**0.07**	0.12
Succinate	***	**6.7**^{ABC}	4.4	**8.6**^{AB}	3.4	**3.2**^{C}	0.48	**13.1**^{A}	1.3	**12.5**^{A}	1.9	**4.9**^{BC}	3.3
Pyruvate	**	**2.8**^{CD}	3.9	**8.2**^{AB}	2.7	**6.1**^{ABC}	1.5	**0.20**^{D}	0.00	**8.9**^{AB}	0.12	**7.4**^{BC}	1.9
Formate (C1)	*	**4.59**^{AB}	0.38	**3.55**^{B}	0.60	**4.04**^{AB}	0.32	**3.59**^{B}	0.19	**4.04**^{AB}	0.17	**4.90**^{A}	0.76
Acetate (C2)	**	**39.0**^{BC}	9.9	**32.3**^{C}	7.0	**44.6**^{AB}	3.6	**50.7**^{A}	0.8	**51.8**^{A}	3.3	**40.2**^{ABC}	2.7
Propionate (C3)	***	**23.9**^{C}	17.1	**30.4**^{C}	5.7	**59.5**^{A}	7.8	**69.9**^{A}	0.7	**53.2**^{AB}	3.1	**36.9**^{BC}	6.5
Butyrate (C4)	***	**0.91**^{B}	0.18	**4.88**^{A}	0.65	**0.94**^{B}	0.19	**1.49**^{B}	1.41	**0.86**^{B}	0.08	**0.92**^{B}	0.04
iso-butyrate (iC4)	ns	**0.011**	0.011	**0.003**	0.004	**0.030**	0.026	**0.031**	0.009	**0.015**	0.007	**0.008**	0.013
iso-valerate (iC5)	ns	**0.061**	0.026	**0.064**	0.070	**0.216**	0.161	**0.101**	0.017	**0.118**	0.029	**0.111**	0.074
Capronate (C6)	*	**0.302**^{A}	0.008	**0.211**^{AB}	0.091	**0.318**^{A}	0.100	**0.095**^{B}	0.010	**0.239**^{AB}	0.026	**0.280**^{AB}	0.069
iso-capronate (iC6)	ns	**0.002**	0.003	**0.006**	0.010	**0.002**	0.004	**0.000**	0.000	**0.004**	0.006	**0.008**	0.001
Total volatile fatty acids	***	**68.7**^{C}	26.7	**71.5**^{C}	13.1	**109.6**^{AB}	11.7	**126.0**^{A}	1.3	**110.3**^{AB}	6.0	**83.4**^{BC}	9.5

see Table 3

ever, there was no correlation between the citrate and the FHL concentrations. "Finland" and "Savoie" had a very high succinate concentration while "Switzerland" had a very low concentration. During propionic acid fermentation, aspartate and indirectly asparagine can be catabolised to succinate. However, a negative correlation was found only with asparagine (r=–0.68, data not shown). "Finland" and "Savoie" showed the highest acetate concentration, "Bretagne" the lowest. Acetate is produced during propionic fermentation and a high negative correlation was found between the acetate and the L+D-lactate concentrations (r=–0.80). In the Bretagne and in Finland, cows are commonly fed with silo forage during the winter months. This agricultural practice is reflected by the much higher butyrate concentration in "Bretagne" than in the other cheese samples. "Finland" also had a high butyrate concentration, but only in one sample (2.49 mmol/kg). The capronate concentration was significantly higher in "Allgäu" and "Switzerland" than in "Finland."

Applying a principal component analysis on the four high discriminating parameters p*H*-value, fat, L-lactate and pyruvate, "Finland," "Savoie" and "Vorarlberg" showed a clear tendency towards separation (Fig. 2). "Switzerland" with even only two parameters (succinate and L-lactate) was quite well separated from the others (Fig. 3).

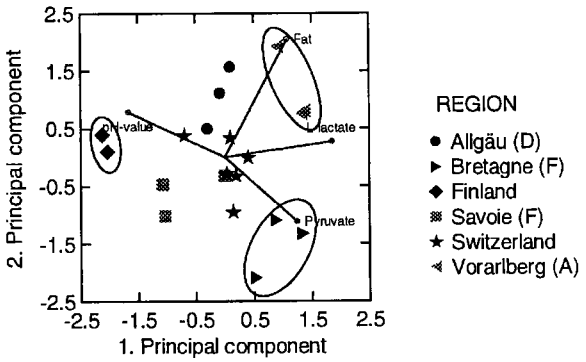

Fig. 2 Principal component analysis of the parameters fat, L-lactate and pyruvate content as well as pH-value. Separation of the groups "Finland," "Vorarlberg" and "Bretagne"

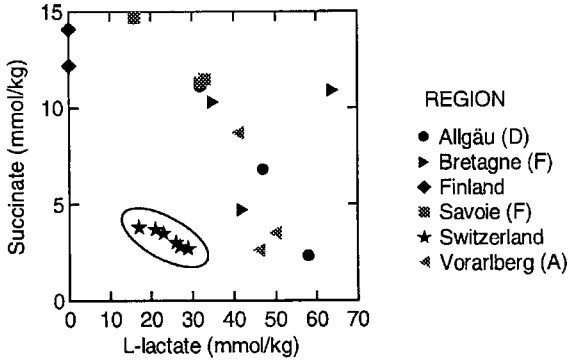

Fig. 3 Discrimination of the Emmentaler Switzerland™ according to L-lactate and succinate

Microbial composition

Table 5 lists the microbial composition of the Emmentalers from the six regions. In general, the microbial counts in Emmentaler Switzerland™ were significantly lower than in cheeses from other regions. All investigated microorganisms were significantly lower in "Switzerland" than in "Savoie." The OHL were significantly

Table 5 Microbial composition of the 20 Emmentaler cheese samples investigated expressed in $\log_{10}$

Region (n=)	ANOVA	AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
Enterococci (cfu/g)	*	**5.18^{AB}**	0.89	**3.3^{AB}**	2.3	**2.1^{B}**	2.3	**3.7^{AB}**	2.4	**6.30^{A}**	0.26	**5.51^{AB}**	0.13
Fac. het. Lb (cfu/g)	*	**8.12^{AB}**	0.26	**8.69^{AB}**	0.92	**7.63^{B}**	0.35	**7.62^{AB}**	0.14	**9.1^{A}**	1.1	**8.09^{AB}**	0.30
Obligate het. Lb (cfu/g)	**	**7.7^{A}**	1.0	**6.03^{A}**	0.58	**2.7^{B}**	2.3	**5.70^{AB}**	0.00	**6.7^{A}**	1.0	**6.37^{A}**	0.58
Propionic acid b. (cfu/g)	ns	**8.31**	0.10	**9.12**	0.70	**8.13**	0.35	**7.99**	0.53	**9.4**	1.3	**8.44**	0.23
Salt tolerant b. (cfu/g)	*	**4.27^{AB}**	0.86	**2.50^{B}**	0.51	**2.5^{B}**	2.0	**3.4^{AB}**	2.3	**5.97^{A}**	0.91	**5.05^{AB}**	0.43
Lb. helveticus (strains/g)	***	**>7^{A}**	n.c.	**>5.8^{A}**	n.c.	**<3.5^{B}**	n.c.	**>5.8^{A}**	n.c.	**>7^{A}**	n.c.	**>7^{A}**	n.c.

see Table 3
n.c.=not calculated

Table 6 Sensory analyses and colour measurement of the 20 Emmentaler cheese samples investigated

Region (n=)	ANOVA	AL(3)		BR(3)		CH(6)		FI(2)		SA(3)		VO(3)	
		$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
Adhesivity	ns	**3.58**	0.48	**2.85**	0.65	**3.15**	0.35	**2.45**	0.35	**3.12**	0.22	**3.17**	0.49
Friability	ns	**3.18**	0.42	**3.14**	0.48	**2.85**	0.37	**2.85**	0.35	**2.75**	0.17	**2.69**	0.32
Elasticity	ns	**3.79**	0.45	**4.19**	0.38	**4.30**	0.28	**4.55**	0.21	**4.44**	0.10	**4.04**	0.21
Firmness	ns	**3.70**	1.06	**4.18**	0.59	**3.59**	0.39	**4.50**	0.85	**3.82**	0.36	**3.40**	0.26
Aroma int.	ns	**3.47**	0.46	**2.97**	0.33	**3.06**	0.32	**3.10**	0.28	**3.27**	0.05	**2.97**	0.34
Odour int.	ns	**3.03**	0.64	**3.14**	0.19	**3.24**	0.24	**3.60**	0.71	**3.16**	0.17	**2.96**	0.04
Bitterness	*	**2.00^{A}**	0.20	**1.54^{C}**	0.17	**1.81^{ABC}**	0.17	**1.95^{ABC}**	0.35	**2.02^{AB}**	0.19	**1.67^{ABC}**	0.08
Saltiness	**	**2.66^{A}**	0.13	**2.27^{AB}**	0.30	**2.04^{B}**	0.27	**2.25^{AB}**	0.07	**2.41^{AB}**	0.05	**1.99^{B}**	0.12
Acidity	*	**2.65^{A}**	0.26	**2.13^{B}**	0.25	**2.05^{B}**	0.16	**2.30^{AB}**	0.28	**2.16^{B}**	0.22	**2.00^{B}**	0.16
Sweetness	ns	**2.45**	0.10	**2.59**	0.10	**2.64**	0.21	**2.90**	0.00	**2.61**	0.33	**2.49**	0.07
L-value[a]	ns	**68.9**	0.45	**69.3**	1.8	**68.9**	1.6	**68.8**	0.57	**67.6**	2.2	**69.4**	1.0
a-value[a]	***	**−2.18^{B}**	0.24	**−2.62^{C}**	0.19	**−2.22^{B}**	0.15	**−1.65^{A}**	0.07	**−2.47^{BC}**	0.23	**−2.32^{BC}**	0.10
b-value[a]	***	**16.63^{AB}**	0.81	**15.57^{B}**	0.45	**15.92^{B}**	0.94	**18.40^{A}**	0.00	**13.87^{C}**	0.40	**16.30^{B}**	0.26

see Table 3
[a] according to Hunter

lower in "Switzerland" compared to all other regions except for "Finland." "Switzerland" showed the lowest concentrations of ECOC. These observations may be explained by research carried out at the Swiss Federal Dairy Research Station. In the 1970s, starter cultures with specifically low values of ECOC, OHL and STB were selected in order to reduce undesirable fermentations. The concentration of PSB was also among the lowest in "Switzerland." The high propionate concentration of these samples indicates however that a part of the PSB must have already died out.

All foreign cheeses contained *Lb. helveticus*. In "Switzerland," the content was below the detection limit of the method applied except for one sample where this species was detected at a level at least one order of magnitude below that of foreign cheeses. In foreign countries, *Lb. helveticus* is commonly used because of its ability to shorten the ripening period. In Switzerland, this bacteria is no longer used in starter cultures, in order to avoid secondary fermentation because of its strong proteolytic activity. Its presence in one Swiss sample may be due to contamination from the environment. The importance of such contamination is not known and further analyses will be necessary to check the occurrence of this contamination.

Lb. helveticus is the single thermophilic lactic acid bacterium producing both L- and D-lactate from lactose. Therefore the absence of this species should induce a decrease of the L/D-lactate ratio. This ratio in Emmentaler Switzerland™ was, however, not significantly different from the others (Table 4).

Sensory analyses

The results of the sensory analyses are listed in Table 6. The observations on texture did not show any difference between the regions of origin. The same was true for the aroma and the odour intensity. "Allgäu" was the most salty, "Switzerland" and "Vorarlberg" were the least salty, in good agreement with the potentiometric chloride measurements carried out (Table 3). "Bretagne" was significantly less bitter than "Allgäu" and "Savoie." "Allgäu" was significantly more acid than all others except "Finland."

The low number of differences in sensory tests explained the difficulty of the panel in determining if a sample originated from Switzerland or not. It must however be pointed out that the panel was not specifically trained on young Emmentaler.

Colour measurements

The parameters (a) and (b) presented considerable differences (Table 6). The Finnish Emmentaler was significantly redder and yellower than the others. "Savoie" on the other hand was significantly less yellow than the others and "Bretagne" more blue. This cannot be explained by the different maturation time of the samples analysed. Namely a decrease in lightness (L), and a slight increase on both reddish (a) and yellowish (b) have been observed [55]. "Finland" and "Bretagne" are, however, the youngest cheese in this study. A possible explanation may be found in the forage. "Finland" uses grass silage whereas "Bretagne" feeds maize silage. In the other regions, silages are not permitted.

Conclusion

Cheese is a living system continuously undergoing microbial, enzymatic and chemical modifications. Therefore, analyses of organic compounds or living microorganisms are only valid for a defined ripening time and data collected on young cheeses may not be valid for more mature samples. For all foreign Emmentaler cheese types, we focused our investigations on cheese samples with a ripening time corresponding to that offered in the stores (i.e. 2.5–4 months). In Switzerland, loaves ripened

for 4 to 6 months are considered to be young cheeses. We limited however our investigation to 4 months Emmentaler Switzerland™ as the risk of confusion is the highest with such young cheeses.

Due to the low number of samples examined, the statistical values presented here are only an indication. General chemical parameters such as fat, fat in dry matter, sodium chloride as well as the pH-value showed significant differences. The concentration of organic acids such as L- and D-lactate, succinate, pyruvate, acetate, propionate, butyrate, capronate and total volatile acid content also were significantly different. The current alkaline phosphatase values (AP) showed too large a variation within a region to be valuable as markers of geographic origin. However, more consistent results of AP could probably be obtained if the samples are taken directly under the rind.

The microbiological ecology of the samples offered very promising results. ECOC, FHL, OHL and STB were all present at significantly lower concentrations ($p<0.05$) in the Emmentaler Switzerland™ than in the foreign cheeses pooled in one group. The presence of *Lb. helveticus* in high amounts in Emmentaler cheese samples appeared to be a reliable indicator of foreign origin since the raw milk starter mixtures used in Switzerland do not contain this species. The importance of possible contamination from the environment must, however, still be investigated. The absence of *Lb. helveticus* does not prove Swiss origin.

Most of the sensory analyses did not show significant differences. Exceptions were the criteria of "bitterness," "saltiness" or "acidity." Cheese from Finland could be easily identified by the colour measurement.

The following analyses were less promising for indication of geographical origin and won't be included in further works: vitamins A and E, water in fat-free matter, citrate, propionic acid bacteria and sensory analyses.

Acknowledgements The authors thank the Swiss Federal Office of Public Health for its financial and logistical support to the project, G. Urbach and G. Gremaud for the reviewing of the publications and J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland™), H. Jatila (Valio Ltd, Finland) and H. Schmallegger (Alma GmbH, Austria) for collecting the samples. A special thank goes to ITFF, Emmentaler Switzerland™, Valio and Alma who kindly offered the samples.

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CHAPTER 6

Analytical methods for the determination of the geographic origin of Emmental cheeses. Free fatty acids, triglycerides and fatty acid composition of cheese fat.

L. Pillonel, M. Collomb, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Mitteilungen aus Lebensmitteluntersuchung und Hygiene, 93, 217-231 (2002)

Introduction

Cow's milk fat is composed of triglycerides consisting of approx. 400 different fatty acids whose composition in milk and milk products is mainly influenced by the forage (1). During summer or by addition of vegetable fats to the fodder, the concentration of long chain unsaturated fatty acids is increased and that of short and medium chain fatty acids (C6-C16) is decreased (2–4). In the case of triglycerides, the group C50-C54 increased as the fodder changed from hay to grass (4) or by addition of vegetable fats (2, 5). Similar results were obtained for the composition of fatty acids and triglycerides by comparing milk fats from cows grazing in the lowlands and highlands (6). In the highlands, the level of unsaturated long chain fatty acids (C18:1, C18:2 and C18:3) was higher, and that of the saturated short and medium chain fatty acids lower (6, 7). Also, the level of short (C28-C36) and medium chain triglycerides (C42-C46) decreased from lowlands to highlands whereas that of long chain triglycerides (C50-C54) increased. The stage of lactation (8, 9) also influenced the composition of milk fat. The content of free fatty acids depended on the lipolytic activity of the microbial flora during cheese making and ripening. Lactic acid bacteria have a very low lipolytic activity (10) whereas the dif-

* Part of the Ph.D. work of Laurent Pillonel

ferent propionic acid bacteria used in the manufacture of Emmental have much higher and differentiated activities (11, 12).

The fatty acid and triglyceride composition could therefore help in discriminating dairy products manufactured in regions of different altitudes or to determine what typical fodder compositions were fed to the cows. The level of free fatty acids may also make it possible to discriminate Emmental cheese making according to the lipolytic activity of the propionic acid bacteria used.

The current work reports an attempt to use free fatty acids and composition of triglycerides and fatty acid to determine the geographic origin of Emmental cheese. It forms the first part of a broad screening test in a 3-year study on the authenticity of Emmental cheese and its geographic traceability (13, 14). During this step, a great number of analytical methods have been tested with respect to their discriminating potential using only two to three cheese samples per region (14–18). It is therefore obvious that the analytical results obtained from such a modest number of cheese samples per region can only give trends which should be confirmed later if they appear valuable for discriminating cheeses produced in Switzerland from those produced in other countries.

Materials and methods

Origin and selection of the cheese samples

The main framework of this study and the sampling have been described in detail in (14). Table 1 summarises the origin, the date of manufacture and the ripening time of these winter Emmental samples as well as the average altitude of the production zones.

Table 1
Origin and ripening time of the 20 cheese samples investigated

Sample	*Region (country)*	*Date of manufacture*	*Ripening time (months)*	*Average altitude (m) of the production sites*
AL 1-3	Allgäu (D)	20.12.2000	4	800
BR 1-3	Bretagne (F)	16.02.2001	2.5	100
CH 1-6	Switzerland (CH)	26.12.2000	4	570
FI 1-2	Middle Finland (FI)	05.02.2001	3	150
SA 1-3	Savoie (F)	08.02.2001	3	470
VO 1-3	Vorarlberg (A)	02.02.2001	3	780

Methods used for the determination of free fatty acid, triglyceride and fatty acid composition of milk fat

The gas chromatographic (GC) determination of the free fatty acids (FFA) was performed according to a method developed by *De Jong and Badings* (19), modified at the FAM. The FFA were extracted in sulfuric acid medium by a solution of

diethylether/heptane 1:1 (v/v). They were retained on an anion-exchange (aminopropyl-) column, the neutral lipids were eluted with hexane/2-propanol 3:2 (v:v) and the FFA with diethyl ether containing 2% of formic acid. For the triglycerides and the composition of fatty acids, the fat was extracted according to an IDF standard (20). For the determination of the triglycerides, the isolated fat was dissolved in heptane and directly injected into the GC according to *Collomb et al.* (21). The fatty acid composition of fat was determined by GC after transesterification to methylesters according to *Collomb and Bühler* (1).

Statistical methods

The averages and standard deviations were calculated for each value. Descriptive statistics, analysis of variance (ANOVA) and pairwise comparisons of mean values with Fisher's LSD test were performed using Systat for Windows version 9.0 (SPSS Inc., Chicago, IL).

Results and discussions

Because of the limited number of cheese samples analysed per region, only compounds showing highly significant inter-region differences (ANOVA, $p \leq 0.001$) have been considered. Consequently, out of the compounds analysed, only two free fatty acids, five triglycerides and 29 fatty acids are discussed. Five groups of fatty acids (medium chain, polyunsaturated, C18:2, conjugated linoleic acids (CLA) and ω-3) showed the required significant differences. We also did not use trained classification techniques such a linear discriminant analysis as they require a large data set in order to be reliable.

Significant differences between the regions of lower and higher altitude

Highly significant differences ($p \leq 0.001$) were found between winter Emmental cheeses produced in the regions of lower altitude Bretagne (BR) and Finland (FI) (100–150 m) compared to those produced in the four other regions of higher altitude (470–800 m). Furthermore, silage was fed only in BR and FI. The concentrations of different fatty acids and triglycerides from cheeses produced in regions of lower altitudes were significantly higher (table 2) or lower (table 3) than those found in cheese from the four regions of higher altitude. Such observations agree with the literature quoted in the introduction.

The concentrations of only one triglyceride (C38) and of six minor C18:1 fatty acids were significantly higher in cheeses produced in BR and FI than in the four other regions of higher altitude (table 2). The concentration of the triglyceride C38 was the highest in cheese produced in BR (13.16 g/100 g), and that from FI (12.93 g/100 g) overlapped with that found in Switzerland (CH) (12.68 g/100 g), BR and Vorarlberg (VO) (12.71 g/100 g). *Precht and Heine* (22) found a mean value of this triglyceride generally higher in winter fat (12.7 g/100 g; $s_x = 0.32$; $n = 283$) than in summer fat (12.13; $s_x = 0.27$; $n = 309$). *Collomb et al.* (6) found similar values to those

Table 2
Compounds where the two lowland regions BR and FI showed significantly ($P \leq 0.001$) higher values than the regions bordering the Alps

Region[1] (n=)	*Overlapping[2]*	*AL (3)*		*BR (3)*		*CH (6)*		*FI (2)*		*SA (3)*		*VO (3)*	
		x	s_x	*x*	s_x	*x*	s_x	*x*	s_x	*x*	s_x	*x*	s_x
Triglyceride (g/100 g triglyceride)													
Triglyceride 38	fi	*12.42^{D}*	0.27	*13.16^{A}*	0.09	*12.68^{BCD}*	0.25	*12.93^{AB}*	0.05	*12.36^{CD}*	0.10	*12.71^{BCD}*	0.07
Fatty acids composition (g/100 g fat)													
C18:1 t12	fi/br	*0.163^{AB}*	0.005	*0.193^{A}*	0.006	*0.136^{BC}*	0.023	*0.197^{A}*	0.006	*0.116^{BC}*	0.025	*0.111^{C}*	0.027
C18:1 t13-14+c6-8	fi	*0.411^{BC}*	0.002	*0.547^{A}*	0.086	*0.393^{C}*	0.053	*0.539^{AB}*	0.015	*0.307^{C}*	0.051	*0.340^{C}*	0.030
C18:1 c11	fi	*0.467^{BC}*	0.029	*0.545^{A}*	0.012	*0.444^{C}*	0.032	*0.505^{AB}*	0.006	*0.455^{BC}*	0.015	*0.458^{BC}*	0.019
C18:1 c12	–	*0.143^{B}*	0.013	*0.208^{A}*	0.006	*0.123^{B}*	0.019	*0.181^{A}*	0.008	*0.124^{B}*	0.002	*0.116^{B}*	0.011
C18:1 c13	fi	*0.049^{BC}*	0.004	*0.064^{A}*	0.002	*0.048^{C}*	0.004	*0.058^{AB}*	0.004	*0.040^{D}*	0.002	*0.040^{D}*	0.005
C18:1 t16+c14	fi	*0.225^{C}*	0.010	*0.338^{A}*	0.027	*0.258^{BC}*	0.017	*0.291^{AB}*	0.004	*0.248^{BC}*	0.008	*0.232^{C}*	0.039

Caption: *x*=mean value; s_x=standard deviation

Production sites: A>B>C>D (=significantly different contents $p \leq 0.001$) or AB=A and B overlap by using an univariate discriminant analysis

[1] AL=Allgäu, BR=Bretagne, CH=Switzerland, FI=Finland, SA=Savoie, VO=Vorarlberg

[2] fi/br=the group "Finland"/"Bretagne" overlaps with at least one other group from the regions bordering the Alps

Table 3
Compounds where the two lowland regions BR and FI showed significantly (P≤0.001) lower values than the regions bordering the Alps

Region (n=)	*Overlapping*	*AL (3)*		*BR (3)*		*CH (6)*		*FI (2)*		*SA (3)*		*VO (3)*	
		x	s_x	*x*	s_x	*x*	s_x	*x*	s_x	*x*	s_x	*x*	s_x
Triglyceride / Free fatty acids													
Triglyceride 48 (g/100 g triglyceride)	br	*9.38*^{A}	0.15	*8.96*^{AB}	0.08	*9.17*^{A}	0.27	*8.53*^{B}	0.08	*9.35*^{A}	0.12	*9.35*^{A}	0.02
Free linolenic acid (mg/kg)	–	*35.7*^{B}	3.3	*17.1*^{C}	2.7	*34.8*^{B}	7.5	*13.4*^{C}	2.6	*35.5*^{B}	0.3	*51.3*^{A}	8.9
Fatty acids composition (g/100 g fat)													
C13 iso	–	*0.137*^{AB}	0.005	*0.088*^{C}	0.004	*0.124*^{B}	0.013	*0.075*^{C}	0.003	*0.157*^{A}	0.007	*0.142*^{AB}	0.003
C14	–	*10.33*^{AB}	0.15	*9.68*^{C}	0.07	*10.13*^{B}	0.23	*9.56*^{C}	0.04	*10.55*^{A}	0.18	*10.24*^{AB}	0.19
C14 iso	–	*0.240*^{B}	0.005	*0.203*^{C}	0.001	*0.240*^{B}	0.018	*0.185*^{C}	0.004	*0.304*^{A}	0.008	*0.245*^{B}	0.006
C14 aiso	–	*0.460*^{AB}	0.020	*0.384*^{C}	0.006	*0.419*^{BC}	0.038	*0.363*^{C}	0.007	*0.479*^{A}	0.017	*0.461*^{AB}	0.013
C14:1 c	fi	*0.913*^{AB}	0.024	*0.806*^{D}	0.015	*0.917*^{A}	0.040	*0.838*^{BCD}	0.023	*0.824*^{CD}	0.021	*0.905*^{ABC}	0.033
C15	–	*1.057*^{A}	0.047	*0.906*^{B}	0.014	*1.060*^{A}	0.060	*0.846*^{B}	0.003	*1.077*^{A}	0.016	*1.071*^{A}	0.038
C15 iso	fi/br	*0.262*^{BC}	0.019	*0.240*^{BCD}	0.011	*0.242*^{CD}	0.023	*0.213*^{D}	0.003	*0.322*^{A}	0.010	*0.279*^{B}	0.013
C16 iso	br	*0.331*^{AB}	0.001	*0.291*^{CD}	0.007	*0.326*^{B}	0.017	*0.277*^{D}	0.004	*0.339*^{AB}	0.008	*0.313*^{BC}	0.006
C18:1 t10–11	fi	*2.05*^{A}	0.04	*1.17*^{D}	0.04	*1.56*^{BC}	0.29	*1.20*^{CD}	0.00	*1.44*^{BCD}	0.18	*1.76*^{AB}	0.05
C18:2 c9t13+(t8c12)	fi/br	*0.172*^{A}	0.016	*0.175*^{A}	0.009	*0.146*^{AB}	0.019	*0.180*^{A}	0.013	*0.118*^{B}	0.008	*0.126*^{B}	0.010
C18:3 c9c12c15	–	*0.884*^{A}	0.063	*0.280*^{C}	0.053	*0.781*^{AB}	0.162	*0.372*^{C}	0.032	*0.673*^{AB}	0.043	*0.915*^{AB}	0.075
C18:2 c9t11	fi	*0.935*^{A}	0.020	*0.410*^{D}	0.010	*0.660*^{B}	0.131	*0.411*^{CD}	0.010	*0.623*^{BC}	0.061	*0.771*^{AB}	0.020
C18:2 c9c11	fi/br	*0.054*^{A}	0.009	*0.013*^{C}	0.004	*0.036*^{B}	0.011	*0.017*^{C}	0.001	*0.029*^{BC}	0.005	*0.045*^{AB}	0.003
C22:5 (DPA) (n-3)	–	*0.104*^{AB}	0.006	*0.051*^{C}	0.003	*0.101*^{AB}	0.008	*0.046*^{C}	0.001	*0.087*^{B}	0.009	*0.108*^{A}	0.014
Groups of fatty acids (g/100 g fat)													
Sum C18:2	fi	*3.29*^{A}	0.03	*2.44*^{D}	0.05	*2.75*^{BC}	0.16	*2.49*^{CD}	0.16	*2.67*^{C}	0.07	*2.97*^{B}	0.08
Sum polyunsaturated FA[1]	–	*4.61*^{A}	0.09	*3.06*^{D}	0.11	*3.97*^{BC}	0.31	*3.13*^{D}	0.14	*3.76*^{C}	0.14	*4.32*^{AB}	0.17

Region (n=)	*Overlapping*	*AL (3)*		*BR (3)*		*CH (6)*		*FI (2)*		*SA (3)*		*VO (3)*	
		$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
Sum CLA[2]	fi	1.01^{A}	0.03	0.44^{D}	0.01	0.72^{B}	0.15	0.45^{CD}	0.01	0.67^{BC}	0.06	0.85^{AB}	0.03
Sum ω-3[3]	fi	1.42^{A}	0.11	0.61^{D}	0.09	1.25^{AB}	0.22	0.66^{CD}	0.01	1.04^{BC}	0.08	1.40^{AB}	0.11

Caption: see table 2; FA=fatty acids; DPA=cis 7, cis 10, cis 13, cis 16, cis 19-docosapentaenoic acid according to reference (1).

[1] C18:2 -ttNMID to -c9c15, C18:3 -c6c9c12+-c9c12c15, C18:2 -c9t11 à C20:2 cc, C20:3 à C22:6

[2] CLA total (sum C18:2 -c9t11, -c9c11, -t9t11)

[3] C18:2 -t11c15+c9c15, C18:3 c9c12c15, C20:3 n-3, C20:5, C22:5 and C22:6

found in BR and FI in summer milk fat (13.4 g/100 g; $n=13$) produced in Switzerland at an altitude of 600–650 m and much lower values (12.0–12.5 g/100 g; $n=36$) at higher altitudes (900–2120 m). However, the concentration of this compound which ranged normally from 11.39 to 13.95 g/100 g ($n=592$) in milk fat (22), is very dependent on the fodder.

The concentrations of the six minor unsaturated fatty acids were the highest in BR and those of the fatty acids C18:1 -t12 and -c12 the highest in BR and FI. These results agreed better with the values generally measured in Switzerland for summer than for winter milk fat (1) and could be due to the influence on milk fat of the silage fed to the cows in FI and BR.

The concentrations of one triglyceride (C48), one free fatty acid (linolenic acid), fourteen fatty acids and four groups of fatty acids (C18:2, polyunsaturated, conjugated linoleic acid and ω-3) were significantly lower in cheese from BR and FI than in samples from the four other regions of higher altitude (table 3). The concentration of triglyceride C48 was the lowest in cheese from FI, and that from BR overlapped with that found in VO, Savoie (SA) and Allgäu (AL). *Precht and Heine* (22) found a mean value for this compound generally higher in winter fat (9.13 g/100 g; $s_x=0.38$; $n=283$) than in summer fat (9.07; $s_x=0.39$; $n=309$). *Collomb et al.* (6) found a trend towards higher values (8.15 to 8.60 g/100 g, $n=49$) as a function of the altitude but the results were not significantly different. In this study, the lower concentration found in BR (8.96 g/100 g) and FI (8.53 g/100 g) compared to that found in other countries (9.17–9.38 g/100 g) could be attributed to the influence of silage and/or to the altitude (lower diversity of plant species in hay (23)). Here again, the concentration of the triglyceride C48 in milk fat normally lies in a much broader range (7.91 to 10.65 g/100 g; $n=592$) (22) than that found in this study.

Except for the C18:2 c9t13+(t8c12), the fatty acids present in a lower concentration in cheeses from BR and FI than in those from other countries were essentially iso, anteiso and unsaturated long chain fatty acids. The concentration of myristic acid (C14) is generally higher in winter fat than in summer fat. That of the iso and anteiso compounds gives generally similar values in the two seasons. That of the unsaturated long chain fatty acids is generally higher in summer milk fat than in winter milk fat (1) and increased with the altitude (6, 7, 23). However, the concentration of fatty acids normally varies within a broader range than that found in this study. For example, *Precht and Molkentin* (24) found in 100 milk fats values ranging from 8.90 to 13.57 g/100 g for the myristic acid and from 0.34 to 1.06 for linolenic acid (C18:3 c9c12c15). For these two compounds, the values found within the six regions considered ranged from 9.56 to 10.55 g/100 g fat for the myristic acid and from 0.28 to 0.88 g/100 g fat for the second compound. The low concentration of esterified as well as free linolenic acid in cheeses from BR and FI can be attributed to a low concentration of this compound in silage fat. The lowest concentration of free linolenic acid in BR (17.1 mg/kg) and FI (13.4 mg/kg) compared to that found in other countries (34.8–51.3 mg/kg) may have the same cause. Another explanation

may be the different lipolytic activity of the propionic acid bacteria used for cheese making. In a study carried out at the FAM, the concentrations of free linolenic acid ranged from 23 to 57 mg/kg Emmental cheese depending on the bacteria used (FAM, unpublished results). However in the current study, the sum of the thirteen free fatty acids analysed (without butyric acid) did not show any significant differences.

The concentrations of groups of fatty acids (Σ C18:2, Σ polyunsaturated, Σ CLA and Σ ω-3) were the highest in AL and VO and the lowest in BR and FI. Table 4 presents the sum of the CLA and the content of the fatty acids C18:1 t10+t11 as a function of the altitude.

Table 4
Influence of altitude on the concentration of CLA and C18:1 t10-11 in Emmental cheese

Forage type	*Region (altitude)*	*CLA (g/100 g fat)*	*C18:1 t10-11 (g/100 g fat)*
Maize and grass silage, hay	Bretagne (100 m)	0.44	1.17
Grass silage, hay	Finland (100–150 m)	0.45	1.20
Hay	Savoie (470 m)	0.67	1.44
Hay	Switzerland (570 m)	0.72	1.56
Hay	Vorarlberg (780 m)	0.85	1.76
Hay	Allgäu (800 m)	1.01	2.05

The contents of both sums of fatty acids increased as a function of the altitude. The elevated concentration of the sum of the t10 and t11 fatty acids is likely to be mainly due to that of the trans vaccenic acid (C18:1 t11) from which the CLA is endogenously synthesised (25). According to *Precht and Molkentin* (26), trans vaccenic acid normally represents approximately 90% of the total of the two acids t10 and t11, which cannot be separated using our chromatographic conditions. These authors showed in feeding tests – where cows stayed in the barn or on pasture or were fed with rape oil, rapeseed wholemeal or pellets – that the increase in the CLA concentration in milk fat usually correlates with the concentration of trans fatty acids. *Collomb et al.* (23, 27) found significantly different values for the sum of CLA and C18:1 t10+t11 fatty acids in milk fat produced from cows grazing in lowlands (600–650 m), mountains (900–1210 m) and highlands (1275–2120 m). The concentrations of CLA, compounds well-known for their positive effects on health (28), increased from 0.87 to 1.61 and to 2.36 g/100 g fat (maximum value: 2.87 g/100 g fat) as a function of the altitude. The concentration of the fatty acids C18:1 t10+t11 increased similarly from 2.11 to 3.66 and to 5.10 g/100 g fat (maximum value: 5.67 g/100 g fat). In the current study, the values obtained were relatively low

compared to those of summer milk fats in high altitude but corresponded to values for summer milk fats from AL and VO.

The lower concentration of the long chain polyunsaturated fatty acids found in BR and FI can be correlated with a silage fodder fat poor in polyunsaturated fatty acids (23). The concentration of the ω-3 compounds ranged from 0.61 to 1.42 g/100 g cheese fat. *Collomb et al.* (23) found increasing values as a function of the altitude (1.39 to 2.09 g/100 g fat). The low value found in BR (0.61 g/100 g fat) and FI (0.66 g/100 g) is due to the low level of linolenic acid (C18:3 c9c12c15), the main ω-3 compound of milk fat.

Significant differences between Finland and other countries

Table 5 presents the concentrations of compounds which were the highest or the lowest in FI cheeses compared to those produced in other countries.

The concentrations of the triglycerides C40 and C54 and those of the fatty acids C18, C18:1 t9, C20 and C20:1 c11 were the highest in FI cheeses. *Precht and Heine* (22) found lower mean values for the triglycerides C40 and C54 in winter fat (C40:9.78 g/100 g; s_x=0.31; n=283; C54:3.99 g/100 g; s_x=0.87; n=283) than in summer fat (C40:10.01; s_x=0.28; n=309; C54:6.58; s_x=0.82; n=309). For the triglyceride C40, *Collomb et al.* (6) found significantly higher values in lowlands (10.4 g/100 g; n=13) compared to the highlands 9.84–10.0 g/100 g; n=24) and much lower values for the triglyceride C54 in the lowlands (4.83 g/100 g) compared to the highlands (7.48–7.78 g/100 g). In this study, the high concentrations of the triglycerides C40 (10.34 g/100 g) and C54 (5.01 g/100 g) in FI cheeses were rather in the range of the values generally obtained for summer fats. These two triglycerides probably consist on a high extent of oleic acid (C18:1 c9) as confirmed by the highest level of this acid (results not tabulated) found in cheese fat from FI (16.72 g/100 g fat) compared to that found in cheese from the other regions (14.22–15.44 g/100 g fat). The concentration of stearic acid (C18) was the highest in cheese from FI (10.20 g/100 g fat) compared to that from the other regions (7.31–8.42 g/100 g fat). This result could be attributed to the high level of oleic acid in grass silage combined with a high biohydrogenation activity in the rumen of the cow (23). The normal range in milk fat lies between 6.12 and 12.50 g/100 g (24).

The concentration of the triglyceride C46 and that of palmitic acid (C16) as well as of other minor fatty acids difficult to interpret were the lowest in FI cheeses. *Precht and Heine* (22) found higher mean values for the triglyceride C46 in winter fat (7.55 g/100 g; s_x=0.51; n=283) than in summer fat (6.82 g/100 g; s_x=0.47; n=309). For this triglyceride *Collomb et al.* (6) found significantly higher values in lowlands (6.67 g/100 g) than in the highlands (6.08–6.31 g/100 g). In this study, the low concentration of the C46 triglyceride (7.07 g/100 g) in FI cheese were once again rather in the range of summer fat and could be due to the silage fodder based on grass. The lower content of the fatty acid C16 found in FI cheese (24.66 g/100 g) supports this

Table 5
Compounds where only FI was significantly different (P≤0.001) from all other regions

Region (n=)	*AL (3)*		*BR (3)*		*CH (6)*		*FI (2)*		*SA (3)*		*VO (3)*	
	x	s_x	*x*	s_x	*x*	s_x	*x*	s_x	*x*	s_x	*x*	s_x
Higher value												
Triglyceride 40 (g/100 g triglyceride)	9.68^{B}	0.12	9.90^{B}	0.10	9.77^{B}	0.24	10.34^{A}	0.04	9.56^{B}	0.05	9.62^{B}	0.06
Triglyceride 54 (g/100 g triglyceride)	3.92^{B}	0.14	3.70^{B}	0.20	3.73^{B}	0.37	5.01^{A}	0.13	3.64^{B}	0.12	3.49^{B}	0.15
C18 (g/100 g fat)	7.81^{BC}	0.16	8.42^{B}	0.25	7.55^{BC}	0.62	10.20^{A}	0.55	7.31^{C}	0.16	7.33^{BC}	0.28
C18:1 t9 (g/100 g fat)	0.213^{B}	0.016	0.231^{B}	0.024	0.199^{B}	0.025	0.292^{A}	0.013	0.209^{B}	0.017	0.192^{B}	0.008
C20 (g/100 g fat)	0.141^{B}	0.008	0.122^{C}	0.006	0.141^{B}	0.009	0.171^{A}	0.009	0.145^{B}	0.008	0.134^{B}	0.002
C20:1 c11[1]	0.052^{AB}	0.003	0.038^{C}	0.002	0.047^{B}	0.003	0.057^{A}	0.002	0.044^{BC}	0.002	0.039^{C}	0.007
Lower value												
Triglyceride 46 (g/100 g triglyceride)	7.74^{AB}	0.18	7.46^{B}	0.06	7.72^{AB}	0.18	7.07^{C}	0.13	7.81^{A}	0.12	7.68^{AB}	0.01
C16 (g/100 g fat)	27.17^{A}	0.49	27.91^{A}	0.78	27.78^{A}	0.75	24.66^{B}	0.26	27.05^{A}	0.27	27.73^{A}	0.92
C17 (g/100 g fat)	0.540^{A}	0.014	0.519^{A}	0.070	0.581^{A}	0.072	0.265^{B}	0.163	0.600^{A}	0.073	0.667^{A}	0.063
C17 iso (g/100 g fat)	0.062^{A}	0.007	0.068^{A}	0.009	0.068^{A}	0.007	0.036^{B}	0.003	0.079^{A}	0.001	0.074^{A}	0.014
C17 aiso (g/100 g fat)	0.212^{A}	0.015	0.207^{A}	0.003	0.221^{A}	0.010	0.16^{B}	0.006	0.222^{A}	0.006	0.217^{A}	0.007
Sum medium chain FA[2]	46.00^{A}	0.79	45.59^{A}	0.87	46.29^{A}	1.11	41.66^{B}	0.22	46.27^{A}	0.54	46.27^{A}	1.23

Caption: see table 2

[1] FI not significantly different from AL

[2] C12 to C16:1 c according to reference (1)

interpretation and corresponds to a concentration found for summer milk fat in lowlands regions (23).

Significant differences between Bretagne and other countries

Table 6 presents the concentrations of compounds which were the highest or the lowest in BR cheeses compared to those produced in other countries.

The concentration of free butyric acid, and minor fatty acids difficult to interpret, were respectively the highest or the lowest in BR. Butyric acid is produced by butyric acid bacteria which are commonly found in silage.

Conclusion

Free fatty acid, triglyceride and fatty acid (FA) composition of Emmental cheese fat were investigated to find potential markers of geographic origin. The fat composition of milk and milk products is strongly correlated with the forage fed to the herd. The latter depends on the season, on regional parameters such as altitude, geology, type of agriculture and on further non-regional parameters such as type of concentrates used and addition of vegetable oils. The cheeses investigated in this study all originated from winter productions. In "Bretagne" and "Finland", feeding with silage is permitted, whereas it is prohibited in the other regions. The differences in altitude and silage feeding are the most likely explanation for the highly significant differences ($P \leq 0.001$) found between these two regions and the others. For groups of fatty acids such as the C18:2, polyunsaturated FA, conjugated linoleic acid and ω-3 FA, "Bretagne" and "Finland" showed the lowest values. The butyric acid content was the highest in those regions. It is thereafter only possible to discriminate between the three groups FI, BR and the remaining regions.

Moreover, these differences may partly disappear during summer production while fodder is based mainly on fresh grass. Consequently, it will not be possible to interpret any fatty acids composition during the transition phases between summer and winter production, which represents almost half a year's production. Finally, the composition of the forage changes during a season. Any addition of a new type of cereal or vegetable fat will modify the fatty acid profile of the milk. Thereafter, apart for the higher content of trans vaccenic acid and conjugated linoleic acids in summer Emmental cheese produced at higher altitude, the fat composition is not adequate for discriminating between the various geographic origins of Emmental cheese.

Acknowledgements

The authors thank the Swiss Federal Office of Public Health for its financial and logistical support to the project, *G. Urbach* and *G. Gremaud* for their reviewing of the publication as well as *J.F. Chamba* (ITFF, France), *M. Carl* (MUVA Germany), *H. Spring* (Emmentaler Switzerland™), *H. Jatila* (Valio Ltd, Finland) and *H. Schmallegger* (Alma GmbH, Austria) for collecting the samples.

Table 6
Compounds where BR was significantly different (P≤0.001) from all other regions

Region (n=)	*AL (3)*		*BR (3)*		*CH (6)*		*FI (2)*		*SA (3)*		*VO (3)*	
	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
Higher value												
Free butyric acid (mg/kg)	*87.2*^{B}	16.3	*425.3*^{A}	41.4	*85.9*^{B}	17.9	*169.5*^{B}	175.0	*83.3*^{B}	5.1	*88.5*^{B}	3.0
C16:1 c (g/100 g fat)	*1.21*^{B}	0.03	*1.37*^{A}	0.04	*1.24*^{B}	0.06	*1.07*^{C}	0.01	*1.19*^{BC}	0.06	*1.16*^{BC}	0.05
Lower value												
C20:1 c9 (g/100 g fat)	*0.119*^{A}	0.007	*0.091*^{B}	0.003	*0.119*^{A}	0.007	*0.125*^{A}	0.006	*0.118*^{A}	0.008	*0.112*^{A}	0.008
C20 (g/100 g fat)	*0.141*^{B}	0.008	*0.122*^{C}	0.006	*0.141*^{B}	0.009	*0.171*^{A}	0.009	*0.145*^{B}	0.008	*0.134*^{B}	0.002

Caption: see table 2

Summary

Free fatty acids, triglycerides and fatty acid composition in cheese fat were investigated on 20 Emmental cheese samples from six regions in Europe to find the best parameters for discriminating between their different geographic origins. These samples originated from a winter production. "Bretagne" and "Finland", where silage feeding is permitted and which are located close to sea level, were easily differentiated from the other regions. The concentration of some groups of polyunsaturated fatty acids such as C18:2 , conjugated linoleic acids and ω-3 was significantly lower. However, the ranges of values obtained in this study were always smaller than those found in broader studies. The butyric acid content was higher in the two regions with silage feeding. These differences may however partially disappear during summer production. The other regions showed almost no significant differences. The high variability of the fatty acid composition due to both seasonal and feeding effects leads to the conclusion that theses methods are not adaquate for discriminating between the geographic origins of Emmental cheeses.

Zusammenfassung

In 20 Emmentalerproben (Winterproduktion) von sechs europäischen Regionen wurden die freien Fettsäuren, die Triglyzeride und die Zusammensetzung der Fettsäuren des Käsefettes studiert. Ziel dieser Untersuchung war, aussagekräftige Parameter zu finden, um diese Käse nach ihrer geographischen Herkunft zu unterscheiden. Die Käse der Regionen «Bretagne» und «Finnland», wo die Verfütterung von Silage erlaubt ist und die in der Nähe des Meeres liegen, sind von den anderen leicht zu unterscheiden. Dabei waren einige mehrfach ungesättigte Fettsäuren wie C18:2, konjugierte Linolsäuren und ω-3 in signifikant geringerer Konzentration vorhanden. Doch ist die Spannbreite der Werte in dieser Studie immer schmaler als in umfangreicheren Studien. Der Buttersäuregehalt war dort höher als in den Käsen der Regionen ohne Silageverfütterung. Diese Unterschiede können während der Sommerproduktion teilweise verschwinden. In den Käsen der anderen Regionen konnten nur geringe Unterschiede festgestellt werden. Die geographische Herkunft der Emmentaler Käse kann mit diesen Methoden aufgrund der grossen saisonal und fütterungsbedingten Variabilität der Fettsäurezusammensetzung nicht unterschieden werden.

Résumé

Les acides gras libres, les triglycérides et la composition en acides gras de la matière grasse du fromage ont été étudiés dans 20 échantillons d'emmental de productions hivernales provenant de six régions d'Europe pour trouver les meilleurs régresseurs susceptibles de discriminer leur origine géographique. Les régions de «Bretagne» et «Finlande», où l'affouragement au silo est autorisé et qui sont proches du niveau de la mer, sont facilement différenciées des autres. La concentration de quelques groupes d'acides gras polyinsaturés tels que C18:2, linoléiques conjugués

et ω-3 était significativement plus faible que dans les autres régions. Cependant les plages de valeurs trouvées étaient toujours moins étendues que celles publiées lors d'études plus larges. La teneur en acide butyrique y était plus haute que dans les régions sans ensilage. De telles différences peuvent pourtant s'atténuer durant les productions estivales. Les autres régions n'ont montré que peu de différences. La forte variabilité de la composition de ces acides, liée tant à la saison qu'à l'affouragement, rend ces méthodes inadéquates pour discriminer l'origine géographique de ces fromages.

Key words
Emmental, Fatty acid, Triglyceride, Authenticity, Traceability

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Corresponding author: Dr. Jacques Olivier Bosset, Federal Dairy Research Station, Liebefeld, CH-3003 Berne, E-mail: jacques-olivier.bosset@fam.admin.ch

CHAPTER 7

Analytical methods for the determination of the geographic origin of Emmental cheeses. Parameters of proteolysis and rheology.

L. Pillonel, B. Albrecht, R. Badertscher, U. Bütikofer, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
J.F. Chamba
ITFF, 74801 La Roche/Foron (France)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Italian Journal of Food Science, 1, 49-62 (2003)

ABSTRACT

Nitrogen fractions, biogenic amines, free amino acids, casein fractions, HPLC-peptide profiles and rheological properties were investigated in 20 Emmental cheeses from six European regions (Switzerland, Vorarlberg (A), Allgäu (D), Finland, Bretagne (F) and Savoie (F)). The ripening times of the samples differed, according to what is found on the market in each region. The non-protein nitrogen and the water-soluble nitrogen fractions showed significant inter-regional differences due

RIASSUNTO

Le frazioni azotate, le ammine biogene, gli amminoacidi liberi, le frazioni caseiniche, i profili dei peptidi-HPLC e le proprietà reologiche sono stati studiati in 20 formaggi Emmental provenienti da sei diverse regioni europee (Svizzera, Vorarlberg (A), Allgäu (D), Finlandia, Bretagne (F) e Savoie (F)). Le frazioni dell'azoto non proteico e dell'azoto solubile in acqua hanno mostrato differenze inter-regionali significative. "Allgäu" e "Savoie" hanno rivelato il più alto tasso di proteolisi mentre

- Key words: authenticity, Emmental cheese, free amino acid, peptide, proteolysis, traceability -

mostly to the different ripening times. "Allgäu" and "Savoie" showed the highest proteolysis rate and "Bretagne" had the lowest. Biogenic amines and rheological properties were of lesser significance for discriminating geographic origin. The relative amounts of five free amino acids allowed "Switzerland" to be separated from the others. The X3 (unknown structure) and α_{s1} casein fractions combined with one peak from the peptide profile allowed three distinct groups to be differentiated: "Finland", "Bretagne" and "Savoie".

"Bretagne" quello più basso. Le ammine di provenienza biologica e le proprietà reologiche si sono mostrate meno importanti nel determinare l'origine geografica dei formaggi. I contenuti relativi di cinque amminoacidi hanno permesso di separare "Switzerland" dagli altri. Le frazioni della caseina X3 (struttura sconosciuta) e α_{s1}, assieme a uno picco del profilo dei peptidi, hanno permesso di differenziare tre gruppi diversi: rispettivamente quello "Finland", quello "Bretagne" e quello "Savoie".

INTRODUCTION

The enzymatic degradation of casein into peptides and amino acids is a very complex process because of the various origins of the proteases and peptidases involved. They may come from the milk itself (plasmin, cathepsin), the coagulant (chymosin, pepsin and other fungal proteases), the starter, as well as non-starter micro-organisms, secondary cultures (e.g., *P. camemberti*) and exogeneous proteinases or peptidases. Proteolysis occurs to a great extent during ripening. The degree of protein breakdown can be expressed by factors such as the content of water soluble nitrogen (WSN), non-protein nitrogen (NPN) or total free amino acids. Proteolysis has been considered as the basis for classifying different cheese types (MARCOS *et al.*, 1979; SMITH and NAKAI, 1990; AISHIMA and NAKAI, 1987; McGOLDRICK and FOX, 1999; FOX, 1993). The evolution of proteolysis, based on peptide mapping at various stages of ripening, has already been studied for Emmental (BICAN and SPAHNI, 1993, BÜTIKOFER *et al.*, 1997) Gruyère, Appenzell (BICAN and SPAHNI, 1993), Parmigiano-Reggiano (ADDEO *et al.*, 1994), Blue cheese (GONZALEZ DE LLANO *et al.*, 1991) and for several artisanal cheeses (GONZALEZ DE LLANO *et al.*, 1995). The influence of specific Lactobacilli strains on the peptide profile has been studied in cheeses such as Emmental (BÜTIKOFER *et al.*, 1997; CHOPARD *et al.*, 2001) and Cheddar (McSWEENEY *et al.*, 1994; PRIPP *et al.*, 1999). Considerable differences were found between peptide profiles of Cheddar cheese made from raw or pasteurised milk (McSWEENEY *et al.*, 1993).

Further degradation of peptides by peptidases leads to small peptides and free amino acids (FAA). The total concentration of FAA is also an indicator of age. RESMINI *et al.* (1985, 1993) identified genuine Parmigiano-Reggiano and Grana Padano cheese by their free amino acid composition. BÜTIKOFER and FUCHS (1997) were able to correctly classify Emmental, Gruyère and Sbrinz with more than 93% accuracy from their relative amounts of free amino acids. Appenzeller and Tilsiter could be correctly classified to 70-75%.

The degradation of the free amino acids through enzymatic decarboxylation may lead to the formation of biogenic amines. In cheese, non-starter bacteria such as enterococci, salt tolerant lactobacilli and enterobacteriaceae may be responsible for the formation of biogenic amines (JOOSTEN and NORTHOLT,

1987). Some of these amines may provoke allergies or illness. The best known amines in cheese are histamine, tyramine, cadaverine, putrescine, tryptamine and β-phenylethylamine (SIEBER and LAVANCHY, 1990).

Rheological characteristics are often correlated with proteolysis (BOSSET *et al.*, 1993; EBERHARD, 1985). Large peptides are important for the development of the correct texture. PIRISI *et al.* (2000) described large differences in mechanical properties of similar PDO ewe's milk cheeses from three different countries. Cheeses from milk produced on mountain pastures exhibited different rheological properties than those produced with milk from valley pastures; they were less elastic and less deformable (BUGAUD *et al.*, 2001).

The present work is part of a broad screening test, which is the first step in a 3-year study on the authenticity of Emmental cheese and its geographic traceability (PILLONEL *et al.*, 2002a; BOSSET, 2001). To date, a large number of analytical methods have been tested for their discriminating potential (PILLONEL *et al.*, 2002a-b; PILLONEL *et al.* 2003a-c), while the number of cheese samples for each region has been limited. Obviously, the analytical results obtained from a modest number of cheese samples from a region can only give trends which will need to be confirmed later if they appear to be of value for discriminating cheeses produced in different countries. The objective of the present paper was to determine whether chemical and physical parameters linked to proteolysis, such as nitrogen fractions, biogenic amines, free amino acids, casein fractions, peptide pattern and rheological analyses make it possible to discriminate between the different geographic origins of Emmental cheese samples. The goal was not to build a detailed model for predicting the origin but rather to observe the trends. The cheese samples, with different ripening times, were chosen from what is sold by retailers in the corresponding regions.

MATERIALS AND METHODS

Origin and selection of the cheese samples

The main framework of this study and the sampling methods are described in detail in a previous work (PILLONEL *et al.*, 2002a). Table 1 summarises the origin, date of production and ripening time of the samples.

Analysis of the compounds produced by proteolysis

The samples were deep-frozen prior to the following analyses: total nitrogen (TN), water-soluble nitrogen (WSN) and non-protein nitrogen (NPN) according to Kjeldahl (COLLOMB *et al.*, 1990); free amino acids (FAA) using HPLC after pre-column derivatisation with o-phthalaldehyde and fluorenylmethyl chloroformate (FMOC) (BÜTIKOFER and ARDÖ,

Table 1 - Origin and ripening time of the 20 cheese samples investigated.

Abbreviation	Region (country)	Number of samples	Date of manufacture	Ripening time (months)
AL	Allgäu (D)	3	25.12.2000	4
BR	Bretagne (F)	3	20.02.2001	2.5
CH	Switzerland (CH)	6	26.12.2000	4
FI	Middle Finland (FI)	2	04.02.2001	3
SA	Savoie (F)	3	05.02.2001	3
VO	Vorarlberg (A)	3	02.02.2001	3

1999); biogenic amines with HPLC after precolumn derivatisation with dansylchloride (BÜTIKOFER *et al.*, 1990); o-phthalaldehyde-value (OPA) photometrically (FRISTER *et al.*, 1989). Casein fractions were determined by SDS electrophoresis (COLLIN *et al.*, 1987). The peptide pattern of the water-soluble peptide fraction was investigated by RP-HPLC (CHOPARD *et al.*, 2001).

Rheological analyses

The following three rheological properties were measured with a universal testing machine (Zwick 1435, Zwick GmbH, Germany) at 15±0.5°C according to the procedure of PESENTI and LUGINBUEHL (1999): i) the stress at 33% deformation, which characterises the elasticity of the body, ii) the strain at fracture, which is a measure of the consistency of the body and iii) the stress at fracture, which corresponds, more or less, to the sensation of hardness in the mouth. The measurements were carried out on a cylindrical, holeless cheese sample (diameter: 12 mm, height: 15 mm).

The penetration depth was determined using a constant force penetrometer (Petrotest PNR-10, Petrolab, USA) under the following conditions: force, 0.63 N; time, 5 s; standard needle.

Statistical analyses

The averages and standard deviations were calculated for each parameter (Table 2-6). Descriptive statistics, analysis of variance (ANOVA), pairwise comparisons of mean values with Fisher's LSD test, principal component analyses of the correlation matrix and correlation test were performed with Systat for Windows version 9.0 (SPSS Inc., Chicago, IL). The correlation coefficients were calculated using the individual values for cheeses from each region.

RESULTS AND DISCUSSIONS

Since the number of samples analysed was very limited, we restricted the discussion of the results to the tests of the mean value differences and untrained classification techniques, such as principal component analysis (PCA). Trained classification techniques were not used because they require larger data sets in order to be reliable.

Nitrogen fractions, OPA-values and biogenic amines

The results for the nitrogen fractions, the OPA-values and the biogenic amines are given in Table 2. The total nitrogen concentration values were only slightly different whereas the differences between the NPN and WSN values were highly significant. The NPN and the OPA-values were strongly correlated ($r = 0.94$). The OPA-values were less discriminating due to the higher relative standard deviations of the results. "Bretagne" had the lowest NPN and WSN values, as expected for a cheese with such a short ripening time. It was followed by "Finland". "Allgäu" and "Savoie", ripened 4 and 3.5 months, respectively, had the highest NPN and WSN values. The values for "Switzerland" fell in the middle. This Emmental, aged for 4 months, is still considered to be very young in Switzerland. In fact, the proteolysis of "Switzerland" is deliberately slowed down to allow a ripening time of up to 12 months, leading to a very characteristic and mature cheese. In the other regions, fast maturation is preferred for economic reasons. The two ratios, WSN/TN and NPN/WSN, are indices of protein and peptide hydrolyses, respectively. The WSN/TN ratio showed exactly the same trend as that of WSN. For the NPN/WSN ratio, the differences were less significant. A point of interest is that the peptide hydrolysis in "Finland" was significantly higher.

Table 2 - Nitrogen fractions and biogenic amines in the 20 Emmental cheese samples investigated.

Analytes	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
TN (g/kg)	*	**44.1^{AB}**	1.7	**45.03^{AB}**	0.72	**44.42^{AB}**	0.85	**45.5^{AB}**	1.3	**46.20^{A}**	0.77	**42.9^{B}**	1.6
WSN (g/kg)	***	**11.2^{A}**	1.1	**6.4^{C}**	1.7	**9.35^{B}**	0.41	**8.31^{BC}**	0.51	**10.72^{AB}**	0.99	**8.77^{B}**	0.38
WSN / TN (%)	***	**25.5^{A}**	1.8	**14.2^{C}**	4.0	**21.1^{B}**	1.0	**18.3^{BC}**	0.6	**23.2^{AB}**	1.8	**20.4^{B}**	0.6
NPN (g/kg)	***	**7.37^{A}**	0.65	**3.98^{D}**	0.90	**5.86^{BC}**	0.55	**6.19^{ABC}**	0.58	**7.20^{AB}**	1.07	**5.42^{CD}**	0.37
NPN / WSN (%)	ns	**65.6**	0.8	**62.9**	8.2	**62.6**	4.3	**74.4**	2.4	**66.9**	4.3	**61.7**	2.6
OPA (mmol/kg)	*	**246^{A}**	63	**136^{B}**	39	**184^{AB}**	20	**222^{AB}**	34	**255^{A}**	61	**187^{AB}**	34
Cadaverine (mg/kg)	**	**44^{A}**	35	**5.6^{B}**	7.0	**0.22^{B}**	0.27	**25^{AB}**	15	**8.6^{B}**	6.7	**1.13^{B}**	0.75
Histamine (mg/kg)	*	**672^{A}**	376	**123^{B}**	149	**12.3^{B}**	7.4	**59^{B}**	26	**478^{A}**	434	**205^{A}**	105
Isopentylamine (mg/kg)	**	**1.80^{AB}**	0.66	**0.93^{B}**	0.55	**2.78^{A}**	1.12	**1.05^{AB}**	0.21	**0.53^{B}**	0.15	**0.17^{B}**	0.29
Putrescine (mg/kg)	*	**15^{AB}**	21	**2.3^{AB}**	1.2	**<2^{B}**	—	**<2^{B}**	—	**24^{A}**	19	**<2^{B}**	—
β-Phenylethylamine (mg/kg)	ns	**6.0**	4.0	**1.1**	1.9	**11**	16	**15**	14	**34**	27	**1.2**	2.1
Tryptamine (mg/kg)	ns	**<2**	—	**<2**	—	**<2**	—	**3.0**	2.2	**<2**	—	**<2**	—
Tyramine (mg/kg)	*	**178^{AB}**	121	**8.6^{B}**	8.4	**54^{B}**	123	**220^{AB}**	45	**403^{A}**	269	**117^{AB}**	91
Total biogenic amines (mg/kg)	*	**920^{A}**	420	**142^{B}**	165	**81^{B}**	134	**325^{AB}**	14	**948^{A}**	709	**325^{AB}**	195

Caption: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

The lowest total concentration of biogenic amines was found in "Switzerland" and the highest in "Allgäu" and "Savoie". These differences were largely due to the degree of proteolysis. Significant differences were found for cadaverine, histamine, isopentylamine, putrescine, tryptamine and tyramine. "Switzerland" had the lowest cadaverine, histamine and putrescine values and slightly higher isopentylamine values. Spermine and spermidine could not be detected in the samples. It should be noted that the biogenic amine concentrations determined did not present any danger to health.

The microbial flora was probably the main factor influencing the amount of biogenic amines. Propionibacterium and thermophilic *Lactobacillus* (the latter being added specifically for this experiment) may account for the formation of histamine in Maasdamer (JOOSTEN, 1987). The influence of Propionibacterium on the formation of histamine and tyramine in Emmental has been reported previously (ANTILA *et al.*, 1984). Non-starter lactic acid bacteria (lactobacilli, enterococci) may cause the formation of tyramine and histamine in Gouda (JOOSTEN and NORTHOLT, 1987). According to the latter authors, salt-tolerant lactobacilli account for a massive formation of putrescine and cadaverine.

Furthermore a concentration gradient was found in Gouda, with the central part of the block containing higher amounts of the investigated amines (tyramine, histamine, putrescine and cadaverine) than samples taken close to the rind (JOOSTEN and STADHOUDERS, 1987). Many chemical, physical and microbial factors also influence the formation of biogenic amines within a cheese variety (SIEBER and BILIC, 1992), leading to very scattered concentrations in samples which come from the same region of production. This was confirmed in "Switzerland" in earlier studies (SIEBER *et al.*, 1988, BOSSET *et al.*, 1992).

Free amino acids (FAA)

The sum of FAA showed the same trend as the WSN and the NPN values but with smaller significant differences between the groups. To produce a discrimination that was less age-dependant, relative amounts were considered (normalisation by the total free amino acids). Out of the 23 free amino acids analysed (Table 3), 16 had a significant difference between at least two groups. No phosphoserine was found. The highest significant differences were found for asparagine, glutamine, methionine, phenylalanine, proline and valine. "Bretagne" could easily be differentiated using the values of methionine and glutamine. With five parameters, asparagine, glycine, lysine, phenylalanine and proline (selection made with the help of a trained classification technique), it was possible to isolate "Switzerland" by PCA (Fig. 1). The other regions could not be clearly separated.

HPLC-peptide profile
and casein fractions

Fig. 2 is a typical peptide chromatogram. The 18 peptide peaks which showed significant differences are listed in Table 4. The highest values for peaks 10.1, 11.3, 12.1, 14, 22 and 24 were recorded for "Switzerland". The use of *L. helveticus* in starter cultures is known to lead to a smaller peak 14 (CHOPARD *et al.*, 2001). These findings were confirmed in this study; the highest peak 14 values were found in "Switzerland", the only sample that did not contained *L. helveticus* (PILLONEL *et al.*, 2002a). Peaks 2 and 3 were only found in "Finland" and peaks 4, 7, 12 and 14.1 had much higher values in "Finland" than in cheeses from the other regions.

The relative proportions of α-, β-, and indirectly γ-caseins in milk are subject to genetic variations between cows within the same breed (VARNAM and SUTHERLAND, 1994). In cheese however, the differences between two samples are more likely to be due to different intensities of proteolysis than to the milk used. The results of the casein fractions are listed in Table 5. All casein fractions showed significant differences except for X1-2, X2-1, X2-2 and β degraded. The fractions coded with an "X" are of unknown structure. X1 and X1-2 migrate after β, X2-1 and X2-2 after αS and X3 before α_{s1}. The α_{s1} fraction was highest in "Bretagne" and "Finland" and lowest in "Switzerland", followed by "Allgäu". In soft cheeses, the α_{s1} fraction is typically degraded to α_{s1}-I by the rennet enzyme chymosin. In cooked cheeses such as Emmental, chymosin is extensively denatured during manufacturing and contributes little to ripening (COLLIN *et al.*, 1988; BOUDJELLAB *et al.*, 1994). It is more likely that the α_{s1} degradation is due to the enzymes of the lactobacilli present in the cheese (OLSON, 1990; CHAMBA, 2000). This type of proteolysis however is relatively slow. The low α_{s1} level in "Switzerland" and "Allgäu" can therefore be explained by the longer ripening time in these regions and/or by a higher protease activity of the lactobacilli. "Bretagne" showed the highest value because of the very short ripening time and probably because of the thermisation applied to the milk (PERREARD and

Table 3 - Free amino acid ratios in the 20 Emmental cheese samples investigated.

Analytes	Nova	Region (n=) AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Total free amino acids (g/kg)	*	**28.2^{A}**	7.1	**17.2^{B}**	5.2	**19.8^{AB}**	3.0	**22.5^{AB}**	7.3	**28.7^{A}**	4.4	**19.3^{AB}**	2.0
Alanine (%)	ns	**3.08**	0.58	**3.84**	1.3	**3.34**	0.17	**3.71**	0.84	**3.26**	0.49	**3.03**	0.21
α-Amino butyric acid (%)	ns	**1.49**	2.10	**0.13**	0.01	**0.18**	0.12	**0.06**	0.02	**0.20**	0.11	**0.06**	0.06
Arginine (%)	ns	**0.10**	0.04	**0.04**	0.01	**0.84**	0.83	**0.86**	0.15	**0.08**	0.03	**0.08**	0.03
Asparagine (%)	***	**3.08^{BC}**	1.17	**4.63^{AB}**	1.95	**7.06^{A}**	0.62	**1.35^{C}**	0.11	**2.51^{BC}**	1.22	**4.77^{AB}**	1.8
Aspartic acid (%)	*	**2.91^{A}**	1.54	**1.27^{AB}**	0.80	**1.78^{AB}**	0.42	**0.16^{B}**	0.00	**0.96^{B}**	0.16	**1.35^{AB}**	1.00
Citrulline (%)	*	**0.89^{B}**	0.39	**2.14^{AB}**	0.52	**3.31^{A}**	0.83	**2.43^{AB}**	0.92	**2.10^{AB}**	1.10	**2.45^{AB}**	0.55
γ-Amino butyric acid (%)	*	**0.40^{A}**	0.34	**0.30^{AB}**	0.28	**0.05^{B}**	0.04	**0.01^{AB}**	0.01	**0.05^{AB}**	0.04	**0.03^{AB}**	0.02
Glutamine (%)	***	**2.30^{B}**	0.55	**4.25^{A}**	0.67	**1.94^{B}**	0.58	**3.20^{AB}**	0.74	**2.81^{B}**	0.26	**2.43^{B}**	0.52
Glutamic acid (%)	ns	**18.38**	0.54	**16.99**	0.24	**18.19**	1.1	**17.63**	0.62	**18.23**	0.41	**19.03**	0.64
Glycine (%)	*	**2.03^{AB}**	0.07	**2.03^{AB}**	0.14	**1.90^{B}**	0.15	**1.83^{B}**	0.01	**1.97^{AB}**	0.02	**2.16^{A}**	0.01
Histidine (%)	**	**0.35^{B}**	0.36	**2.81^{A}**	0.09	**1.84^{A}**	0.32	**3.24^{A}**	0.17	**2.20^{A}**	1.8	**1.87^{AB}**	0.41
Isoleucine (%)	**	**5.12^{A}**	0.72	**3.87^{ABC}**	0.65	**2.86^{C}**	0.28	**3.77^{ABC}**	0.66	**4.50^{AB}**	0.78	**3.53^{BC}**	0.73
Leucine (%)	**	**12.14^{B}**	1.2	**12.19^{B}**	0.84	**13.90^{A}**	0.53	**11.87^{B}**	1.3	**11.94^{B}**	0.59	**13.87^{A}**	0.55
Lysine (%)	*	**12.58^{A}**	0.34	**10.82^{B}**	1.6	**11.34^{AB}**	0.43	**12.69^{A}**	0.26	**12.69^{A}**	0.07	**11.60^{AB}**	0.35
Methionine (%)	***	**2.50^{A}**	0.11	**1.95^{B}**	0.09	**2.44^{A}**	0.07	**2.57^{A}**	0.03	**2.28^{A}**	0.22	**2.45^{A}**	0.09
Ornithine (%)	*	**5.09^{A}**	0.31	**3.63^{AB}**	0.49	**2.47^{B}**	1.0	**2.99^{AB}**	1.7	**4.33^{AB}**	1.8	**3.73^{AB}**	0.82
Phenyalanine (%)	***	**6.26^{AB}**	0.72	**5.33^{C}**	0.18	**6.96^{A}**	0.35	**6.06^{BC}**	0.34	**5.95^{BC}**	0.09	**6.53^{AB}**	0.29
Proline (%)	***	**6.28^{BC}**	2.74	**9.44^{AB}**	1.00	**4.05^{C}**	0.35	**9.98^{AB}**	0.78	**10.21^{A}**	0.74	**5.27^{C}**	2.4
Serine (%)	ns	**2.10**	0.10	**2.20**	0.47	**1.82**	0.20	**2.35**	0.38	**1.80**	0.37	**1.98**	0.28
Threonine (%)	ns	**2.90**	0.07	**2.57**	0.44	**2.77**	0.08	**3.16**	0.34	**2.83**	0.28	**2.97**	0.07
Tryptohphan (%)	*	**0.34^{B}**	0.05	**0.37^{B}**	0.10	**0.41^{AB}**	0.04	**0.53^{A}**	0.07	**0.45^{AB}**	0.09	**0.39^{AB}**	0.00
Tyrosine (%)	ns	**2.21**	0.65	**2.42**	0.37	**2.76**	1.0	**2.48**	0.99	**1.30**	0.57	**2.63**	0.72
Valine (%)	***	**7.49^{AB}**	0.20	**6.80^{C}**	0.16	**7.78^{A}**	0.19	**7.08^{BC}**	0.00	**7.34^{AB}**	0.40	**7.77^{A}**	0.13

Caption: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

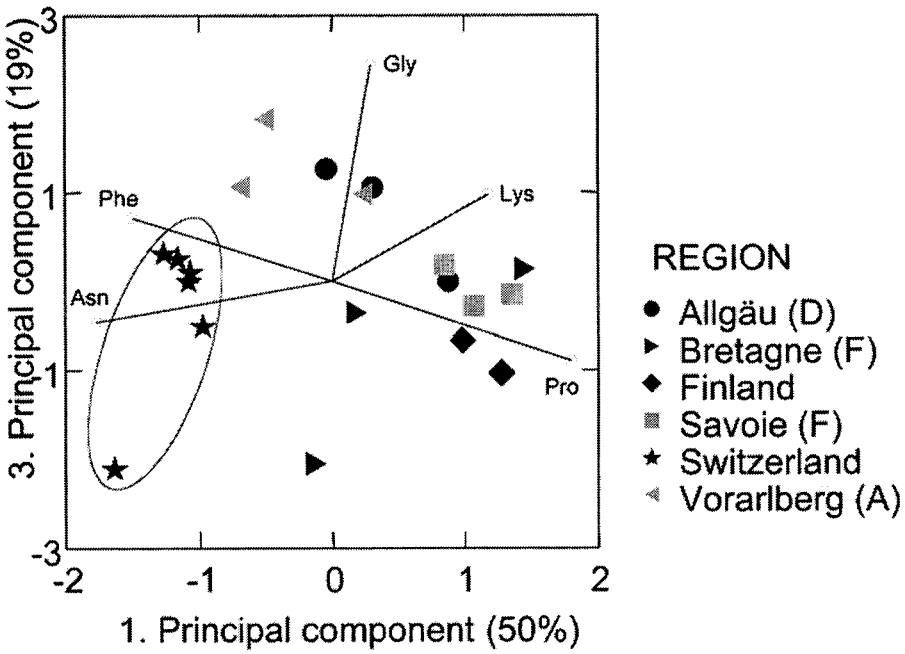

Fig. 1 - Principal component analysis using relative concentrations of asparagine, glycine, lysine, phenylalanine and proline. Separation of the region "Switzerland".

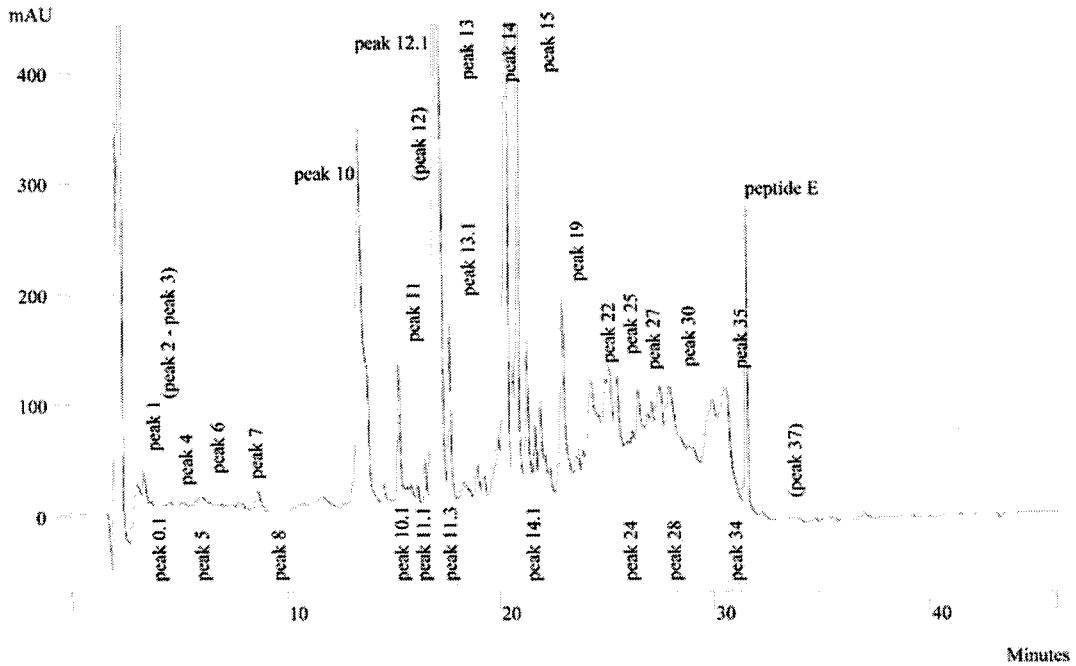

Fig. 2 - Peptide chromatogram of an Emmental sample from Switzerland. Peptide E is a synthetic standard added.

Table 4 - Peptide pattern of the 20 Emmental cheese samples investigated using HPLC (peak area, arbitrary units).

Peak number	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
1	*	**158^{AB}**	138	**459^{A}**	59	**190^{B}**	51	**251^{AB}**	8	**262^{AB}**	58	**154^{B}**	176
2	***	**n.d.^{B}**	—	**n.d.^{B}**	—	**n.d.^{B}**	—	**107^{A}**	20	**n.d.^{B}**	—	**n.d.^{B}**	—
3	***	**n.d.^{B}**	—	**n.d.^{B}**	—	**n.d.^{B}**	—	**122^{A}**	18	**n.d.^{B}**	—	**n.d.^{B}**	—
4	*	**n.d.^{B}**	—	**26^{B}**	45	**29^{B}**	71	**193^{A}**	83	**32^{B}**	55	**n.d.^{B}**	—
7	***	**n.d.^{B}**	—	**n.d.^{B}**	—	**12^{B}**	29	**528^{A}**	32	**30^{B}**	53	**n.d.^{B}**	—
8	**	**212^{B}**	120	**269^{B}**	60	**434^{A}**	51	**466^{A}**	8	**279^{B}**	90	**351^{AB}**	38
10.1	**	**74B^{C}**	69	**27^{C}**	47	**184^{A}**	67	**177^{AB}**	13	**24^{C}**	42	**129^{ABC}**	18
11	*	**1,276^{A}**	73	**595^{AB}**	587	**1,144^{A}**	429	**554^{AB}**	297	**322^{B}**	398	**687^{ABC}**	248
11.1	*	**367^{A}**	227	**55^{B}**	96	**91^{B}**	115	**283^{AB}**	91	**31^{B}**	53	**278^{AB}**	206
11.3	**	**215^{AB}**	19	**79^{BC}**	138	**309^{A}**	110	**101^{ABC}**	143	**n.d.^{C}**	—	**145^{ABC}**	13
12	*	**n.d.^{B}**	—	**447^{AB}**	496	**n.d.^{B}**	—	**783^{A}**	696	**263^{AB}**	77	**n.d.^{B}**	—
12.1	***	**8,827^{AB}**	8,275	**1,537^{BC}**	1,347	**12,380^{A}**	2,285	**1,178^{BC}**	1,666	**n.d.^{C}**	—	**8,122^{ABC}**	667
13.1	*	**1,402^{AB}**	340	**908^{B}**	399	**1,745^{AB}**	379	**2,187^{A}**	249	**829^{B}**	823	**1,722^{AB}**	419
14	***	**5,707^{BC}**	3,774	**2,554^{C}**	1,246	**14,755^{A}**	3,873	**955^{C}**	585	**683^{C}**	58	**9,649^{AB}**	1,641
14.1	**	**268^{B}**	326	**558^{B}**	401	**202^{B}**	170	**2,020^{A}**	480	**1,043^{AB}**	866	**227^{B}**	242
22	*	**997^{B}**	818	**589^{B}**	300	**2,791^{A}**	1,069	**2,219^{AB}**	1,937	**536^{B}**	292	**1,135^{AB}**	387
24	*	**970^{AB}**	984	**234^{AB}**	17	**1,832^{A}**	816	**492^{AB}**	188	**98^{B}**	22	**819^{AB}**	709
37	*	**n.d.^{B}**	—	**53^{A}**	47	**n.d.^{B}**	—	**n.d.^{B}**	—	**n.d.^{B}**	—	**n.d.^{B}**	—
Total area	***	**70,092^{A}**	8,108	**37,076^{B}**	2,338	**81,370^{A}**	12,527	**73,532^{A}**	1,769	**35,576^{B}**	24,376	**68,974^{A}**	15,313

Caption: x = mean value; sx = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.
n.d.: below the detection limit.

Table 5 - Casein fractions using SDS-PAGE-electrophoresis in the 20 Emmental cheese samples investigated (peak area, arbitrary units).

Fraction	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Para K	*	**8.87^{A}**	0.85	**6.60^{B}**	0.56	**8.02^{AB}**	0.83	**7.05^{AB}**	0.92	**7.33^{AB}**	0.58	**7.87^{AB}**	0.55
β degraded	ns	**3.40**	0.66	**1.93**	0.71	**2.72**	0.58	**1.85**	0.07	**3.87**	0.32	**3.8**	1.2
γ1	*	**12.40^{A}**	0.96	**8.4^{B}**	1.7	**11.1^{AB}**	1.4	**9.6^{AB}**	2.6	**10.13^{AB}**	0.87	**9.57^{AB}**	0.23
γ2	**	**7,1^{A}**	1.0	**3.73^{B}**	0.80	**6.53^{A}**	0.69	**6.0^{A}**	1.2	**5.83^{A}**	0.32	**5.93^{A}**	0.81
γ3	*	**3.46^{ABC}**	0.76	**2.00^{C}**	0.10	**4.23^{A}**	0.88	**3.05^{ABC}**	0.78	**3.93^{AB}**	0.72	**4.0^{AB}**	1.2
X1	ns	**3.53**	0.38	**2.40**	0.50	**3.50**	0.42	**3.00**	0.57	**3.37**	0.15	**2.87**	0.86
X1-2	ns	**n.d.**	—	**n.d.**	—	**0.40**	0.67	**n.d.**	—	**n.d.**	—	**n.d.**	—
β	**	**14.4^{C}**	1.4	**21.5^{A}**	1.6	**16.8^{B}**	1.5	**17.6^{AB}**	2.7	**17.1^{B}**	1.6	**18.1^{AB}**	1.4
X2-1	ns	**1.87**	0.57	**2.37**	0.72	**1.97**	0.27	**1.90**	0.85	**2.43**	0.25	**2.73**	0.90
X2-2	ns	**8.67**	0.83	**7.77**	1.21	**8.68**	0.37	**7.70**	0.28	**8.30**	0.92	**7.80**	0.61
α_{s2}	**	**n.d.^{B}**	—	**2.7^{A}**	2.4	**n.d.^{B}**	—	**n.d.^{B}**	—	**n.d.^{B}**	—	**n.d.^{B}**	—
α_{s1}	***	**8.7^{BC}**	1.5	**20.6^{A}**	1.4	**6.68^{C}**	0.96	**19.70^{A}**	0.28	**11.7^{B}**	1.8	**10.5^{B}**	1.7
α_{s1}-I	**	**19.5^{ABC}**	1.8	**15.7^{C}**	1.8	**22.3^{A}**	1.8	**16.9^{BC}**	2.2	**19.47^{ABC}**	0.92	**21.1^{AB}**	2.1
X3	***	**8.00^{A}**	0.66	**4.23^{C}**	0.60	**7.1^{AB}**	1.2	**5.70^{BC}**	0.14	**6.60^{AB}**	0.17	**5.53^{BC}**	0.23

Caption: x = mean value; sx = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.
X1, X1-2: fractions with unknown constitution migrating after β.
X2-1, X2-2: fractions with unknown constitution migrating after α_{s1}.
X3: fractions with unknown constitution migrating before α_{s1}.
n.d.: below the detection limit.

CHAMBA, 1999). The significantly higher α_{s1} level in "Finland" compared with "Savoie" and "Vorarlberg", though all three were ripened for approximately three months, may be explained by the higher cooking temperature in the former (54°C vs. 52°-54°C) and by the milk thermisation process applied in "Finland" and not in the other two countries (information held by the producers).

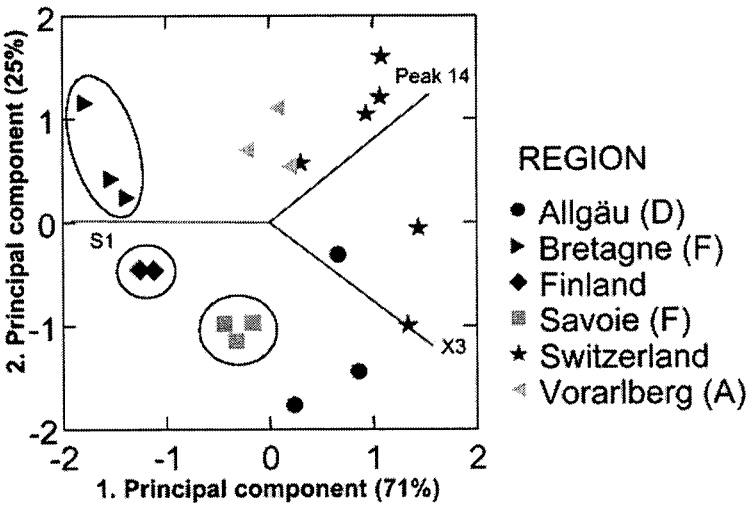

Fig. 3 - Principal component analysis using values of α_{s1}, X3 (unknown structure) and peak 14. Separation of the regions "Bretagne", "Finland" and "Savoie".

Unlike chymosin, the endogenous milk enzyme, plasmin, is not damaged by the high temperature used in cooked cheese. The plasmin activity even elevated in "high cooked" cheese varieties is due to thermal inactivation of inhibitors of plasminogen activators. This results in the increased conversion of plasminogen to the active form, plasmin (LU and NIELSEN, 1993; FARKYE and FOX, 1990). Plasmin degrades α_{s2} and β casein. α_{s2} commonly disappears in Emmental after 45 days of ripening (CHAILLET, 2002). In the current study, two samples from "Bretagne" still contained α_{s2}, probably due to the very short ripening time. The degradation of the β casein leads to the γ1 to γ3 fractions. The same tendency was observed in β as in α_{s1}. "Bretagne" had the highest value followed by "Finland", whereas "Allgäu" and "Switzerland" showed the lowest values. The likely explanation is once again the longer ripening time. The X1-2 fraction was only found in two Swiss samples. The parameters α_{s1} and X3, which gave the best discrimination, combined with peak 14 from the HPLC-peptide profile, gave an interesting separation profile (Fig. 3). The ratio of casein fractions to TN gave a similar separation profile.

Rheology

No significant differences were found between the cheeses from any of the regions except for the penetration depth (Table 6). "Switzerland" showed the highest penetration depth, while "Savoie", "Bretagne", and "Finland" had the lowest. The penetration depth is supposed to be correlated with the water and fat content (BOSSET *et al.*, 1993). This was not observed in this case with water and the correlation with the fat content was poor (r = 0.40). The penetration depth was negatively correlated with the stress at 33% deformation (r = -0.873), which corresponds to the firmness of the body. The stress at fracture was positively correlated with the strain at fracture (r = 0.704), which corresponds to the length of the body.

CONCLUSION

Compounds linked to proteolysis allowed some of the Emmental cheese samples to be separated according to

Table 6 - Rheological analyses of the 20 Emmental cheese samples investigated.

Parameters analysed	Nova	Region (n=)											
		AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Depth of penetration (mm)	*	**10.6^{AB}**	1.6	**9.4^{B}**	1.4	**12.0^{A}**	1.2	**9.5^{AB}**	1.3	**9.3^{B}**	0.77	**10.9^{AB}**	1.3
Stress at fracture (N)	ns	**68**	14	**74**	7.7	**74**	8.7	**65**	3.1	**64**	2.3	**71**	5.8
Strain at fracture (%)	ns	**56**	19	**104**	46	**68**	31	**79**	3.6	**56**	5.7	**60**	25
Stress at 33% deformation (N)	ns	**18**	5.4	**19**	4.8	**14**	1.4	**20**	3.1	**18**	3.7	**17**	2.4

Caption: x = mean value; sx = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$.
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.

their geographic origin. The NPN, WSN and WSN/TN fractions showed highly significant differences which were partly due to differences in ripening times. These differences are useful because each region produces Emmental with a particular ripening time before being put on the market. The high standard deviations of the biogenic amine concentrations made them unusable as markers of origin. The relative amounts of the free amino acids asparagine, glycine, lysine, phenylalanine and proline made it possible to separate "Switzerland". A clear differentiation of "Bretagne" was achieved using the methionine and glutamine values. The casein fractions (α_{s1} and X3) and the peptide pattern (peak 14) provided a good separation of the "Finland", "Bretagne" and "Savoie" groups. The rheological results were of less interest for the geographic discrimination of Emmental cheese.

ACKNOWLEDGEMENT

The authors thank the Swiss Federal Office of Public Health for its financial and logistical support of the project, G. Urbach and G. Gremaud for reviewing of the publication as well as J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland™), H. Jatila (Valio Ltd, Finland) and H. Schmallegger (Alma GmbH, Austria) for collecting the samples.

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CHAPTER 8

Analytical methods for the determination of the geographic origin of Emmental cheeses. Mid- and Near-Infrared spectroscopy.

L. Pillonel, W. Luginbühl, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
D. Picque
INRA/LGMPA, 78850 Thiverval Grignon (France)
E. Schaller
Büchi Labortechnik AG, 9230 Flawil (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

European Food Research and Technology, 216, 174-178 (2003)

Abstract Four different Fourier transform infrared spectroscopic techniques (near infrared diffuse reflection (NIR/DR), mid-infrared attenuated total reflection (mIR/ATR) using two different instruments and mid-infrared transmission (mIR/Tr) spectroscopy) in combination with multivariate chemometrics were investigated for their potential for discriminating Emmental cheese of various geographic origins. A total of 20 Emmental cheese samples produced in winter from Switzerland (n=6), Allgäu (Germany) (n=3), Bretagne (France) (n=3), Savoie (France) (n=3), Vorarlberg (Austria) (n=3) and Finland (n=2) were analysed. The normalised spectra or their 2nd derivatives were analysed by principal component analysis (PCA) and linear discriminant analysis (LDA) of the PCA-scores. Despite the few samples in this preliminary investigation clear trends were observed. The mIR transmission spectra achieved 100% correct classification in LDA when differentiating the Swiss Emmental from the other samples pooled as one group. The NIR/DR spectroscopy allowed a classification by the six regions of cheese origin.

Keywords Authenticity · Traceability · Emmental cheese · NIR · MIR · IR

L. Pillonel · W. Luginbühl · J.O. Bosset (✉)
Federal Dairy Research Institute (FAM), Liebefeld,
3003 Bern, Switzerland
e-mail: jacques-olivier.bosset@fam.admin.ch
Tel.: 41 31 323 81 67, Fax: 41 31 323 82 27

D. Picque
INRA/LGMPA, 78850 Thiverval Grignon, France

E. Schaller
Büchi Labortechnik AG, 9230 Flawil, Switzerland

R. Tabacchi
Universitiy of Neuchâtel, 2007 Neuchâtel, Switzerland

Introduction

Infrared (IR) spectroscopy is widely used for determination of organic constituents in foods and pharmaceutical products. The advantages of IR measurements are obvious: they are rapid, cheap, non-destructive and multiparametric. The use of IR spectrometry for the analysis of dairy products has been reviewed [1, 2, 3]. Mid- and near- infrared (mIR and NIR) spectroscopy of dairy products has in practice been restricted to measurements of main components such as fat, protein, moisture, lactose and urea. Some applications for cheese have been published [4, 5, 6, 7, 8, 9, 10]. In addition, Sorensen et al. [11] found correlation between certain sensory properties and NIR spectra. Dufour et al. [12] were able to discriminate between four different ripening stages by combining mIR and fluorescence spectra. Infrared spectroscopy found application for identification and/or authentication of various products such as meat [13, 14, 15], honey [16], coffee [17, 18, 19] and fruit juice [20, 21, 22].

The present investigation is part of a broad screening test, which is the first step in a 3-year study into the authenticity of Emmental cheese and its geographic traceability [23, 24]. At this point, a large number of analytical methods have been tested for their discriminating potential [24, 25, 26, 27] and the number of cheese samples for each region has been limited. It is therefore obvious that the analytical results obtained from such a modest number of cheese samples from each region can only give trends that should be confirmed later if they appear valuable for discriminating cheeses produced in different countries. The aim of the present paper is to find out whether NIR and mIR make possible a discrimination between the different geographic origins of Emmental cheese samples.

Materials and methods

The sampling procedure and the background of the project have been described in the first publication of the series [24]. Table 1

Table 1 Origin, date of manufacture and ripening time of the 20 cheese samples investigated

Abbreviation	Region (country)	Number of samples	Date of manufacture	Ripening time (months)
AL	Allgäu (D)	3	25 Dec 2000	4
BR	Bretagne (F)	3	20 Feb 2001	2.5
CH	Switzerland (CH)	6	26 Dec 2000	4
FI	Middle Finland (FI)	2	04 Feb 2001	3
SA	Savoie (F)	3	05 Feb 2001	3
VO	Vorarlberg (A)	3	02 Feb 2001	3

summarises the origin, the date of manufacture and the ripening time of the samples. They were chosen with different ripening time according to the reality of the market. Each region produces a cheese with typical characteristics, one of which is the ripening time, which can vary from 6 weeks to several months. Three samples originated from each of Bretagne, Savoie, Vorarlberg and Allgäu. Two samples originated from Finland and six from Switzerland. All instruments were of type Fourier transform.

NIR diffuse reflection (NID/DR)

The samples were kept at –18 °C until analysis. Approx. 150 g grated cheese were placed in a glass Petri dish and measured by diffuse reflection on a Büchi NIRLab N-200 spectrometer (Flawil, Switzerland). The Petri dish rotates on itself during the measurement. For each sample, 64 scans were recorded from 4000 cm^{-1} to 10 000 cm^{-1} with a spectral resolution of 2 cm^{-1}.

mIR attenuated total reflection (mIR/ATR) using two different instruments

Slices of approx. 6×1×0.4 cm were cut from the fresh cheese samples avoiding eyes of the body. All analyses were carried out within a few days. The cheese slices were then pressed with the help of a clamp on the horizontal ZnSe crystal plate of an ATR sampling accessory (Graseby-Specac benchmark 6 reflection horizontal ATR with clamp assembly). The pressure applied on the cheese sample ensured an homogeneous contact between the cheese surface and the crystal. The measurements were carried out using an mIR spectrometer equipped with a DTGS detector (FTS-7, Bio-Rad Digilab) at room temperature.

The same analyses were carried out in another laboratory with a second instrument using an ATR accessory designed for 12 reflections (Nicolet Magna 750 FTIR spectrometer).

With both instruments the spectral resolution was 4 cm^{-1}, 32 scans were averaged for each spectrum in the range of 3500 cm^{-1} to 700 cm^{-1}. The spectra of three replicates of each cheese sample were recorded.

mIR transmission (mIR/Tr)

The analyses were carried out on fresh samples within a few days. Grated samples (5 g) were dispersed in 100 mL water with a Polytron PT2100 at 15 000 rpm for 60 s. An aliquot of 60 µL was applied to a polyethylene card (Spectra Tech Nicolet) and dried overnight. The prepared cards were analysed in transmittance on a Nicolet Magna 750 IR spectrometer.A total of 32 interferograms with a resolution of 4 cm^{-1} were recorded and averaged. Each cheese sample was analysed in triplicate and the average spectrum was introduced into the data set.

Statistical methods

For each type of spectrum, spectral regions were selected to eliminate zones with either low signal-noise ratios or no significant spectral information. All spectra were normalised between 0 and 1. For the spectra obtained using the Büchi NIR spectrometer and the Bio-Rad instrument, principal component scores from the correlation matrix were computed with PLSPlus/IQ Vs. 5.07 (Thermo Galactic, Salem, NH). Linear discriminant analysis (LDA) was performed on these scores with Systat Vs. 9.0 (SPSS Inc., Chicago, IL).

The spectra from the Nicolet instrument were not used directly. Their 2nd derivatives were first calculated before principal component scores were computed with Statistica VS 5.5 (Satasoft France, Paris). LDA was carried out with Systat.

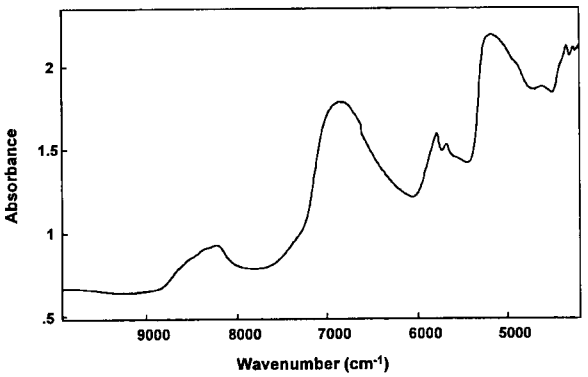

Fig. 1 Typical NIR/DR diffuse reflection absorption spectrum using a Büchi NIRLab N-200 instrument

Results

Typical IR spectra and the selected spectral windows are illustrated in Figs. 1, 2 and 3 for the three techniques mentioned above. The mIR spectra showed narrower and more abundant absorption zones than NIR, especially in the mIR/ATR technique. The interpretation of the results focused on the differentiation of "Switzerland" from the other regions. However, discrimination between the other regions was also possible.

Two grouping possibilities were investigated: in the first case, the Swiss samples were compared to a pool including all the others (two groups); in the second case, each region was one group within six groups. Principal component analysis was applied to each set of spectra to reduce the number of variables for further statistical analysis. Linear discriminant analyses were carried out on the principal component scores selected by stepwise backward elimination to evaluate the potential for separation between the groups. The goal was not to build a model for prediction but only to study the feasibility of

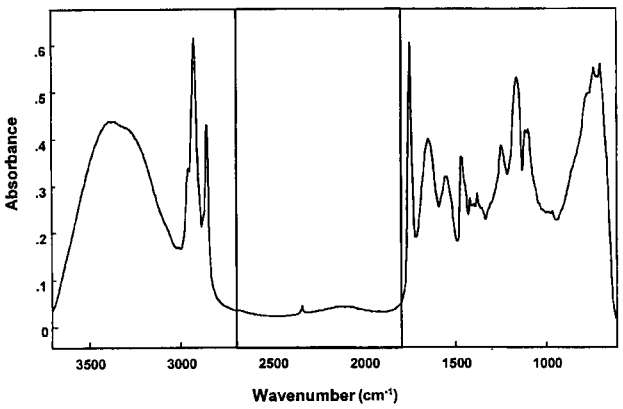

Fig. 2 Typical mIR/ATR attenuated total reflection absorption spectrum using a Bio-Rad FTS-7 instrument; range of interest 3700–2700 and 1800–620 cm^{-1}. Area between the *vertical lines* was omitted due to the low signal-noise ratio

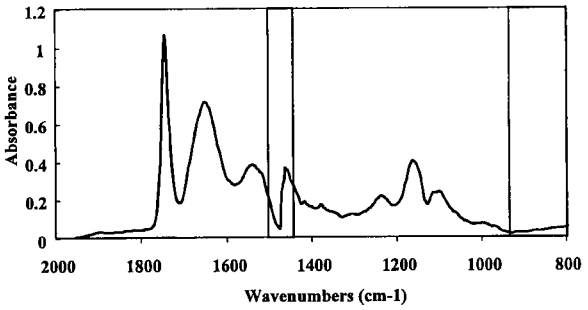

Fig. 3 Typical mIR/Tr transmission absorption spectrum using a Nicolet Magna 750 instrument; range of interest 900 – 1450 and 1500–1800 cm^{-1}. Area between the *vertical lines* was omitted due to the low signal-noise ratio

the method. The number of principal components used was reduced to a minimum by backward elimination to diminish the problem of model over-fitting. The percentage of correct classification, as well as the median, minimal, maximal and normalised Mahalanobis distances between the samples of one region and the centroid of the group "Switzerland", are listed for the four methods in Table 2.

The NIR/DR and mIR/ATR (Magna 750) spectra showed the best discrimination with 100% correct classification by individual region. "Finland" was always the easiest region to separate (highest median distance from Switzerland). It was not possible to classify all Swiss samples correctly (78%) using the mIR/ATR (FTS-7) spectra. To improve the comparison of the methods, the

Table 2 Classification rates by linear discriminant analysis according to two types of grouping

	Switzerland vs. other regions		Separation of each individual region					
	Switzerland	Others	Switzerland	Allgäu (D)	Bretagne (F)	Finland	Savoie (F)	Vorarlberg (A)
NIR/DR								
PC for LDA*	4		7					
% Correct	100	90	100	100	100	100	100	100
Distance**	3 (1, 5)	11 (4, 25)	8 (5, 12)	25 (17, 27)	38 (25, 48)	95 (67, 118)	25 (10, 32)	25 (7, 28)
Median normalised***	1	4	1	3	5	12	3	3
mIR/ATR(FTS7)								
PC for LDA*	1	9						
% Correct	72	81	78	89	100	100	100	89
Distance**	1 (0.1, 9)	5 (0.9, 19)	6 (3, 20)	24 (15, 54)	42 (25, 55)	295 (250, 318)	46 (29, 66)	12 (3, 23)
Median normalised***	1	5	1	4	7	49	8	2
mIR/ATR (Magna 750)								
PC for LDA*	3	10						
% Correct	100	71	100	100	100	100	100	100
Distance**	2 (0.7, 4)	5 (0.8, 14)	9 (4, 11)	150 (103, 156)	264 (242, 300)	439 (414, 464)	30 (12, 39)	20 (6, 27)
Median normalised***	1	3	1	17	29	49	3	2
mIR/Tr								
PC for LDA*	4	6						
% Correct	100	100	100	67	100	100	100	100
Distance**	2 (0.6, 8)	12 (4, 33)	5 (2, 8)	16 (9, 16)	23 (10, 32)	71 (40, 101)	22 (22, 25)	26 (15, 27)
Median normalised***	1	6	1	3	5	14	4	5

* Number of principal components used for the linear discriminant analysis

** Median distance to the centroid of the group "Switzerland" (minimum, maximum)

*** Median distance normalised by the median distance of "Switzerland"

median distances from "Switzerland" were normalised by the median distance of the Swiss samples from their centroid. With NIR/DR and mIR/Tr, the median distances normalised to Switzerland were generally lower than with the other techniques. However, the closest groups to "Switzerland" were at a distance of at least three in NIR/DR and mIR/Tr vs. two for the others techniques. This may explain the good discrimination ability of these two techniques when separating "Switzerland" from the others. The mIR/Tr classified 100% correctly, NIR/DR 100% for "Switzerland" and 90% for the non-Swiss groups. The mIR/ATR (FTS-7) spectra only achieved 72% correct classification for the Swiss samples.

Discussion and conclusion

Multivariate statistics is a very powerful tool for distinguishing groups of objects with very similar properties. The more properties (variables) used for classification, the more objects (samples) are needed to get a robust, unambiguous statistical model. With only 20 samples, the current model tends to suffer from over-fitting and is not very robust against the inclusion or exclusion of samples. However, the goal of the current work was only to evaluate the basic discriminating potential of IR spectroscopy with respect to cheese authenticity and not, at this point, to build a detailed model for the prediction of the origin. LDA made it possible to compare the various techniques used.

A simple method for checking the robustness of the discriminant functions of an LDA is the Jackknife classification test. In this re-sampling test, the discriminant functions are computed consecutively leaving out one sample. The group membership of the removed sample is then predicted by the classification functions obtained without the sample excluded in the training step (this procedure is also known as cross validation). If the model is robust, the classification pattern will not depend to a great extent on the inclusion or exclusion of a given sample in the analysis and the percentages of well-classified samples will be similar for both methods. Table 3 compares the results of the total correct classification rate in the normal and the Jackknife mode.

For the classification by regions, only the NIR/DR technique gave satisfactory results: 100% correct classifications were achieved in both modes. The very good classification obtained using mIR/ATR (Magna 750) broke down to 65% with the Jackknife test. Similar poor results were obtained using the two remaining techniques.

For the classification into two groups, the best results were achieved using mIR/Tr: 100% could be classified correctly in the normal mode and 85% in the Jackknife test. NIR/DR did not do badly with 93% and 90%, respectively. The mIR/ATR (FTS-7) technique is interesting because almost all variation is contained in only one principal component. However, the correct classification rate is poor.

Table 3 Percentage of total correct group assignments by normal and Jackknife linear discriminant classification

	By region		CH vs. Others	
	Normal	Jackknifed	Normal	Jackknifed
NIR/DR	100	100	93	90
mIR/ATR(FTS-7)-	90	78	88	85
mIR/ATR(Magna 750)	100	65	80	70
mIR/Tr	95	65	100	85

In conclusion, of the four techniques tested, two may be retained for their promising results. NIR/DR gave 100% discrimination by grouping into the six individual regions chosen for this preliminary study, whereas mIR/Tr achieved 100% correct classification when comparing "Switzerland" with the other regions pooled as one group. Both techniques made possible the analysis of grated cheese, which is an essential condition if one wants to test the authenticity of prepackaged grated Emmental cheese. Further analyses with more samples will be necessary to confirm these results and to build a validated model for prediction.

Acknowledgement The authors thank the Swiss Federal Office of Public Health for its financial and logistical support for the project, G. Urbach and G. Gremaud for their reviewing of the publication as well as J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland), H. Jatila (Valio Ltd, Finland) and H. Schmallegger (Alma GmbH, Austria) for collecting the samples.

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CHAPTER 9

Analytical methods for the determination of the geographic origin of Emmental cheeses. Volatile compounds by GC/MS-FID and electronic nose.

L. Pillonel, S. Ampuero, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

European Food Research and Technology, 216, 179-183 (2003)

Abstract The volatile compounds of Emmental cheese of different origins were investigated to check their suitability as markers of geographic origin. A total of 20 Emmental cheese samples from Switzerland, Allgäu (D), Bretagne (F), Savoie (F), Vorarlberg (A) and Finland (produced in winter with a ripening time according to that sold in the corresponding regions) were analysed using dynamic headspace gas chromatography followed by flame ionisation and mass spectrometry. All regions were easily differentiated by using compounds occurring only in the corresponding region or by combining a few compounds by principal component analysis (PCA). Further analyses were carried out using a mass spectrometry-based electronic nose. PCA achieved 90 and 91% of correct classifications for the Emmental from Switzerland and other regions, respectively.

Keywords Emmental cheese · Authenticity · Traceability · Volatile compound · Gas chromatography · GC-MS · Electronic nose

Introduction

The origins of volatile compounds found in cheese are diverse and can be classified into two groups: the first one contains native volatile compounds already present in milk which are not transformed during cheese manufacturing while the second group includes components produced in the cheese itself during manufacture or maturation. Forage is an important factor influencing the composition of the volatiles of the first group. Feeding cows with e.g. highland grass (rich in dicotyledones) during mountain pasture leads to higher concentrations of terpenes, sesquiterpenes and esters, as described in literature for different cheese types [1, 2, 3, 4]. This information may be useful for recognising a typical highland cheese (e.g. protected denomination of origin) from a lowland cheese. However, they are not adequate as geographic markers for a given cheese type produced in different countries. Moreover, the composition of forage undergoes dynamic changes over the year and so will any parameter strongly related to it [5]. Local manufacturing practices can also modify the composition of volatiles in milk. When curd is cooked on an open log fire, significantly higher amounts of polycyclic aromatic hydrocarbons are found in cheese [6]. Once again, such markers are restricted to very specific products within a small scale production.

The volatile compounds of the second group are formed during manufacture and ripening of cheese by microbial, enzymatic and (bio)chemical transformations. Among these, proteolysis, lipolysis and lactose fermentation are major biochemical events. Degradation of amino acids leads to amines, aldehydes, alcohols, acids and sulphur compounds. Breakdown of fatty acids produces esters, methyl ketones and secondary alcohols [7, 8, 9]. The occurrence of the various pathways depends on the manufacturing technology, including the choice of the starter and non-starter bacteria used. A manufacturing process is often typical of a defined region and, therefore, the resulting composition of volatile compounds could be an interesting indicator of Emmental origin.

Different techniques and types of apparatus [10] as well as preconcentration methods [11] have been proposed for analysis of food volatiles. GC-MS with purge & trap technique is one of the most widely used techniques and represents the method of choice for the current work because of its high reproducibility. This technique is, however, expensive and very time-consuming. A promising alternative is the use of electronic noses because of their rapid screening capacity. In recent years, electronic noses have been gaining attention as useful tools, especially for quality control in the food industry

L. Pillonel · S. Ampuero · J.O. Bosset (✉)
Federal Dairy Research Institute (FAM), Liebefeld, 3003 Bern, Switzerland
e-mail: jacques-olivier.bosset@fam.admin.ch
Tel.: +41 31 323 81 67, Fax: +41 31 323 82 27

R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel, Switzerland

[12] as they are ideally suited for rapid discrimination. An electronic nose based on mass spectrometry has already been successfully used to differentiate and recognise four different processed cheeses [13] in our laboratory.

The present investigation forms part of a broad preliminary screening test of discriminatory analytical tools within a 3-year study on the authenticity of Emmental cheese and its geographic traceability [14,15]. This study is especially concerned with distinguishing cheeses produced in Switzerland from those produced in other countries. At this point in the investigation, a great number of analytical methods already have been tested with respect to their discriminating potential, using three cheese samples from the regions Allgäu, Vorarlberg, Savoie, Bretagne, two from Finland and six from Switzerland [5, 15, 16, 17]. It is therefore obvious that the analytical results obtained from such a modest number of cheese samples per region can only give trends that will have to be confirmed later by tests performed on a larger number of samples. The objective of the present investigation was to check the possibility of using volatile compounds to discriminate Emmental cheese from different European countries, especially the Emmentaler Switzerland, by using two different analytical techniques: a dynamic headspace GC-MS plus FID and an MS-based electronic nose.

Materials and methods

Origin and selection of the cheese samples

The main framework of this study and the sampling procedure have been described in detail earlier [15]. Table 1 summarises the origin, the date of manufacture and the ripening time of the samples. They were chosen with different ripening time according to the reality of the market. Each region produces a cheese with typical characteristics, one of which is the ripening time, which can vary from 6 weeks to several months. At the end of the ripening time, all samples were stored deep frozen (–20 °C) until analysis.

Purge & trap analysis

Grated sample (20 g) and 80 g of boiled Milli-Q water were homogenised with a Polytron PT3000 running at 10 000 rpm for 1 min. An amount of 10 g of the suspension obtained was introduced into a 25-mL non-fritted glass sparger immersed in a water bath at 45 ± 0.5°C. The purge & trap system 3100 (Tekmar, Cincinnati, OH, USA) including a Tenax trap was op erated under the following conditions: prepurge time of 1 min; nitrogen as purge gas; purge flow of 30 mL/min; purge pressure of 150 kPa; purge time of 15 min, dry purge time of 6 min; desorb preheat at 220 °C; desorption at 225 °C for 4 min; cryofocus temperature at –145 °C; injection temperature program: within 1 min from –125 to 225 °C; bake 7 min at 230 °C; 6-port valve at 150 °C; line at 150 °C; transfer line from P&T to GC at 150 °C; mount temperature at 60 °C.

A Hewlett-Packard (HP) 5890 GC, series II was used. Separations were performed on a 30 m×0.32 mm i.d.×4 µm SPB1 sulphur column (Supelco). Helium was employed as carrier gas with an inlet pressure of 50 kPa. Following sample transfer, the oven temperature was maintained at 45 °C for 13 min and then heated at 5 °C/min to 240 °C, which was held for 5 min.

Two detectors were mounted in parallel by splitting the flow at the end of the capillary column; one stream (0.99 mL/min at 45 °C) led to a flame ionisation detector (FID), the other (1.05 mL/min at 45 °C) to a mass-selective detector (MSD model HP 5972). The latter operated in the scan mode (TIC) from 26 to 250 amu at 1.1 scan/s, ionisation was by EI at 70 eV by autotuning.

The FID signal was used for the semi-quantitative determination of the peak height. Only compounds with a peak height greater than the value of 3.6 arbitrary units (selected detection threshold) have been considered for this study. This value corresponds approximately to an MSD signal-noise ratio of five. Peaks suffering from overlapping (asymmetric shape, poor resolution) have been excluded.

Statistical methods (static headspace)

The averages and standard deviations were calculated for each value. The pair-wise comparison of mean values with Fisher's LSD test as well as the principal component analysis were performed using Systat for Windows version 9.0 (SPSS Inc., Chicago, IL).

Electronic nose

Aliquots of 4 g per sample were grated and filled into 10-mL vials and closed with a butyl/PTFE septum and a cap. A Smart Nose (LDZ, Marin, Switzerland) equipped with a Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) was used. Main operating conditions were as follow: incubation temp at 90 °C, incubation time of 30 min, injection volume of 2.5 mL, syringe temp at 110 °C, injector temp at 170 °C, nitrogen as purge gas, with a purge flow of 350 mL/min; syringe purge time of 2 min, mass spectrometer scan speed of 0.5 s/amu, mass range of 10–110 amu, ionisation at 45 eV. The total acquisition time was set to 150 s so that three cycles were measured for each injection.

Generally, four replicates were measured for each sample. The analyses occurred in a randomised order. By introducing each sample into the incubator exactly 30 min (incubation time) before its programmed injection time, the effective measuring/sampling time was reduced to 4.5 min per vial. This was possible due to the programming capabilities of the auto-sampler and its capacity to incubate six vials at a time. Data were treated using the software supplied with the Smart Nose. For more details see [13].

Table 1 Origin, date of manufacture and ripening time of the 20 cheese samples investigated

Abbreviation	Region (country)	Number of samples	Date of manufacture	Ripening time (months)
AL	Allgäu (D)	3	25 Dec 2000	4
BR	Bretagne (F)	3	20 Feb 2001	2.5
CH	Switzerland (CH)	6	26 Dec 2000	4
FI	Middle Finland (FI)	2	04 Feb 2001	3
SA	Savoie (F)	3	05 Feb 2001	3
VO	Vorarlberg (A)	3	02 Feb 2001	3

Table 2 Concentration of volatile compounds showing significant differences (ANOVA, difference test on mean values) using purge & trap gas chromatography (peak height given in arbitrary units)

Peak no. (Fig.1)	Analytes	Retention index[1]	ANOVA[2]	Region (n)											
				AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
				x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
	Ketones and aldehydes														
1	Propan-2-one[a]	466	*	78^{B}	29	341^{AB}	230	572^{A}	260	552^{AB}	200	280^{AB}	370	89^{B}	52
5	Butan-2,3-dione[a]	556	*	20^{AB}	10	60^{A}	29	35^{AB}	17	31^{AB}	7	30^{AB}	7	12^{B}	3
6	Butan-2-one[a]	567	***	44^{BC}	27	41^{BC}	9	21^{C}	7	118^{A}	68	74^{AB}	13	21^{BC}	10
10	3-Hydroxybutanone[b]	683	***	$u.d.l.^{B}$	–	3.4^{B}	2.6	18.7^{A}	7.9	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–
18	Hexanal[a]	780	***	24^{AB}	10	4.8^{C}	1.6	26.9^{A}	8.2	30.4^{A}	2.7	7.3^{C}	2.4	13.2^{BC}	1.9
	Alcohols														
2	Propan-2-ol[a]	481	***	131^{BC}	64	167^{B}	80	62^{C}	35	38^{C}	19	291^{A}	56	66^{BC}	11
4	Propan-1-ol[a]	535	*	530^{A}	310	148^{B}	42	143^{B}	94	115^{B}	51	271^{B}	140	144^{AB}	120
7	Butan-2-ol[a]	583	***	83^{AB}	53	21^{BC}	20	$u.d.l.^{C}$	–	7^{BC}	6	116^{A}	55	18^{BC}	23
8	2-Methylpropanol[a]	616	*	11.8^{AB}	2.5	4.3^{B}	2.5	7.8^{AB}	3.9	7.1^{AB}	2.4	13.5^{A}	2.1	6.5^{AB}	3.8
9	Butan-1-ol[a]	652	**	33^{B}	18	143^{A}	61	19^{B}	21	52^{B}	62	18^{B}	4	24^{B}	32
11	Pentan-2-ol[a]	685	***	100^{B}	75	45^{B}	38	11^{B}	7	$u.d.l.^{B}$	–	291^{A}	92	23^{B}	7
14	2-Methylbutanol[a]	725	*	77^{AB}	12	61^{AB}	9	61^{B}	25	100^{AB}	51	129^{A}	58	43^{B}	13
24	Hexan-1-ol[a]	852	***	8.6^{A}	3.7	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–
	Esters														
12	Butanoic acid ethyl ester[a]	695	**	54^{BC}	30	23^{C}	10	169^{AB}	66	122^{ABC}	30	216^{A}	110	82^{ABC}	25
13	Acetic acid propyl ester [a]	697	***	25^{A}	14	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–
25	Acetic acid 3-methylbutylester[b]	859	**	5.1^{A}	2.6	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	3.3^{AB}	1.1	$u.d.l.^{B}$	–
	Hydrocarbons														
3	Pentane[a]	499	**	14.9^{A}	1.7	9.2^{B}	4.5	8.2^{B}	0.9	9.6^{AB}	2.1	10.8^{AB}	2.1	6.9^{B}	1.0
15	2,3,4-trimethylpentane[b]	756	**	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	9^{AB}	11	$u.d.l.^{B}$	–	12.0^{A}	5.7
16	Toluene[a]	761	**	12.6^{B}	2.3	12.0^{B}	2.8	10.6^{B}	3.7	25^{AB}	22	11.0^{B}	3.6	37^{A}	13
17	3-Methylheptane[a]	776	*	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	6.0^{A}	2.7	6.6^{A}	8.0	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–
19	2,2-Dimethylheptane[b]	787	**	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	7.9^{AB}	9.7	$u.d.l.^{B}$	–	10.9^{A}	4.9
20	Oct-1-ene[b]	790	***	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	22.1^{A}	9.4
21	Oct-?-ene[b]	803	*	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	7.3^{A}	8.9	$u.d.l.^{B}$	–	5.8^{AB}	2.5
22	Alcene (C8H16)[b]	808	***	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	5.5^{A}	2.2
23	Oct-?-ene[b]	813	*	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	$u.d.l.^{B}$	–	5.2^{A}	5.9	$u.d.l.^{B}$	–	4.9^{A}	2.1

Mean values within same row with at least one identical letter in the superscript are not significantly different. A>B>C>D (= significantly different contents) by using an univariate discriminant analysis

x mean value, s_x standard deviation, u.d.l. under the detection limit

AL Allgäu, *BR* Bretagne, *CH* Switzerland, *FI* Finland, *SA* Savoie, *VO* Vorarlberg

[a] Confirmed by comparing retention indices

[b] Identification with MassLib

[1] SPB1 chromatographic column; [2]* $p \leq 0.5$, ** $p \leq 0.1$, *** $p \leq 0.01$

Results and discussions

As the number of samples analysed was very limited (20 in total from six regions), we restricted the discussion of the results to tests of mean value differences and untrained classification techniques such as principal component analysis (PCA). In this technique, a linear combination of n parameters is calculated to maximise the distance between the points or samples in the n-dimensional space created. PCA shows natural group tendencies as the system does not know how to build the groups (untrained). PCA is less subject to over-fitting than trained classification techniques such as linear discriminant analysis, which would require a larger data set to be reliable.

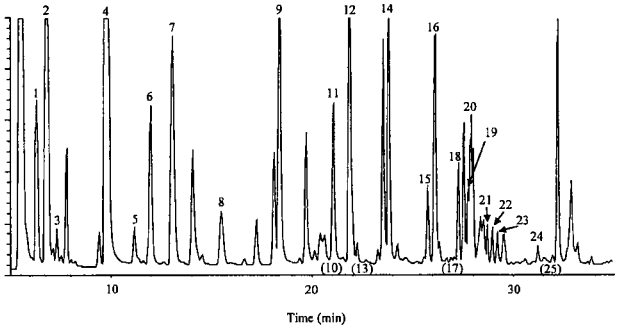

Fig. 1 Typical GC-FID of the volatile fraction of Emmental cheese (the peak numbering is listed in Table 2). Peaks 10, 13, 17 and 25 are not visible on this chromatogram

Purge & trap GC/MS-FID system

Figure 1 shows a typical chromatogram. The corresponding peak numbering is indicated in Table 2. For the Emmentaler Switzerland, four compounds showed extreme concentrations. The value of 3-hydroxybutanone was significantly higher in "Switzerland" than in all other regions. The propan-2-one concentration was the

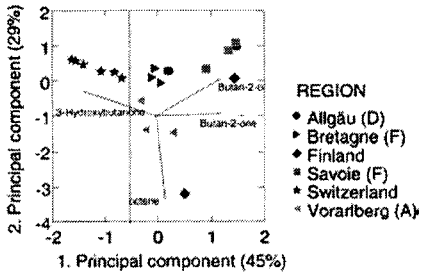

Fig. 2 Principal component analysis using the concentrations of butan-2-one, 3-hydroxybutanone, butan-2-ol and octene (RI=803). 100% separation of the Emmentaler Switzerland

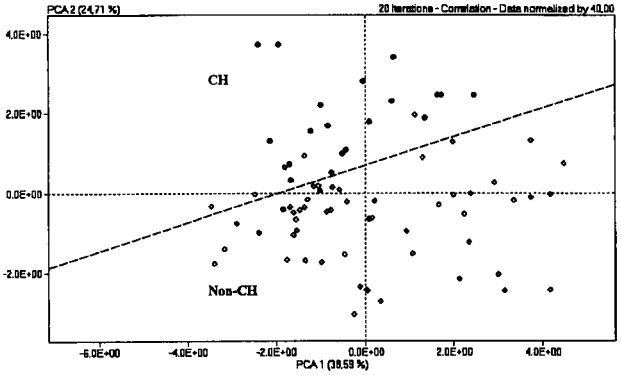

Fig. 3 Principal component analysis using an electronic nose. 90% correct classification for the Emmentaler Switzerland (*CH*) and 91% for the other samples grouped as one region (*non-CH*).

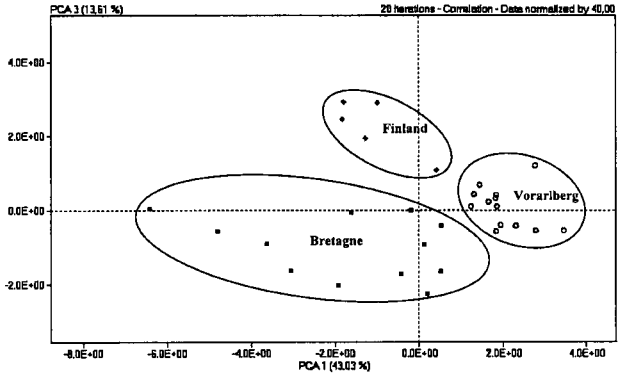

Fig. 4 Principal component analysis using an electronic nose. The three regions *Finland, Bretagne* and *Vorarlberg* are well separated

highest in "Switzerland;" however, not significantly different from "Bretagne," "Finland" and "Savoie." The concentrations of butan-2-one and butan-2-ol were the lowest in "Switzerland." The former was significantly lower than in "Finland" and "Savoie," the latter significantly lower than in "Allgäu" and "Savoie." 3-Methylheptane was found only in "Finland" and in "Switzerland" and at similar concentrations. These semi-quantitative results can unfortunately not be compared with those from previous studies due to the different traps used for preconcentration (e.g. Tenax vs. Carbosieve SIII/Carbopack B60/80 in [18]).

Certain compounds seemed highly specific to a given region. Hexan-1-ol and acetic acid propyl ester were present only in "Allgäu." 3-Methylbutylester of acetic acid occurred only in "Allgäu" and "Savoie." "Savoie" showed significantly higher concentrations of propan-2-ol and pentan-2-ol than in the cheeses from the other regions. "Finland" and "Vorarlberg" showed some similarities: 2,3,4-trimethylpentane, 2,2-dimethylheptane as well as two different octenes were detected only in cheeses from these two regions. It must be pointed out that only the sample FI1, and not FI2, contained the latter compounds. Furthermore, "Vorarlberg" was the only region where oct-1-ene and a further alkene were found. It was therefore very easy to separate "Allgäu," "Vorarlberg," "Savoie" and "Finland" one by one from the other regions using their specific compounds. The concentration of butan-1-ol was significantly higher in "Bretagne." This region may be separated from the others using the concentrations of butan-1-ol and hexanal. "Switzerland" was the single region where butan-2-ol was not detected in the samples. Combining the concentration of butan-2-ol, butan-2-one, 3-hydroxybutanone and octene (RI=803) by PCA, Switzerland" formed a separated group (Fig. 2). A tendency of grouping by the other regions (except "Finland") was also observed on the latter figure. These preliminary results will naturally have to be confirmed with a larger number of samples.

Electronic nose

The statistical analyses were carried out using the Software supplied with the Smart Nose. Though PCA is not a classification method, the program gives the possibility of making a group assignment by Euclidean distances in the multidimensional space created by the PCA. For each separation pattern, a new set of parameters was chosen to calculate the principal component scores.

A PCA on 76 analyses (in general 4 replicates of the 20 samples) showed a good classification of cheese samples as Swiss and non-Swiss (Fig. 3). Correct classification was achieved for 90% of the Swiss replicates and 91% of the non-Swiss replicates. The analysis of the results showed that out of the 21 Swiss replicates, two belonging to two different samples were misclassified. This result is out-weighted by the fact that the other 3 or 4 replicates of the respective samples were correctly classified. The same considerations were valid for the misclassified non-Swiss samples, where one replicate each from Finland, Vorarlberg and Savoie, and two from Germany were misclassified.

As expected, performing the statistical analysis with a limited number of regions enhances the quality of the classification. For instance, considering only samples from Bretagne, Vorarlberg and Finland, 100% correct classification was achieved (Fig. 4). Table 3 gives the

Table 3 Classification results from a PCA using an electronic nose

	Region	% Correct classification	
Switzerland vs	Other regions	CH: 90	non-CH: 91
Switzerland vs	Allgäu	CH: 100	AL: 92
Switzerland vs	Bretagne	CH: 95	BR: 100
Switzerland vs	Finland	CH: 90	FI: 100
Switzerland vs	Savoie	CH: 90	SA: 83
Switzerland vs	Vorarlberg	CH: 95	VO: 100

percentage of correctly classified cheese samples from Switzerland versus one of the other regions, one at a time. In this region-by-region analysis, Swiss replicates were at least 90% correctly classified. As for the rest of the regions, only "Allgäu" and "Savoie" showed less than 100% correct classification (92 and 83%, respectively).

Conclusion

The volatile compounds of Emmental cheese samples from different European regions were investigated by GC-MS. Each region could be separated from the others using compounds which were more or less specific to one or two regions. For instance, the concentrations of butan-2-one, 3-hydroxybutanone, butan-2-ol and octene made it possible to separate "Switzerland" from the other cheeses. These investigations showed the potential of volatile compounds to discriminate cheese samples with different origins. However, this analytical tool is very expensive and time-consuming and a less costly method would be preferable.

The use of an MS-based electronic nose turned out to be an interesting alternative to the GC-MS with purge & trap technique whose main advantage consists in a clear identification of the discriminating compounds. By separating Swiss and non-Swiss samples using PCA, 90% of "Switzerland" and 91% of the remaining samples were correctly classified via the Euclidean distances. By discriminating each non-Swiss region one at a time from "Switzerland", correct classification rates of 90–100% were obtained for "Switzerland" and 83–100% for the other regions. The use of a trained classification technique, such as linear discriminant analysis or differential factor analysis, in combination with a larger database, should improve the discrimination. Moreover, the preconcentration of the headspace before injecting it into the MS detector should enhance the sensivity of the analysis and hence the potential of this analytical technique.

Acknowledgement The authors thank the Swiss Federal Office of Public Health for its financial and logistical support to the project, G. Urbach and G. Gremaud for their reviewing of the publication, R. Gauch for his technical support as well as J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland), H. Jatila (Valio Ltd, Finland) and H. Schmallegger (Alma GmbH, Austria) for collecting the samples.

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CHAPTER 10

Analytical methods for the determination of the geographic origin of Emmental cheeses. Stable isotope ratios, major, trace and radioactive elements.

L. Pillonel, R. Badertscher, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
P. Froidevaux
Institut de radiophysique appliquée (IRA), 1007 Lausanne (Switzerland)
G. Haberhauer
ARC Seibersdorf research GmbH, 2444 Seibersdorf (Austria)
S. Hölzl, P. Horn
LM-Universität München, 80333 München (Germany)
A. Jakob
Labor Spiez, 3700 Spiez (Switzerland)
E. Pfammatter
Laboratoire cantonal du Valais, 1950 Sion (Switzerland)
U. Piantini
Ecole d'ingénieur du Valais, 1950 Sion, (Switzerland)
A. Rossmann
Isolab GmbH, 85301 Schweitenkirchen (Germany)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Food Science and Technology, 36, 615-623 (2003)

Abstract

Twenty Emmental cheeses from six European regions (Allgäu (D), Bretagne (F), Finland, Savoie (F), Switzerland and Vorarlberg (A)) were analysed for stable isotope ratios such as $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, D/H and $^{87}Sr/^{86}Sr$, as well as major (Ca, Mg, Na, K), trace (Cu, Mn, Mo, I) and radioactive elements (^{90}Sr, ^{234}U, ^{238}U). The discriminating potential of these parameters was evaluated using difference tests of mean values and principal component analysis (PCA). "Finland", "Bretagne" and "Savoie" cheeses were well separated using $\delta^{13}C$-, $\delta^{15}N$-, $\delta^{2}H$- and $\delta^{87}Sr$-values. Concentrations of molybdenum and sodium allowed the groups "Switzerland", "Vorarlberg" and "Allgäu" to be separated. ^{90}Sr activity was highly correlated with the altitude of the production zone. Using this parameter, "Finland" and "Bretagne" were separated from "Vorarlberg" cheese. The results of the current screening test will help selecting the best tracers of origin for the remainder of the project.

Keywords: Authenticity; Geographic traceability; Emmental cheese; Stable isotope ratio; Trace element; Radioactivity

1. Introduction

Analysis of stable isotopes has often been used to determine the authenticity of different food products, e.g. fruit juices, wines, spirits, essential oils, milk and milk products, honey and olive oil (Hener et al., 1998; Rossmann, 2001). In milk products, this technique was basically used for the origin assignment of milk (Kornexl, Werner, Rossmann, & Schmidt, 1997; Rossmann, Kornexl, Versini, Pichlmayer, & Lamprecht, 1998), butter (Rossmann et al., 2000) and cheese (Manca et al., 2001). The stable isotope ratios of various elements provide complementary information, which can be combined with multivariate statistics.

*Corresponding author. Tel.: +41-31-323-8167; fax: +41-31-323-8227.
E-mail address: jacques-olivier.bosset@fam.admin.ch (J.O. Bosset).

The $^{13}C/^{12}C$ ratio for both milk fat and cheese protein gives information on the type of forage fed to the cows. The carbon isotope value of plants depends on their photosynthetic cycles for CO_2 fixation (Smith & Epstein, 1971). C_4 plants such as maize show higher $\delta^{13}C$ values than C_3 plants, which constitute the major part of a cow's fodder.

Differences in the $^{15}N/^{14}N$ ratio also result essentially from forage. Organic fertilisers and intensive farming methods increase the level of ^{15}N in the soil and consequently in the plants, in milk, and in cheese (Mariotti et al., 1981). In addition, climate and soil conditions can influence $^{15}N/^{14}N$ in soil (Farrell, Sandercock, Pennock, & Van Kessel, 1996) and therefore the type of plants growing on it. Nitrogen-fixing plants (leguminosae) show lower $\delta^{15}N$ than non-nitrogen-fixing plants (Delwiche & Steyn, 1970).

The $^{18}O/^{16}O$ ratio of milk depends on the water ingested and the proportion of fresh vs. dry fodder. Isotope ratios of precipitation and groundwater depend largely on temperature, latitude, altitude and distance from the sea (e.g. Moser & Rauert, 1980). In grass proper, enrichment in ^{18}O occurs due to fractional evaporation of water. Therefore, during summer, when cows feed almost exclusively on fresh grass, a higher $^{18}O/^{16}O$ ratio is observed (Kornexl et al., 1997; Rossmann et al., 1998). Similarly, D/H gives the same climate and weather information as does $\delta^{18}O$.

The $^{34}S/^{32}S$ ratio is more difficult to interpret due to the numerous factors influencing it: nature of soil, industrial emissions and sulphur-containing fertilisers (Rossmann, 2001). It was not investigated in the current study.

The $^{87}Sr/^{86}Sr$ ratio can also be useful for origin assignments as it is dependent only on the types of rocks and soils, and not on human activity, climate or season of production (Rossmann et al., 2000). ^{87}Sr is produced from ^{87}Rb by radioactive β-decay, whereas the abundance of primordial ^{86}Sr remains virtually constant in a given rock. Old acidic rocks such as granite show the highest ratios, mafic and carbonate-rich rocks the lowest.

Several major and trace elements have also been used successfully for determination of the authenticity of wine (e.g. Baxter, Crews, Dennis, Goodall, & Anderson, 1997). In cheese, trace elements mainly have been measured for descriptive purposes (Favretto, 1990; Gambelli, Belloni, Ingrao, Pizzoferrato, & Santaroni 1999; Karadjova, Girousi, Iliadou, & Stratis 2000). Coni, Bocca, Ianni, and Caroli (1994) and Coni, Caroli, Ianni, and Bocca (1995) investigated trace elements in milk and dairy products to find correlations between animal fodder, time of year, environmental conditions, and level of elements in raw milk. Concentrations of Ba, Cd, Co, Cr, Pb and Sr were highest in winter samples; those of Al and Mn were lowest. No particular trend was observed for Cu, Mg, Ni and Zn. In a further study, significant differences in the selenium content of nonfat dry milk in various countries were found (Varo et al., 1984). Favretto, Marletta, Bogoni, and Favretto (1989) observed correlations between some trace elements and the geographic origin of Italian milk.

Measurement of radioactive constituents may also give important information on the geographic origin of a food product. For instance, environmental ^{90}Sr in Central Europe stems essentially from atmospheric nuclear bomb tests performed in the second half of the 20th century and to a lesser extent from the Chernobyl accident.[1] Sr follows calcium in the food chain and could therefore be an interesting element in cheese (Geering, Friedli, & Lerch, 1990). Various deposition intensities have been observed depending on the geomorphology of the country and weather conditions (Froidevaux, Geering, Schmittler, Barraud, & Valley, 2001, Chap. 7.1; Gastberger, Steinhäusler, Gerzabeck, & Hubmer, 2001). Further uranium series disequilibria such as $^{230}Th/^{234}U$ or $^{234}U/^{238}U$ are highly dependant on the geology of soil and rock alteration processes (e.g. Asmerom & Edwards, 1995; Chabaux, O'nions, Cohen & Hein, 1997, Suksi et al., 2001, and references therein) and could probably also be used as indicators of the geographic origin of cheese.

Authentication procedures based on inorganic parameters as described above are fundamentally different and complementary to "organic" parameters (e.g. microbiology, volatile compounds, organic acids). With the exception of some trace elements, such as Cu, the former are not influenced by cheese manufacture (milk transformation process) and hence are primary geographic indicators. The latter parameters are highly dependant on the culture used, the manufacturing process, etc. and can be considered as secondary geographic *indicators* because the technology used in the cheese making process is bound to regional or national traditions.

The current work belongs to the first part of a broad screening test in a 3-years study into the authenticity of Emmental cheese and its geographic traceability (Bosset, 2001; Pillonel et al., 2002a). During the first year, a great number of analytical methods were tested with respect to their discriminating potential using only two to six cheese samples per region (Pillonel et al., 2002a–c; Pillonel et al., 2003a, b). It is therefore obvious that the analytical results obtained from such a modest number of cheese samples per region can only give trends, which should be confirmed later if they appear valuable for discriminating cheeses produced in Switzerland from those produced in other countries. The aim of the present paper is to find out whether stable isotope ratios, trace elements and radioactive elements allow a discrimination between the various geographic origins of Emmental cheese samples to be made.

2. Material and methods

Details of reagents and analytical instruments are not given where a reference to these methods is available. The values of the isotope ratios are expressed in δ (‰) and correspond to international standards (V-SMOW for $\delta^{18}O$ and $\delta^{2}H$, V-PDB for $\delta^{13}C$, AIR for $\delta^{15}N$, and a laboratory water standard with $^{87}Sr/^{86}Sr = 0.7093$ for calculating $\delta^{87}Sr$-values) according to the relation:

$$\delta‰ = 1000[R_{sample} - R_{standard}]/R_{standard},$$

[1] The much lower temperatures encountered during the emission of radioactive material in Chernobyl, in contrast to a nuclear explosion, produced strontium oxides with relatively high solubility. They therefore moved faster from the soil surface into deeper zones.

where R represents the ratio of the higher mass to the lower mass isotopes.

The absolute reproducibilities of the isotope measurements were: $\pm 0.4‰$ for $\delta^{18}O$ in cheese water and $\pm 0.1‰$ in glycerol, $\pm 0.1‰$ for $\delta^{13}C$ in glycerol and pH 4.3-insoluble fraction, $\pm 0.2‰$ for $\delta^{15}N$, $\pm 2‰$ for $\delta^{2}H$ and $\pm 0.04‰$ for $\delta^{87}Sr$, all in pH 4.3-insoluble fraction.

2.1. Origins and selection of the cheese samples

The main framework of this study and sampling have been described in detail by Pillonel et al. (2002a). Table 1 summarises origin, date of manufacture and ripening time of the samples. Emmental cheeses were collected at different ripening times according to the availability in the market. Each region supplies cheese with typical characteristics depending among other factors on the ripening time which can vary from several weeks to several months. Three samples came from each region of Bretagne, Savoie, Vorarlberg and Allgäu, two samples came from Finland and six from Switzerland.

2.2. Preparation and measurement of $^{18}O/^{16}O$ ratios in cheese water

Fifty grams of grated cheese were lyophilised using a freeze-dryer lyolab G-GII (LSL Secfroid, Aclens, Switzerland). The quantitatively extracted water was cold-trapped for analysis. The measurements were done using a Finnigan Delta-Plus XL mass spectrometer equipped with a gas bench II system. The $\delta^{18}O$ analyses were performed according to the water equilibration method (Klimmek, Preiss-Weigert, & Wittkowski, 2000).

2.3. Preparation and measurement of $^{18}O/^{16}O$ and $^{13}C/^{12}C$ in glycerol

Fat was extracted from the above-mentioned lyophilised cheese with two times 100 mL of diethyl ether. After evaporation of the solvent, glycerol was obtained from the fat extract by hydrolysis according to Weber, Kexel, and Schmidt (1997) and Fronza et al. (1998).

Table 1
Origin and ripening time of the 20 cheese samples investigated

Sample	Region (country)	Date of manufacture	Ripening time (months)
AL 1-3	Allgäu (D)	20.12.2000	4
BR 1-3	Bretagne (F)	16.02.2001	2.5
CH 1-6	Switzerland (CH)	26.12.2000	4
FI 1-2	Middle Finland (FI)	05.02.2001	3
SA 1-3	Savoie (F)	08.02.2001	3
VO 1-3	Vorarlberg (A)	02.02.2001	3

Measurements were carried out on a Micromass MM 903 IRMS with an on-line coupled PDZ Europa Roboprep CN elemental analyser modified to perform pyrolysis of organic samples for oxygen isotope analysis on CO at 1100°C using glassy carbon as catalyst (Werner, Kornexl, Rossmann, & Schmidt, 1996).

2.4. Preparation and measurement of $^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{2}H/^{1}H$ and $^{87}Sr/^{86}Sr$ ratio in pH 4.3-insoluble fraction

The pH 4.3-insoluble fraction was obtained by acidifying the above-mentioned defatted lyophilised cheese to pH 4.3, washing the solid phase with water and drying it in vacuo (Manca et al., 2001). Measurements were carried out using a Micromass MM 903 IRMS with an on-line coupled PDZ Europa CN elemental analyser with standard operating combustion furnace at 1020°C. Quantities of ~0.8 and ~1.6 mg of pH 4.3-insoluble fraction for the measurement of $\delta^{15}N$ and $\delta^{13}C$, respectively, were weighed in 4×6 mm tin capsules and introduced into the elemental analyser, as described by Kornexl et al. (1997).

The $^{13}C/^{12}C$ ratio was investigated in two further fractions: (i) fat was extracted from the fresh cheese samples by centrifugation and (ii) carbon dioxide was extracted from cheese body and precipitated as barium carbonate according to Pauchard, Flückiger, Bosset, and Blanc (1980). However, the standard deviations within a region were much higher in these extracts than in pH 4.3-insoluble fraction or glycerol. The corresponding results were, therefore, not presented.

The $^{15}N/^{14}N$ ratio was also measured in a further fraction: total nitrogen from a Kjeldahl digestion fixed as ammonium chloride. However, the results (not shown) were not interesting because of their strong scattering within a region.

The $^{2}H/^{1}H$ ratio for pH 4.3-insoluble fraction was determined using a high temperature pyrolysis system working at 1450°C on the basis of ceramic tubes with glassy carbon reactor filling coupled with a Thermo Finnigan Delta-Plus XL IRMS system, designed for continuous flow isotope ratio mass spectrometry (CF-IRMS) of hydrogen isotopes in helium carrier gas. Three replicates of a sample, 250–300 µg each, were weighed into silver capsules. Measurements were made using calibration of the system with reference hydrogen gas and working standard material (sucrose) of known isotopic composition in regular repetitions (Kelly, Parker, Sharman, & Dennis, 1998).

For the strontium measurements, 0.2 g from the pH 4.3-insoluble fraction were ashed in a resistance furnace at 650°C. After dissolving the ash in nitric acid (3 mol/L), strontium was separated by SrSpec® (Eichrom Industries, USA). Isotope ratios were measured in a thermion mass spectrometer (Finnigan MAT261). All reagents

were prepared by sub-boiling. Analytical blanks were negligible.

2.5. *Major and trace elements measured by atomic absorption spectroscopy (AAS)*

The cheese samples were grated with a plastic rasp. For the determination of calcium, magnesium and sodium, approximately 1 g of material was digested in 5 mL suprapur nitric acid (650 g/kg) at normal pressure. For copper, potassium and zinc, 2.5 g of material were used. The solutions were analysed with an air–acetylene flame spectrometer (Badertscher & Kuhn, 1998a; Badertscher & Kuhn, 1998b, FAM accredited method). For the determination of manganese, 0.5 g of material were digested in 5 mL suprapur nitric acid (650 g/kg) under pressure in Teflon bombs. The solutions were analysed in a graphite furnace spectrometer (Badertscher & Liniger, 1999, FAM accredited method). CV were below the following values: Ca, 2.3%; Na, 4.7%; Cu, 4.8%; Zn, 1.0%; Mn, 3.6%; K, 6.4%; Mg, 2.4%.

2.6. *Preparation and measurement of trace elements using inductively coupled plasma mass spectrometry (ICP-MS)*

Grated cheese (plastic rasp, 0.4 g) was placed with 8 mL sub-boiled nitric acid (650 g/kg) and 4 mL ultrapure water in a quartz vessel. The vessels, closed with their lids, were placed in a Teflon bomb containing 4 mL of the same sub-boiled nitric acid, 2 mL ultrapure water and 0.5 mL oxygenated water (300 g/kg). Samples were digested under pressure in a microwave oven MLS ETHOS Plus up to 170°C for 20 min. The blue-green solutions obtained were diluted to 20 mL with ultrapure water and the solutions were transferred into PFA (perfluoro alkoxyl alkane) tubes for analysis. The microwave oven could process ten pressure bombs at once. For each batch, six samples, two references (milk powders, BCR-063R and BCR-150, IRMM, Geel, Belgium) and two blanks were prepared.

The solutions were analysed using a Perkin Elmer ELAN 6000 ICP-MS, calibrated with a ICP Multielement Standard Solution VI (Merck No 1.10580.0100). A solution of 0.05 mg/L Rh was added on-line (1:1) to the calibration and sample solutions as an internal standard with a peristaltic pump. Major operating conditions were as follow: RF power, 1100 W; plasma gas flow, 15 L/min; auxillary gas flow, 1 L/min; nebulizer gas flow, 1 L/min; solution pump rate, 0.5 mL/min; sample introduction system, micro-uptake concentric nebulizer, mini-cyclonic spray chamber; rinse time, 120 s at 12 rpm; sample uptake time, 15 s at 24 rpm; equilibration time, 120 s at 12 rpm; scanning mode, peak hopping; dwell time, 25 ms.

Analyses were carried out with the analytical program TotalQuant® (Perkin Elmer) which provides semi-quantitative results with a typical measurement uncertainty ($P = 0.95$) of about $\pm 10\%$. The precision was assured by three replicates, allowing a comparison between the different regions of origin. Therefore, only elements showing highly significant inter-region differences (ANOVA, $P \leqslant 0.001$) are reported.

2.7. *Preparation and measurement of radioactive elements*

γ-spectra (^{137}Cs) were recorded on a Canberra HPGe GCW4523 well detector in a 250 mL cylindrical geometry on fresh samples or in a 4 mL well geometry on ashed samples. After calcinations at 550°C for 24 h, half of the ash was used for the analysis of ^{90}Sr and half for $^{234}U/^{238}U$.

^{90}Sr activity was determined by measuring its daughter product, ^{90}Y (Geering et al., 1990). Briefly, a pure strontium source was obtained after dissolution of ash in concentrated hydrochloric acid, followed by a separation on a Dowex AG 50w x8 cation exchange column. After a 15 days growing period for ^{90}Y to reach secular equilibrium, yttrium was separated on a cation exchanger, precipitated as yttrium oxalate and counted in a gas proportional counter (Tennelec LB 4100w, 0.2 cpm background). Natural strontium and yttrium were used as carrier and measured by atomic absorption at the end of the chemical separation to determine the overall yield of the separation procedure. The precision was better than 0.008 Bq. Calcium and strontium have similar chemical properties. The results were normalised by the calcium concentrations to minimise the influence of the fabrication (e.g. curdle washing).

^{238}U and ^{234}U activity was determined by alpha spectrometry (Canberra Alpha Analyst) after microwave dissolution of the samples of ash (Milestone Ethos Plus) in 8 mol/L nitric acid, extraction on an Eichrom UTEVA cartridge (2 mL) and electrodeposition on a stainless steel disk in sulphate media at pH 1.9 (1.2 A, 6 V). ^{232}U (50 mBq) was used as an internal standard to determine the overall yield of the separation process (for more details on uranium determination by α-spectrometry see Haldimann et al., 2001). Coefficients of variation were smaller than 15% except for "Finland", with values up to 30%. The results were normalised by the calcium concentrations too. They are quite representative for the ash content and easier to handle.

2.8. *Statistical analyses*

The averages and standard deviations for cheeses from the same region were calculated for each parameter. Descriptive statistics, analysis of variance (ANOVA), pairwise comparisons of mean values with

Fisher's LSD test and principal component analyses (PCA) were performed with Systat for Windows version 9.0 (SPSS Inc., Chicago, IL). Natural grouping was visualised by PCA. The goal of the current study was not to build a model for the prediction of a group assignment, but to check the capacity of a series of parameters to discriminate Emmental cheeses according to their region of origin. Therefore, trained classification techniques such as linear discriminant analysis were not used because they require a larger data set for reliability.

3. Results and discussion

3.1. Stable isotope ratios

The results of 8 different isotope ratios are listed in Table 2. $\delta^{13}C$ values for pH 4.3-insoluble fraction and glycerol were strongly correlated ($r = 0.98$), indicating that they may have the same origin. As already observed for butter, δ-values were systematically higher in pH 4.3-insoluble fraction than in fats (Rossmann et al., 2000). The depletion of ^{13}C during the synthesis of lipids is a well-known phenomenon (Melzer & Schmidt, 1987).

Bretagne was the only region where maize is fed during winter months. Accordingly, samples from this region had by far the highest $\delta^{13}C$-values. Significant differences between the other regions were also found. Especially, δ-values in "Finland" were significantly lower than the others. An explanation for this shift may be the colder climate and the higher latitude of this country. Results on this point are, however, contradictory in the literature. Troughton and Card (1975) found no pronounced trends in the $\delta^{13}C$ values of C3 plants as a function of temperature (14–40°C). Measured $\delta^{13}C$ values in tree rings gave a temperature coefficient of +2.4‰/°C (Libby & Pandolfi, 1974) while Smith, Herath, and Chase (1973) observed a coefficient of −1.0‰/°C in C3 plants at low temperature (14°C) but no effect over the range 18–30°C.

Nitrogen ratios were investigated in the pH 4.3-insoluble fraction. "Bretagne" and "Finland" showed the highest $\delta^{15}N$ values, which reflect the conditions of more extensive use of organic fertilisers in these parts of Europe. The significantly lower values obtained in "Savoie" may be explained by extensive agricultural practices typical for such regions of the Pre-Alps or by a higher proportion of leguminosae in the fodder.

Oxygen ratios were investigated in two different fractions. In glycerol no significant differences were observed between the regions of origin. In cheese water, the ratios were significantly higher in "Bretagne". The proximity to the sea is responsible for the higher $\delta^{18}O$ in the rain and hence in ground water. The samples originating from the Alpine regions (Allgäu, Savoie, Switzerland and Vorarlberg) were depleted in ^{18}O due to their higher altitude and greater distance from the sea. The values in "Finland" were even lower, confirming results already obtained for butter (Rossmann et al., 2000). The analysis of cheese water is not very robust for authentication goals as it would be very easy to add water with a specific $\delta^{18}O$ value during cheese-making. An alternative is offered by D/H ratios, which generally follow the same trend as $^{18}O/^{16}O$, and can be measured in pH 4.3-insoluble fraction.

The D/H ratio of pH 4.3-insoluble fraction was determined by CF-pyr-IRMS. A good correlation was found with the results from oxygen in water ($r = 0.85$). Because the pH 4.3-insoluble fraction is also required for ^{13}C and ^{15}N measurements, ^{2}H measurements would be preferred to ^{18}O measurements.

The stable isotope ratios mentioned above all undergo seasonal variations which will have to be taken into account within the next step of the project. The isotope ratio of the element strontium ($^{87}Sr/^{86}Sr$) provides means by which regions may be subdivided further

Table 2
Stable isotope ratios in the 20 Emmental cheeses investigated

Region (n =)	ANOVA	AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Stable isotope ratios (δ‰[a])													
$\delta^{13}C$ in pH 4.3-insoluble fraction	***	-25.2^{C}	0.29	-17.8^{A}	0.77	-24.8^{C}	0.52	-26.38^{D}	0.05	-23.9^{B}	0.35	-24.9^{BC}	0.14
$\delta^{13}C$ in glycerol	***	-31.9^{CD}	0.14	-21.6^{A}	0.28	-31.6^{C}	0.42	-32.4^{D}	0.13	-29.7^{B}	0.39	-31.31^{C}	0.04
$\delta^{15}N$ in pH 4.3-insoluble fraction	***	5.3^{AB}	0.37	6.4^{A}	0.72	5.8^{AB}	0.70	6.4^{A}	0.33	3.8^{C}	0.16	5.1^{B}	0.20
$\delta^{18}O$ in cheese water	**	-7.8^{B}	1.0	-4.9^{A}	1.7	-8.8^{BC}	1.0	-11.2^{C}	0.92	-8.1^{BC}	1.0	-7.7^{B}	1.2
$\delta^{18}O$ in glycerol	ns	16	1.2	19.9	0.69	17	1.1	16.0	0.86	17	1.7	18	2.1
$\delta^{2}H$ in pH 4.3-insoluble fraction	***	-121^{BC}	1.1	-102^{A}	4.1	-122^{C}	1.9	-132^{D}	6.3	-115^{B}	1.8	-122.8^{C}	0.57
$\delta^{87}Sr$ in pH 4.3-insoluble fraction	***	-1.6^{B}	0.42	-1^{B}	1.6	-1.2^{B}	0.39	4.9^{A}	0.70	-1.1^{B}	0.26	-1.6^{B}	0.24

x = mean value, s_x = standard deviation, ANOVA, ns = not significant, $*p \leqslant 0.05$, $**p \leqslant 0.01$, $***p \leqslant 0.001$.
Production sites: A > B > C > D (= significantly different contents) or AB = A and B overlap by using a univariate discriminant analysis.
AL = Allgäu, BR = Bretagne, CH = Switzerland, FI = Finland, SA = Savoie, VO = Vorarlberg.
[a] $\delta‰ = 1000[R_{sample} - R_{standard}]/R_{standard}$, where R represents the ratio of the higher mass to the lower mass isotopes.

according to their geological conditions, independent of the season. The mean $\delta^{87}Sr$ values laid between –1.64‰ and –1.00‰ for all regions except for "Finland" with a mean value of 4.85‰. Finland is the only region investigated which is covered homogeneously with an old acidic rock bed. This led to this significantly higher ratio.

Combining the results of the four values, $\delta^{13}C$, $\delta^{15}N$, $\delta^{2}H$ and $\delta^{87}Sr$ (all for pH 4.3-insoluble fraction), in a principal component analysis (PCA), "Finland", "Bretagne" and "Savoie" were distinctly separated (Fig. 1). On the other hand, the regions "Switzerland", "Allgäu" and "Vorarlberg" were too similar (climate, geology, agriculture) to be differentiated.

3.2. *Major and trace elements*

Results obtained for the 7 elements analysed by AAS are listed in Table 3. All elements showed significant differences according to the geographic origin of the cheeses. The calcium content of cheeses from the two

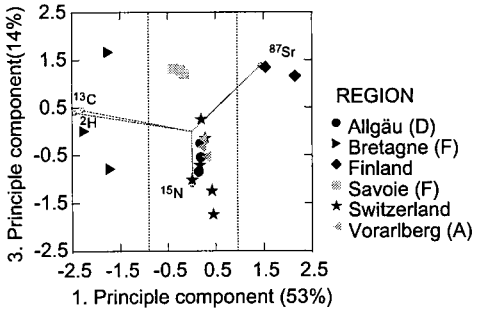

Fig. 1. PCA of the parameters $\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$, $\delta^{87}Sr$. Separation of the groups "Finland", "Savoie" and "Bretagne".

French regions (SA and BR) were the highest, those from Finland the lowest. This may be explained by the fact that no curd washing is applied in France whereas the washing rate is the highest in Finland with up to 26% water addition.

The regions Bretagne and Finland have in common that their Emmental cheese is manufactured in stainless-steel vats instead of the traditional copper vats encountered in most European regions. The copper content was therefore the lowest in "Bretagne". In "Finland", the values found were biased by the addition of copper sulphate into the milk during manufacture. It was not possible to explain the relatively low copper concentration in "Vorarlberg".

Differences obtained for magnesium and potassium were less significant than for the other elements and more difficult to interpret. Manganese and zinc were found at highest concentrations in "Bretagne" followed by "Finland". These higher concentrations could be explained by a different diet fed to the cows (type of concentrates). Sodium content correlated well with chloride concentration ($r = 0.95$, Pillonel et al., 2002a), confirming its origin as sodium chloride from the salt bath.

From the ICP-MS analyses carried out, four elements were retained for their high significance (Table 3). Two of them, copper and manganese, were already analysed by AAS. The results from both techniques were in very good agreement for copper ($r = 0.99$) and satisfactory for manganese ($r = 0.82$).

Iodine was a further interesting element. Significantly lower values were found in "Allgäu" and in "Vorarlberg", and significantly higher values in "Finland". The iodine content of milk products varies according to the season of the year and to the geographic origin of the milk (Larsen, Knuthsen, & Hansen, 1999). The

Table 3
Major and trace elements in µg/g cheese in the 20 Emmental investigated

Region (n=)	ANOVA	AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Elements by AAS													
Calcium	***	9496^{B}	153	10498^{A}	209	9472^{B}	193	8929^{C}	136	10020^{A}	357	9345^{BC}	134
Copper	***	9.0^{AB}	2.0	$< 1^{C}$	—	8.5^{B}	1.6	13.1^{A}	2.7	8.0^{B}	1.3	4.0^{C}	1.4
Magnesium	**	385^{A}	11	390^{A}	4	348^{B}	18	358^{AB}	21	365^{AB}	13	336^{B}	11
Manganese	***	0.28^{B}	0.02	0.40^{A}	0.03	0.26^{B}	0.03	0.30^{AB}	0.08	0.27^{B}	0.07	0.21^{B}	0.02
Potassium	*	931^{AB}	120	953^{AB}	63	837^{B}	77	800^{B}	12	977^{A}	38	868^{AB}	3
Sodium	***	2475^{A}	329	1700^{AB}	630	1316^{B}	217	2561^{A}	39	1482^{B}	375	1316^{B}	355
Zinc	***	43.5^{C}	1.3	47.8^{A}	2.5	42.2^{C}	0.8	47.1^{AB}	0.5	44.4^{BC}	1.5	41.3^{C}	0.7
Elements by ICP-MS[a]													
Copper	***	8.12^{B}	2.06	0.32^{C}	0.12	8.18^{B}	1.97	12.74^{A}	2.42	7.34^{BC}	0.89	3.59^{C}	1.38
Iodine	***	0.01^{C}	0.00	0.07^{BC}	0.01	0.10^{AB}	0.05	0.17^{A}	0.03	0.06^{BC}	0.01	0.02^{C}	0.00
Manganese	***	0.21^{B}	0.00	0.34^{A}	0.05	0.21^{B}	0.03	0.20^{B}	0.00	0.21^{B}	0.04	0.20^{B}	0.01
Molybdenum	***	0.11^{B}	0.03	0.07^{C}	0.00	0.15^{A}	0.01	0.07^{BC}	0.00	0.08^{BC}	0.02	0.07^{BC}	0.01

See Table 2.
[a] The results of the ICP-MS are semi-quantitative.

values are higher in winter, probably due to the use of iodine-enriched fodder during these months. The geographic variation can be ascribed to differences in the natural iodine content of potable water. In cheese, the brine represents a further source of iodine.

Finally, molybdenum was very interesting for separating "Switzerland" from the other regions as its concentration was significantly higher only in this country. The origin of these differences will be investigated in future work.

By reporting the concentration of molybdenum against sodium, it was possible to distinguish between the three geographically very close regions, "Switzerland", "Allgäu" and "Vorarlberg". "Switzerland" was even clearly separated from all other cheese samples (Fig. 2).

3.3. *Radioactive elements*

No ^{137}Cs activity could be detected in the 20 cheese samples analysed. Activities of the three isotopes, ^{90}Sr, ^{234}U and ^{238}U, as well as $^{234}U/^{238}U$ ratios are presented in Table 4.

The activity of ^{90}Sr, mainly due to aerosol deposition, depends on the geomorphology encountered. In the northern alpine regions, geomorphology may be expressed as altitude. A correlation coefficient of $r = 0.71$ was found between the ^{90}Sr activity in cheese and the altitude of the production zones (Fig. 3). However, it only allowed a clear separation to be made of "Vorarlberg" from "Bretagne" and "Finland".

Preferential leaching of ^{234}U due to alpha recoil leads to $^{234}U/^{238}U$ ratios less than those in soil. As a result, groundwaters are ^{234}U enriched, as well as the uranium accumulation zones (Suksi et al., 2001). Surprisingly, all samples showed a $^{234}U/^{238}U$ ratio higher than one, in contrast to values generally found in soil and vegetation. This ^{234}U enrichment of cheese is not yet fully understood, even if these results tend to prove that the cow's drinking water must be the main pathway of contamination of the milk with uranium. Further details may be found in Froidevaux, Geering, Pillonel, Bosset, & Valley (submitted).

The concentration of uranium in all analysed samples was lower than 32 mBq/kg cheese (= 2.6 µg/kg). "Switzerland" showed the highest values. The Finnish samples had unusually low uranium concentrations considering the high uranium content of soil and water in Finland.

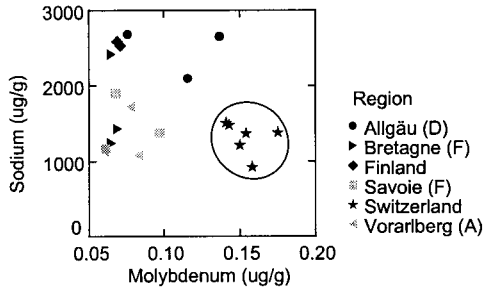

Fig. 2. Concentration of molybdenum and sodium by ICP-MS and AAS, respectively. Separation of "Switzerland" from all other samples. "Vorarlberg" and "Allgäu" are distinctly separated one from the other using these two parameters.

4. Conclusion

Primary geographic *indicators* for the origin of Emmental cheese were investigated in this screening

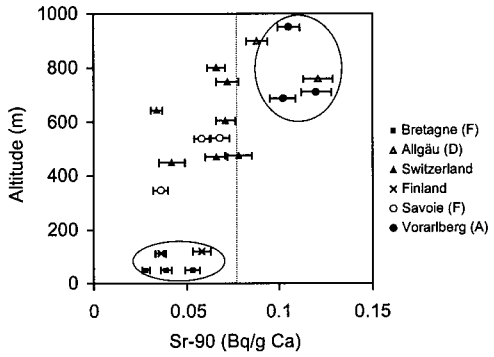

Fig. 3. Relation between the ^{90}Sr activity and the altitude of the production zone. "Finland" and "Bretagne" are separated from "Vorarlberg".

Table 4
Radioactive elements in the 20 Emmental cheeses investigated

Region (n =)	ANOVA	AL (3)		BR (3)		CH (6)		FI (2)		SA (3)		VO (3)	
		x	s_x	x	s_x	x	s_x	x	s_x	x	s_x	x	s_x
Radioactive elements													
Bq/g calcium													
^{90}Sr	**	0.09^{AB}	0.028	0.04^{C}	0.013	0.06^{BC}	0.018	0.05^{BC}	0.016	0.05^{BC}	0.016	0.11^{A}	0.010
^{234}U	**	1.2^{BC}	0.27	1.7^{ABC}	0.76	2.5^{A}	0.73	0.36^{C}	0.057	1.3^{ABC}	0.18	2.1^{AB}	0.73
^{238}U	*	0.90^{AB}	0.17	1.1^{AB}	0.52	1.9^{A}	0.76	0.275^{B}	0.007	1.1^{AB}	0.23	1.7^{A}	0.65
$^{234}U/^{238}U$	ns	1.33	0.06	1.53	0.09	1.4	0.23	1.3	0.17	1.2	0.12	1.20	0.09

See Table 2.

study. With only 20 samples analysed, the goal of the current study was only to establish potential indicators, and not to build a model for prediction. This will be the next objective of this 3-years project. Analyses of the stable isotope ratios in pH 4.3-insoluble fraction made it possible to separate geographically distant regions. PCA of the parameters $\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$, $\delta^{87}Sr$ delivered a good separation of the groups "Finland", "Bretagne" and "Savoie". Major and trace elements such as calcium, copper, molybdenum, iodine, manganese, sodium and zinc showed highly significant differences. Copper concentrations were strongly correlated with the technology used (stainless steel vs. copper vat). Using concentrations of molybdenum and sodium, it was possible to separate the three relatively close regions "Switzerland", "Vorarlberg" and "Allgäu". Stable isotope ratios and major/trace elements are complementary and very useful for the determination of origin. Most of these parameters are however influenced by the season due to variation in the forage composition. Seasonal effects will be studied in the next step of this project. Information obtained from the radioactivity results were of limited interest for authentication except for the correlation between ^{90}Sr and the altitude of the production zone in the northern Alps. ^{137}Cs was not detected in cheese.

Acknowledgements

The authors thank the Swiss Federal Office of Public Health for its financial and logistical support, G. Urbach and G. Gremaud for their reviewing of the publication as well as J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland™), H. Jatila (Valio Ltd, Finland) and H. Schmallegger (Alma GmbH, Austria) for collecting the samples and S. Duc for his technical support. PF thanks J.-J. Geering and F. Barraud (IRA) for their technical help and the Swiss Federal Office of Public Health for its help through the Contract No. 3189.001.4.

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CHAPTER 11

Analytical methods for the determination of the geographic origin of Emmental cheeses. Summary of a screening study.

L. Pillonel, J.O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Mitteilungen aus Lebensmitteluntersuchung und Hygiene, 94, 60-69 (2003)

Introduction

Food authenticity is a broad analytical challenge for food chemists, including many different types of misrepresentations (1). Determination of the geographic origin is one aspect which is receiving more attention with the increasing mobility and the low transport costs of our society. Consumers are used to agricultural products coming from all over the world, but what about Emmental cheese from Bretagne, Finland, Ireland or even New Zealand? It may sound strange to most Swiss consumers but less than 10% of the Emmental produced in Europe is Swiss (2).

The imminent opening of the Swiss cheese market will promote importation of less expensive cheeses produced in countries with low-cost milk. Emmental cheese, often called "Swiss cheese", is the example par excellence of a cheese variety that is produced in many countries around the world. It is often difficult or sometimes impossible to differentiate a prepacked young Emmentaler Switzerland™ from a foreign one by sensory analyses. The risk of finding mislabelled cheeses in stores should therefore not be underestimated. For the protection of the consumers, canton chemists need efficient analytical tools, in addition to rigorous accounting controls, to check the origin of cheese. The goal of the current 3-years research project, carried out at the Federal Dairy Research Station with the financial support of the

*Paper dedicated to Mrs. Helene Griessen on the occasion of her retirement in gratitude for her valuable processing of many submitted manuscripts.

Swiss Federal Office of Public Health (3), is to deliver such analytical tools to canton chemists.

Many analytical methods have been proposed for solving problems of authenticity (4). For instance, the techniques used in meat authentication were recently reviewed (5). The matrix of cheese is very complex and no single method could solve the problem. In order to find which is the best set of analytical methods, a preliminary study in the form of a screening test was carried out in a first step. This work, which focuses on Emmental cheese, may be extended in the future to other cheese types such as raclette or PDO (protected denomination of origin) cheeses.

Twenty Emmental cheese samples from six regions were investigated using more than 30 analytical methods. All results have been or will be published in various journals (6–11). This paper examines the use of these methods for the differentiation between Emmentaler Switzerland™ and those Emmental cheeses produced in other countries. The results of this preliminary study are shortly presented here along with the methods that will be used for the remainder of the project.

Material and methods

Table 1 summarises the origin, the date of manufacture and the ripening time of the samples used in the screening test. Six samples originated from Switzerland, three each from Allgäu (D), Vorarlberg (A), Bretagne (F) and Savoie (F) and two from Finland. The cheese samples were chosen with different ripening time (2.5–4 months) according to availability at the retailers in the corresponding regions. For most regions, this means a ripening time of less than three months. In Switzerland however, a 4-months old Emmentaler is still considered very young. We limited our investigations to young Emmental because there the risk of confusion is highest. For more details see (6).

Table 1
Origin and ripening time of the 20 cheese samples investigated

Abbreviation	*Region (country)*	*Number of samples*	*Date of manufacture*	*Ripening time (months)*
AL	Allgäu (D)	3	25.12.2000	4
BR	Bretagne (F)	3	20.02.2001	2.5
CH	Switzerland (CH)	6	26.12.2000	4
FI	Middle Finland (FI)	2	04.02.2001	3
SA	Savoie (F)	3	05.02.2001	3
VO	Vorarlberg (A)	3	02.02.2001	3

The analytical methods tested are listed in tables 2–4. For details, see the corresponding references indicated therein.

To evaluate the potential of each parameter to discriminate between cheeses, a difference test on the mean value of each region was calculated. Furthermore princi-

Table 2
Methods which will no longer be used

Methods/Parameters	*Reason for rejection*	*Reference*
Biogenic amines	Strong variation within a region	7
OPA[1]	Redundance with the NPN-fraction	6
Propionic acid bacteria	No significant differences	6
Enterococci, facultative hetero-fermentative und salt tolerant lactobacilli	Poor significant differences	6
Water, alkaline phosphatase, citrate, vitamin A und E	No significant differences	6, 7
Rheology	No significant differences except for penetration depth	7
Colour measurement	Strong dependence on the season	6
Fat chemistry, triglycerides, free fatty acids	Time-consuming, strong dependence on the season	9
Radioactive compounds	Poor significant differences, high cost	11
ICP-MS[2]	High cost	11

[1] o-phthalaldehyd-value
[2] Inductively Coupled Plasma Mass Spectrometry

Table 3
Promising but time-consuming methods

Methods or parameters	*Separated*	*Not separated*	*Reference*
HPLC[1] – peptide profile	FI, (CH+VO), (BR+SA)	AL	7
SDS-PAGE[2]	(BR, FI)	all others	7
Volatile compounds using GC[3]	CH, AL, VO, SA, FI	BR	10
Free amino acids	CH, VO, BR, AL, FI	SA	7

AL=Allgäu, BR=Bretagne, CH=Switzerland, FI=Finland, SA=Savoie, VO=Vorarlberg
Regions within brackets are not separated from one another
[1] High Performance Liquid Chromatography
[2] Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
[3] Gas Chromatography

pal component analysis (PCA) was carried out to visualise the natural groupings generated by the selected parameters.

Results and discussion

Two types of geographic indicators may be defined: the primary and secondary ones. Primary indicators are independent of the technology used. They are only

Table 4
Selected methods for the remainder of the project

Methods or parameters	*Separated*	*Not separated*	*Reference*
Infrared spectroscopy[1]	All regions		8
Volatile compounds using electronic nose	All regions[2]		10
Obligate heterofermentative Lb.	CH[2]	All others	6
Lb. helveticus	CH	All others	6
pH-value, L-lactate, succinate, pyruvate, N-fractions[3], fat, sodium chloride	VO, FI, BR, CH, AL[2], SA[2]		6
Volatile short-chain acids	BR, FI[2]	AL, CH, SA, VO	6
Atomic absorption spectroscopy[4]	BR, FI, CH[2], VO[2], AL[2]	SA	11
Isotope ratio mass spectrometry[5]	FI, BR, SA	CH, VO, AL	11

[1] Using a linear discriminant analysis on the spectra.
[2] A clear grouping tendency was observed for these regions. They are however not perfectly separated.
[3] including total nitrogen, water soluble nitrogen and non protein nitrogen
[4] including the elements calcium, copper, magnesium, manganese, molybdenum, sodium and zinc.
[5] D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{87}Sr/^{86}Sr$

influenced by the origin and the composition of the milk. Forage and drinking water, influenced by e.g. nature of soil, climatology and human activity, are the two factors influencing these indicators. Stable isotope ratios, radioactive elements and certain trace elements belong to this group. The secondary indicators are dependant on the technology used. They include all fabrication steps, starters and non-starters bacteria and ripening conditions. Each region produces a typical Emmental that, hand-made or industrial, expresses a character of origin which may be defined by secondary indicators. This group contains mainly organic compounds such as peptides, free amino acids, organic acids, enzymes, volatile compounds and microorganisms.

Primary geographic indicators

Stable isotope ratios have already been successfully used for the determination of origin of Swiss wines (12). In the current investigation, the following five isotope ratios were investigated using isotope ratio mass spectrometry (IRMS): D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{87}Sr/^{86}Sr$. "Bretagne" showed the highest $^{13}C/^{12}C$ ratio because of the maize forage fed to the cows during the winter months. Maize belongs to the group of C4-plants which are enriched in ^{13}C because of their specific pathway for fixing carbon dioxide. Levels of D/H and $^{18}O/^{16}O$ are often related to each other and depend on the distance from the sea, the altitude and the climatic conditions. They both presented the highest values in "Bretagne" due to the prox-

imity to the sea. The $^{15}N/^{14}N$ ratio is normally higher when organic fertilisers are intensively used. This ratio was lower in "Savoie", probably relating to the more extensive use of the pastures in this prealpine region. The four parameters mentioned above, though independent of the manufacturing process, undergo seasonal variation which must be taken into account. $^{87}Sr/^{86}Sr$ ratio is complementary and very useful for origin assignment as it is only dependent on the type of rock where the cows grazed and not on human activity, climate or season of production. The supplementation with imported forage (maize, concentrates, etc.) can, of course, modify the results. The Sr-ratio was the highest in "Finland" because of the old acidic rock bed (granite) underlying this country. The three geographically close regions "Switzerland", "Allgäu" and "Vorarlberg" could not be separated using stable isotope ratios (fig. 1).

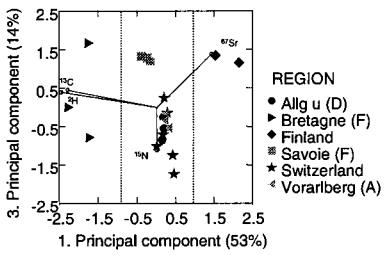

Figure 1 **Principal component analysis of the parameters $\delta^{13}C$, $\delta^{2}H$, $\delta^{15}N$, $\delta^{87}Sr$.** Clear separation of the groups "Finland", "Savoie" and "Bretagne" from the other regions

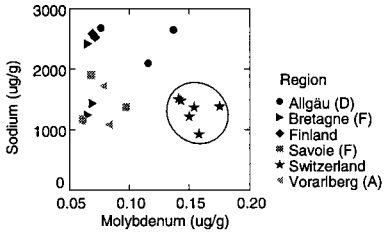

Figure 2 **Concentration of molybdenum and sodium by ICP-MS and AAS respectively.** Separation of Emmentaler Switzerland™ from all other samples. "Vorarlberg" and "Allgäu" are distinctly separated one from the other using these two parameters

Some trace elements also showed significant differences. The concentrations of sodium and molybdenum made possible the discrimination of the three latter regions. Emmentaler Switzerland™ was even completely separated from the other samples (fig. 2). The radioactive isotopes ^{137}Cs, ^{90}Sr, ^{234}U and ^{238}U showed few significant differences between the regions.

Secondary geographic indicators

This category contains more than 20 methods or parameters. The microbiological analyses delivered interesting information on the Emmentaler Switzerland™.

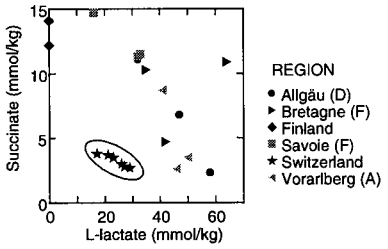

Figure 3 **Discrimination of the Emmentaler Switzerland™ according to L-lactate and succinate concentrations**

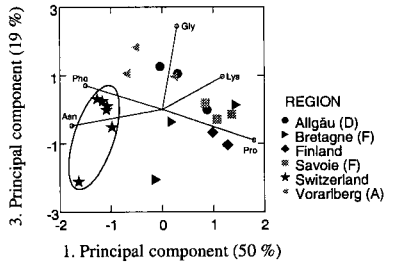

Figure 4 **Principal component analysis of the relative concentration of asparagine, glycine, lysine, phenylalanine and proline (total free amino acids =100 %).** Separation of the regions "Switzerland"

Significantly fewer obligate heterofermentative Lactobacilli and *Lb. Helveticus* were found in this cheese. The former are kept low with specific cultures while the latter are not used in starters at all because both have a high proteolytic activity that may lead to failures in texture and aroma. With the two parameters L-lactate and succinate, it was possible to clearly separate the Swiss products from the others (fig. 3).

Combining the five free amino acids asparagine, glycine, lysine, phenylalanine and proline by principal component analysis, a good separation of the group "Switzerland" was achieved (fig. 4).

Further parameters showed significant differences between the regions. A PCA of the values obtained for pyruvate, L-lactate, fat and pH-value led to the separation of "Finland", "Vorarlberg" and "Bretagne" (fig. 5).

Infrared spectroscopy made possible a 100 % correct classification of all groups using linear discriminant analysis (LDA) of the principal component scores. However, the separation is not visible on the 2-dimensional representation. Furthermore, it must be emphasised that trained classification techniques such as LDA require a large data set to be reliable. These promising results must be confirmed by further investigations.

Fat composition, which is strongly related to the type of forage, was less interesting because of the high seasonal variations and the difficulty of interpreting the results. For certain parameters or groups of parameters such as the conjugated linoleic acid (CLA), a correlation between the altitude of production zone and the concentration in cheese was however observed.

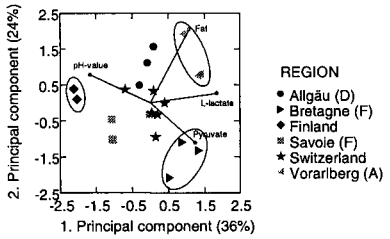

Figure 5 **Principal component analysis of the parameters fat, L-lactate and pyruvate as well as pH-value.** Separation of the groups "Finland", "Vorarlberg" and "Bretagne"

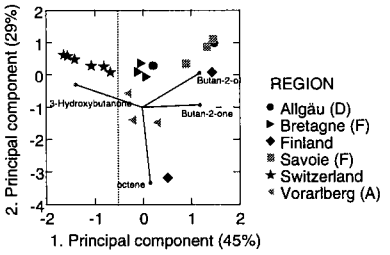

Figure 6 **Principal component analysis using the concentrations of butan-2-one, 3-hydroxybuta none, butan-2-ol and octene.** 100% separation of the Emmentaler Switzerland™

Valuable information was obtained from the volatile compounds investigated using gas chromatography. A combination of the peak heights of the four compounds butan-2-one, 3-hydroxybutanone, butan-2-ol and octene by PCA made possible a good separation of the Emmentaler Switzerland™ (fig. 6). However such analyses are time-consuming and expensive. The performance of a mass spectrometry-based electronic nose was therefore tested. The results, not presented here, were promising and could be enhanced in the future by incorporating a preconcentration step of the volatiles prior to injection into the spectrometer. Further investigations will be carried out along these lines.

Finally, some major and trace elements may be assigned to the group of secondary indicators. The best example is copper, which has very different concentrations depending on the type of vat used. In Bretagne, where Emmental is made in stainless steel closed vats, copper concentrations are approximately one order of magnitude lower than in Emmental manufactured in the traditional copper vats.

Conclusions

To select the criteria to be used for the remainder of the project, the methods tested within the current screening programme were divided into three groups according to the results obtained. The first group contains the methods which will no longer be used (table 2). The differences were either not significant enough or the dependence on the season was too strong. ICP-MS (Inductively Coupled Plasma Mass Spectrometry) will no longer be applied because of the difficulty of finding appropriate reference materials and its high cost. The interesting molybdenum concentrations will be investigated using atomic absorption spectroscopy (AAS).

Table 3 lists the methods that provided good results but were time-consuming and expensive. These methods could be exploited if the ones used from the third group do not allow for sufficient separation.

The methods listed in table 4 were chosen for the remainder of the project. It is not very likely that one of these methods alone will make possible a perfect separation and identification of all regions. However, by combining various methods it should be possible to recognise the origin of each of the samples. Some of these methods are rapid and inexpensive but may lack robustness in case of variation in the cheese production. The methods dealing with primary indicators (AAS, IRMS) require more time and skill but are less subject to variations. Both types of methods are therefore complementary and should be considered jointly. The investigations on the geographic situation and the manufacturing method together warrant a high confidence level for the classification of the origin.

During the year 2002–2003, the selected parameters will be tested on a new set of samples from the same six regions. This time, approximately 80 samples from winter and summer production will be investigated. This will then make possible the evaluation of any seasonal influence on the individual parameters measured. Also, powerful statistical analyses such as linear discriminant analysis or artificial neural networks will be used to classify the 160 samples analysed. A mathematical model for the control of the origin (Swiss or non-Swiss) will be proposed.

Acknowledgements

The authors thank the Swiss Federal Office of Public Health for its financial and logistical support (Contract Nr. 01.001323/2.26.01.-81), J. Casey, G. Urbach and G. Gremaud for their reviewing of the publication as well as J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland™), H. Jatila (Valio Ltd, Finland) and H. Schmallegger (Alma GmbH, Austria) for collecting and shipping the samples.

Summary

The current paper presents the summary of results obtained during the first part of a 3-year research project on the authenticity and geographic traceability of Emmental cheese. The aim of this study was to select the best methods or parameters for the discrimination between the different regions of origin. A screening test was carried out on more than 30 analytical methods or parameters with a limited number of samples ($n=20$) from six regions in Europe: Bretagne (F), Savoie (F), Allgäu (D), Vorarlberg (A), Finland and Switzerland. The following methods or parameters were selected for the remainder of the project: infrared spectroscopy, MS-based electronic nose, short-chain fatty acids as well as other organic acids, major and trace elements, stable isotope ratios, pH-value, gross chemical composition as well as microbiology, especially with regard to *Lb. helveticus.*

Zusammenfassung

Diese Arbeit stellt die Synthese der Resultate dar, die während des ersten Teils eines Forschungsprojektes über drei Jahre zur Authentizität und geographischen Rückverfolgbarkeit von Emmentaler Käse erhalten wurden. Das Ziel war, die besten Methoden oder Kriterien auszuwählen, um die verschiedenen Ursprungsregionen unterscheiden zu können. Ein Screeningtest wurde mit mehr als 30 analytischen Methoden oder Kriterien an einer begrenzten Anzahl von Proben ($n=20$) aus sechs Regionen Europas durchgeführt: Bretagne (F), Savoyen (F), Allgäu (D), Vorarlberg (A), Finnland und Schweiz. Die folgenden Methoden oder Parameter wurden für den weiteren Verlauf des Projektes ausgewählt: Infrarot-Spektroskopie, elektronische Nase basierend auf der Massenspektrometrie, kurzkettige Fettsäuren und andere organische Säuren, Mineralstoffe, Spurenelemente, Verhältnis der stabilen Isotope, pH-Wert, grobchemische Zusammensetzung und die Mikrobiologie, im speziellen *Lb. helveticus.*

Résumé

Cette publication présente la synthèse des résultats obtenus durant la première partie d'un projet de recherche de trois ans portant sur l'authenticité et la traçabilité géographique de fromages Emmental. Le but de cette première étude était de sélectionner les méthodes et critères les plus discriminants pour reconnaître des fromages d'origines différentes. Un test de criblage a été effectué avec plus de 30 techniques ou critères analytiques mais sur un nombre limité d'échantillons ($n=20$) provenant de six régions en Europe: Bretagne (F), Savoie (F), Allgäu (D), Vorarlberg (A), Finlande et Suisse. Les méthodes ou paramètres suivants ont été retenus pour la suite du projet: spectroscopie infrarouge, nez électronique basé sur la spectrométrie de masse, acides gras à courte chaîne et autres acides organiques, éléments minéraux et de traces, rapports d'isotopes stables, valeur de pH, composition chimique globale ainsi que microbiologie, spécialement pour *Lb. helveticus.*

Key words

Emmental cheese, Authenticity, Analytical traceability, Screening test

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Corresponding author: Jacques Olivier Bosset, Federal Dairy Research Station, CH-3003 Berne, E-mail: jacques-olivier.bosset@fam.admin.ch

CHAPTER 12

Geographic origin of European Emmental cheese. 1. Characterisation and descriptive statistics.

L. Pillonel, R. Badertscher, M. Casey, J. Meyer, J. O. Bosset
Federal Dairy Research Station, 3003 Berne (Switzerland)
A. Rossmann
Isolab GmbH, 85301 Schweitenkirchen, Germany
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel, Switzerland

International Dairy Journal, (submitted)

Abstract

To survey the authenticity of Emmental cheese, samples from the main European manufacturers were collected, corresponding to 110 winter and 73 summer samples. From a preliminary study, a series of promising analytical methods were selected and applied to the 183 samples: total nitrogen, water soluble nitrogen (WSN), 12% TCA soluble nitrogen (TCA-SN), pH-value, volatile short-chain acids, chloride, organic acids, enterococci, obligate heteroferm. Lb. (OHL), *Lb. helveticus*, sodium, copper, zinc, magnesium and stable isotope ratios (δ^2H, δ^{13}C, δ^{15}N, δ^{34}S). The data were analysed by univariate statistic according to the geographic origin and the season of production. Significant differences between the regions of origin were found for all investigated parameters (ANOVA with $P \leq 0.001$). Some regions showed very specific properties such as low leucine aminopeptidase and *Lb. helveticus* in Switzerland, low lactate and pyruvate in Finland or high δ^2H and δ^{34}S in Bretagne. However, for the correct assignment of all regions, several parameters combined by multivariate analysis will be required. Seasonal differences were observed in certain regions for the parameters acetate, propionate, caproate, WSN, TCA-SN, pyruvate, OHL, zinc and δ^{13}C.

Keywords: Authenticity, Emmental cheese, season effect, stable isotope

1. Introduction

Food authenticity and traceability of origin have become a theme of great interest during the last decade. Economic pressure, combined with new manufacturing technologies and low transport costs, have led to increasing numbers of food scandals and frauds. Various products with high added value are subject to adulteration or false denomination, e.g.: meat, olive oil, cheese, wine, coffee, honey.
Among cheeses, Emmental represents a very interesting case. Also known as Swiss cheese, Emmental is a worldwide widespread cheese type manufactured in almost all industrialised countries. Its added value depends on the technology used. Only a few regions such as Switzerland, the East part of France, Austria and South Germany manufacture a traditional Emmental using e.g. raw milk and copper vats. Some consumers are ready to pay more for these traditional/regional products. Switzerland is specially sensitive to the question because of the high milk price. Swiss Emmental is by far the most expensive, making fraudulent substitution the most lucrative in this case (Bosset, 2001). Therefore it is necessary to have

analytical means of checking whether a particular piece of Emmental corresponds with its labelling.
To reach this goal, a screening study using more than 20 analytical methods was first carried out on 20 authentic cheese samples collected in France, Switzerland, Germany, Finland and Austria. The aim of this preliminary study was to select pertinent analytical methods or parameters for discriminating the geographic origin of the samples analysed before using them in the current large scale study. The corresponding results have already been detailed in various publications (Pillonel et al., 2002; Pillonel et al., 2003a-b; Pillonel, Luginbühl, Picque, Schaller, Tabacchi & Bosset, 2003; Pillonel, Collomb, Tabacchi & Bosset, 2002; Pillonel, Ampuero, Tabacchi & Bosset, 2003). The selection of the methods from the screening was presented in a further paper (Pillonel, Tabacchi & Bosset, 2003).
The current work is the follow-up of these preliminary studies, taking advantage of the knowledge acquired so far. It was carried out with 110 samples from a winter production and 73 from a summer production using the analytical tools selected from the screening study. The current discussion of the results is limited to descriptive statistics and difference tests on the mean values for each region. Appropriate mathematical models using multivariate statistical analysis that allows the identification of the geographic origin are presented in a further paper (Pillonel, Tabacchi & Bosset, 2004).

2. Materials and methods

2.1. Cheese samples

Table 1 summarises the geographic origin, ripening time and season of production of the 183 samples analysed. To compare and discriminate the cheese samples according to their origin, the following seven regions were defined (Figure 1): Switzerland (CH), France Savoie (raw milk, FR), France Bretagne (thermised milk, FT_b), France East-Central (thermised milk, FT_e), Germany Allgäu (D), Western Austria (A) and Finland (FI). The results of the 20 samples used in the preliminary study were included wherever the corresponding analyses were carried out (this was not the case for LAP and $^{34}S/^{32}S$). The regions where thermisation and/or bactofugation is applied correspond to those where silage is used. The ripening time of the selected samples varied greatly depending on their geographic origin. According to manufacturing usage, Emmental may be sold with a maximum of 6-7 weeks (Bretagne) or a minimum of three months (Switzerland). The samples were selected for their ripening time to agree with what consumers may expect in the stores. The scattering within a given region is partly explained by the diversity in ripening of Emmental offered (from young to very mature). In Switzerland for instance, it was very difficult to find Emmental ripened for only 90 days. Most of the samples had a ripening time varying from four to six months, some of them reaching 12 months or even more. We focused our attention on young and medium aged samples (only 8 samples had more than 7 months) for which the risk of confusion is highest.
The value for enterococci, obligate heterofermentative lactobacilli, copper, magnesium, sodium and zinc were missing for the sample D13.

Table 1 Origin, ripening time, season of manufacture and number of samples investigated

Country of origin	Abbre-viation	Processing of the milk	Median ripening time (Min., Max.)	Number of summer samples[1]	Number of winter samples[2]
Austria	A	Raw	80 (60, 155)[1]	5	10
Switzerland	CH	Raw	175 (90, 260)	32	38
Germany	D	Raw	101 (71, 210)[3]	6	17
Finland	FI	Therm.[**]	90 (81, 90)	4	8
France	FR	Raw	106 (81, 215)	13	19
France	FT_b[*]	Therm., Bact.[**]	53 (42, 73)	7	12
France	FT_e[*]	Therm., Bact.[**]	74 (41, 149)	6	6
TOTAL				73	110

[1] Production 15 April-30 September
[2] Production 15 October-30 March
[3] Data only available for 7 samples
[*] FT_b = from the region Bretagne; FT_e = from the region East-central
[**] Therm. = Thermisation; Bact. = Bactofugation

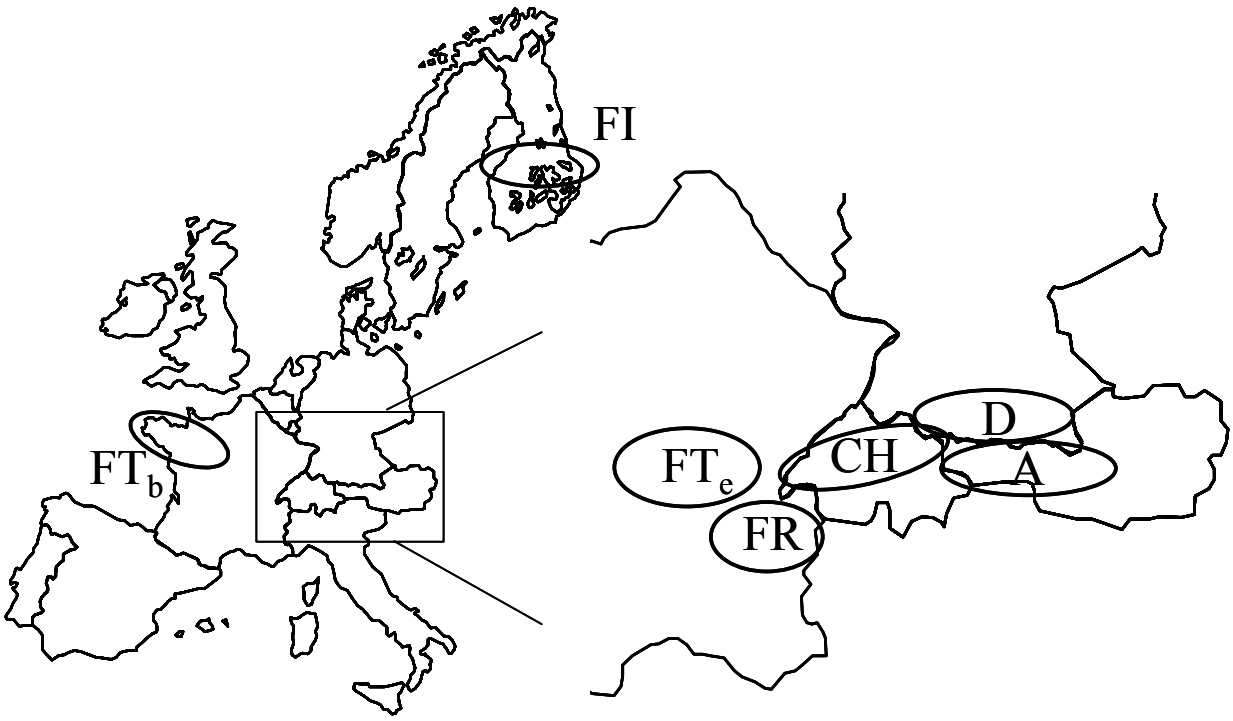

Figure 1 Origin of the samples investigated
Caption: A = Western Austria, CH = Switzerland, D = Germany Allgäu, FI = Finland, FR = France Savoie, FT_b = France Bretagne, FT_e = France East-Central

2.2 Sample preparation

Unless otherwise specified, the samples were deep-frozen (-20°C) until analysis. The samples were purchased as blocks, and 2 cm from the rind were discarded. The five first centimetres from the side (talon) were also not used. The samples to be delivered to the various laboratories were cut into slices across the whole height of the block. This helped to avoid misinterpretation of results due to gradients in the block.

2.3 Chemical and biochemical analyses

The following analyses were carried out: volatile short-chain acids using gas chromatography (Badertscher, Liniger & Steiger, 1993); fat according to Gerber van Gulik (anonymous, 1975); total nitrogen (TN), water-soluble nitrogen (WSN) and 12 % TCA soluble nitrogen (TCA-SN) according to Kjeldahl (Collomb, Spahni-Rey & Steiger, 1990); sodium chloride with a potentiometric titration using a silver electrode (anonymous, 1988); L- and D-lactate, succinate and pyruvate with enzymatic test kits after extraction (Anonymous, 1989); enzymatic activity of L-leucine-aminopeptidase using L-leucin-4-nitroanilide as substrate (LAP). The pH-value was determined at room temperature using a penetrometric glass electrode (Mettler-Toledo, no. 104063123).

2.4 Microbial analyses

Enterococci (ECOC) (according to Mossel, 1978) and obligate heterofermentative lactobacilli (OHL) (according to Isolini, Grand and Glättli, 1990) were determined on fresh samples. The occurrence of *Lb. helveticus* was investigated by the polymerase chain reaction (PCR) after extraction of the DNA using the High Pure PCR Template Preparation Kit (Roche, Switzerland). PCR amplifications were carried out using Taq DNA polymerase (Applied Biosystems). The specific primers for the detection of *L. helveticus* were 5'-CCGAAGGAACNCCTAATCTCTTA-3' and 5'-AGCAGATCGCATGATCAGCT-3' which correspond to internal sequences of the 16S rDNA gene. The conditions for the PCR were as follows: 10 min at 95°C; 33 cycles (30 s at 95°C, 30 s at 55 °C, 1 min at 72 °C); 10 min at 72 °C. Quantification of the 854 bp amplicon was carried out by electrophoresis using an Agilent 2100 Bioanalyser (Agilent, Waldbronn, Germany).

2.5 Inorganic compounds and isotope ratios

For the quantification of magnesium and sodium, approximately 1 g of grated material was digested with 5 mL nitric acid (650 g/kg, suprapur Merck) at normal pressure. For copper and zinc, 2.5 g of material were used. The solutions were analysed, after suitable dilution, with an air-acetylene atomic absorption flame spectrometer of type Varian SpectrAA-800 (Basel, Switzerland) (FAM accredited method no. ME05101O.620 (Mg), ME3902O.620 (Na), ME04002O.620 (Cu), ME04202O.620 (Zn)).
$^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{2}H/^{1}H$ and $^{34}S/^{32}S$ ratios were determined in a protein fraction obtained as follows: the grated cheese samples were freeze-dried and afterwards defatted with petroleum ether (Merck AG, Darmstadt, analytical grade) in a soxhlet apparatus. In the preliminary study, a further operation was included. The non-fat fraction was brought to pH 4.3 and the insoluble fraction washed with water. However no significant differences were observed between both preparation modes so that the shorter method was used. C, N and S were measured by Elementar elemental analyser Vario EL III coupled to an isotope ratio mass spectrometer (IRMS) AP 2003 (Elementar Analysensysteme GmbH, Hanau, Germany, and

GVI Instruments Ltd. Manchester, UK). D/H ratios were measured on a Thermo Instruments delta XL plus IRMS coupled with a Thermo Instruments high temperature pyrolysis unit.
The following standards with known ratios were used: a standard casein (Sigma-Aldrich, analytical grade) which had been calibrated in a European research project (SMT4-CT2236-1998) for $^{13}C/^{12}C$ and $^{15}N/^{14}N$, and later on for $^{2}H/^{1}H$ and $^{34}S/^{32}S$ vs. official reference materials (PEF-1 and NIST-22, V-CDT and silver sulfide, respectively). The values were reported in the δ-scale (‰) according to the corresponding international standards (PDB, NBS-22, V-CDT, air N_2). To check the reliability of the analyses, additional IHRM (in house reference materials) of known isotopic composition were used (wheat flour, lactose, sucrose).

2.6 Statistical analyses

The decimal logarithm of the microbiological counts was used for the calculations. The averages and standard deviations by regions of origin were calculated for each parameter. Descriptive statistics, box plots, analysis of variance (ANOVA), pairwise comparisons of mean values with Fisher's LSD test and t-test were performed with Systat for Windows version 9.0 (SPSS Inc., Chicago, IL). Fisher's LSD test was used to compare the various regions of origin ($P \leq 0.001$) and t-tests were used to investigate the influence of the season ($P \leq 0.01$).

3. Results and discussion

In the following analysis of variance (ANOVA), only parameters with $P \leq 0.001$ are presented. In the pairwise comparison, two groups were considered as different only if $P \leq 0.001$ in the Fisher's LSD test. Furthermore, a box plot was designed for each parameter to visualise the data, with the special aim of locating outliers which could lead to erroneous interpretations of the Fisher's test.
Indicators of origin for manufactured products may be subdivided into primary and secondary indicators. Primary indicators are not influenced by the technology applied for manufacture. In the case of cheese, compounds acting as primary indicators are transferred from the forage and the water consumed by the herd into the milk and finally the cheese. Primary indicators are not influenced by cheese making or ripening conditions but depend only on the feed of the cows. The latter undergoes natural variation over the year. Furthermore, some of the forage may be imported from distant countries, requiring careful interpretation of the results.
Secondary indicators do not depend directly on the geographic origin but mainly on the technology used for the transformation of a product, i.e. the milk used. Cheese making is related to local, regional or national traditions leading to differences within a cheese type. Starters, heating temperature of the curd, brining and ripening time are some of the manufacturing parameters which are typical for a defined region and lead to chemical, physical or microbial secondary indicators.

3.1 Primary indicators
Two trace elements were investigated in this category (Table 2). The magnesium concentration was significantly higher in FT_b than in all other regions except for D. Zinc also showed the highest concentration in FT_b whereas the lowest were found in CH, D and FR. The origin of such variations between the regions is very difficult to determine. Investigations on grass and concentrates fed in the respective regions would be necessary to help understand these differences. No indications on such differences could be found in the literature.

Table 2 Primary chemical indicators of origin measured in the 183 Emmental samples

	A		CH		D		FI		FR		FT_b		FT_e	
	x	s_x	**x**	s_x	**x**	s_x	**x**	s_x	**x**	s_x	**x**	s_x	**x**	s_x
Magnesium (µg/g)	**338**^{BC}	16	**339**^{BC}	23	**353**^{AB}	33	**331**^{BC}	19	**333**^{C}	18	**369**^{A}	23	**340**^{BC}	20
Zinc (µg/g)	**44.2**^{BC}	2.7	**42.4**^{C}	2.2	**43.1**^{C}	4.0	**46.4**^{AB}	1.2	**43.4**^{C}	1.7	**47.1**^{A}	1.9	**47.0**^{AB}	1.7
δ^2H (‰)*	**-122.0**^{E}	3.5	**-115.4**^{D}	5.6	**-117.5**^{D}	4.0	**-124.4**^{E}	4.9	**-111.2**^{C}	4.3	**-96.0**^{A}	4.1	**-102.2**^{B}	5.1
$\delta^{13}C$ (‰)*	**-25.52**^{CD}	0.59	**-25.09**^{C}	0.57	**-25.17**^{CD}	0.79	**-26.27**^{D}	0.10	**-24.64**^{C}	0.97	**-18.92**^{A}	1.92	**-20.78**^{B}	1.46
$\delta^{15}N$ (‰)*	**5.27**^{CD}	0.40	**5.94**^{AB}	0.66	**5.42**^{CD}	0.41	**6.53**^{A}	0.27	**4.45**^{E}	0.42	**5.84**^{ABC}	0.49	**4.98**^{DE}	0.28
$\delta^{34}S$ (‰)*	**3.19**^{C}	1.06	**3.91**^{C}	0.83	**3.83**^{C}	0.91	**5.12**^{B}	0.38	**4.85**^{B}	0.83	**7.52**^{A}	0.60	**4.97**^{B}	0.50

Caption: x = mean value; s_x = standard deviation;
Production sites: A>B>C>D (=significantly different contents) or AB = A and B overlap by using a univariate discriminant analysis.
*δ (‰) = 1000 [$R_{sample} - R_{standard}$] / $R_{standard,}$ where R represents the ratio of the higher mass to the lower mass isotopes

Four isotopic ratios were measured: $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ (Table 2). The box plots are displayed in Figure 2. Bretagne is a region very close to the sea, leading to the highest $\delta^{2}H$ values in FT_b, logically followed by FT_e, a region located in central France (Figure 1). The values for FR and CH lay in the middle, the values for A and D being still lower, because of their greater distance from the sea. The high latitude of Finland also explains the low $\delta^{2}H$ values found for this country (Moser & Rauert, 1980). Similar results were obtained for ^{18}O by Rossmann, Haberhauer, Hölzl, Horn, Pichlmayer and Voerkelius (2000). The regions where maize silage is fed are characterised by a significantly higher $\delta^{13}C$ value (Pillonel, Badertscher *et al.*, 2003). This was found in FT_b and FT_e. Smaller differences were observed in the other regions, which can be explained by such climatic conditions as temperature and humidity, which are known to cause shifts in the carbon isotope ratios in plant materials between regions. However one German sample showed a too high value (-22.3 ‰) to be only due to climate. Most probably, maize was used there too. The $\delta^{15}N$ values were the highest in FI, possibly due to a lower proportion of leguminosae or a higher level of organic fertilizers (e.g. with animal manure) in these northern parts of Europe. The lowest values found in D, A, and especially FR, reflected a probable low N fertilisation in these pre-Alpine regions (Rossmann, Kornexl, Versini, Pichlmayer & Lamprecht, 1998). There is no satisfactory explanation for the low $\delta^{15}N$ found in FT_e. In the case of $\delta^{34}S$, FT_b showed the highest values due to sulfate seaspray. Marine sulfate is known to be highly enriched in ^{34}S as compared with soil sulphate (Krouse, Steward & Grinenko, 1991). Further sources of ^{34}S fractionation are the nature of the bedrock (Rossmann *et al.*, 1998) and to a lesser extent anthropogenic emissions (Pichlmayer, Schöner, Seibert, Stichler & Wagenbach, 1998). The interpretation of the remaining $\delta^{34}S$ values was therefore more difficult.

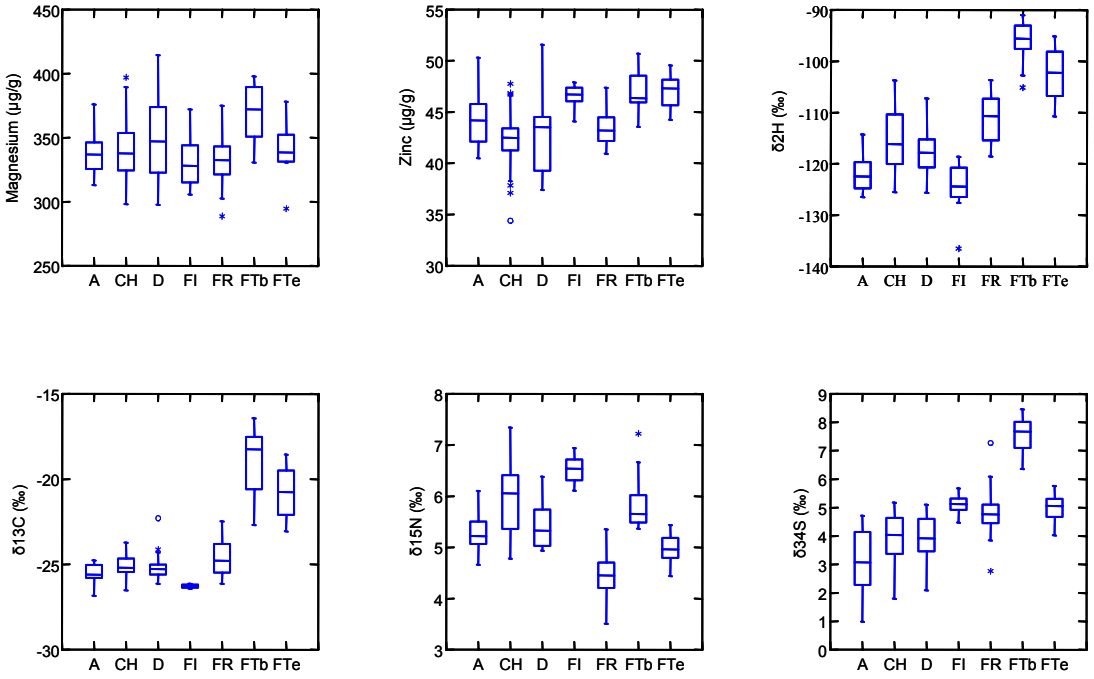

Figure 2 Box plots of the primary indicators of origin.
Caption: the regions A to FT_e are defined in Figure 1

3.2 Secondary chemical indicators

Table 3 and Figure 3 report the results of the parameters investigated in this category. The formate concentrations in FT_b were significantly lower than in CH, D and FI. Formate is normally catabolised from citrate by facultative heterofermentative bacteria. However in the preliminary study, no correlation was found between those two parameters (Pillonel *et al.*, 2002). The highest acetate concentrations were found in FR and FI, the lowest in FT_b and FT_e. Acetate is produced stoichometrically parallel to propionate during lactate fermentation. Therefore propionate followed the same trend as acetate ($r = 0.80$). The high values in FR and FI indicate a stronger propionic acid fermentation. As expected, the highest butyrate concentrations were found in both FT_b and FT_e, regions where silage feed is allowed. In Finland, though silage is also used, the concentrations encountered were not significantly different from the silage-free regions. This is probably due to a very strict production hygiene in the feeding and milking zone of the farms. In all other regions, outliers were found, indicating the difficulty of producing a spore-free milk. Regarding caproate, the concentration was significantly higher in A only because two samples showed extremely high values (4.36 and 3.15 mmol/kg). The median value was even lower than for CH.

Sodium chloride, which finds its origin almost exclusively in the brining, was significantly higher in D than in all other regions. The pH-value in CH and FT_b was significantly lower than in the remaining regions except in FT_e. No correlation was found between the sum of the investigated acids and the pH-value, probably due to the buffering effect of free amino acids which are strongly dependent on the degree of proteolysis.

The total nitrogen (TN) content of the cheeses were quite similar. Only FR had significantly higher values than FT_b, CH and D. Water soluble nitrogen (WSN) and 12 % TCA soluble nitrogen (TCA-SN) showed much greater differences. The highest degree of proteolysis was found for CH, D and FR. In the case of CH and FR, these results could partly be explained by the longer ripening time. The lowest value was found in FT_b where the blocks are ripened for only 6 to 7 weeks. Ratios such as WSN/TN and TCA-SN/WSN did not offer any additional information.

Among the elements investigated, two belonged to the group of secondary indicators, i.e. copper and sodium. The copper concentration in cheese was naturally significantly higher in the regions still using the traditional copper vats (A, CH, D, FR). However some very low values were also found in D and FR (Figure 3). It is difficult to define a concentration limit for the use of copper vat due to lack of sufficient information from the individual manufacturers. The concentration also depends on the way the milk is stored before processing. In this study, FI contained even more copper because of copper sulphate addition to the milk prior to cheese making. However, Finnish Emmental is also made without copper sulphate addition (H. Jatila personal communication). This cheese was not included in the study. The outlier sample from Austria was probably also manufactured with copper sulphate addition. Sodium originated largely from brining and therefore correlated strongly with the chloride measurements ($r = 0.73$).

Table 3 Secondary chemical indicators of origin measured inthe 183 Emmental samples

	A		CH		D		FI		FR		FT_b		FT_e	
	x̄	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x
Formate (mmol/kg)	**3.41**^{AB}	1.11	**3.74**^{A}	0.97	**3.68**^{A}	1.31	**4.23**^{A}	0.93	**3.24**^{AB}	0.77	**2.52**^{B}	1.25	**3.40**^{AB}	1.23
Acetate (mmol/kg)	**44.0**^{BC}	3.8	**46.4**^{BC}	4.6	**40.1**^{CD}	13.0	**50.4**^{AB}	4.4	**56.6**^{A}	8.0	**33.8**^{D}	9.1	**35.1**^{D}	16.7
Propionate (mmol/kg)	**46.1**^{AB}	12.4	**59.6**^{A}	9.8	**33.7**^{B}	22.5	**56.0**^{A}	19.1	**60.7**^{A}	16.4	**34.3**^{B}	11.2	**30.5**^{B}	25.0
Butyrate (mmol/kg)	**2.14**^{BC}	3.49	**0.97**^{C}	0.44	**1.87**^{C}	2.58	**1.40**^{C}	1.22	**0.99**^{C}	0.35	**3.79**^{AB}	1.33	**5.02**^{A}	3.45
Caproate (mmol/kg)	**0.71**^{A}	1.26	**0.29**^{B}	0.10	**0.18**^{B}	0.12	**0.12**^{B}	0.12	**0.26**^{B}	0.09	**0.20**^{B}	0.08	**0.15**^{B}	0.11
NaCl (g/kg)	**4.99**^{B}	1.92	**4.26**^{B}	1.36	**7.43**^{A}	3.00	**5.05**^{B}	2.20	**3.75**^{B}	1.19	**4.40**^{B}	1.34	**4.38**^{B}	1.50
pH	**5.68**^{ABC}	0.08	**5.62**^{D}	0.04	**5.75**^{A}	0.10	**5.70**^{ABC}	0.07	**5.73**^{AB}	0.07	**5.58**^{D}	0.04	**5.63**^{CD}	0.08
TN (g/kg)	**44.6**^{AB}	1.5	**44.4**^{B}	1.5	**44.0**^{B}	1.6	**44.9**^{AB}	1.3	**45.7**^{A}	1.0	**44.3**^{B}	0.8	**44.8**^{AB}	1.0
WSN (g/kg)	**8.8**^{B}	0.9	**10.2**^{A}	1.2	**10.4**^{A}	1.3	**8.4**^{BC}	1.8	**10.3**^{A}	1.4	**6.8**^{C}	1.2	**8.1**^{BC}	2.3
TCA-SN (g/kg)	**6.3**^{BC}	1.2	**7.1**^{AB}	1.2	**6.8**^{AB}	1.5	**6.1**^{BC}	1.1	**7.6**^{A}	1.6	**5.1**^{C}	1.1	**6.1**^{BC}	2.1
Copper (µg/g)	**5.5**^{C}	3.1	**8.1**^{B}	1.8	**7.9**^{BC}	4.6	**14.3**^{A}	2.4	**5.9**^{C}	3.5	**0.8**^{D}	0.1	**1.7**^{D}	1.3
Sodium (µg/g)	**2110**^{AB}	820	**1660**^{AB}	730	**2760**^{A}	1030	**2140**^{AB}	730	**1610**^{B}	510	**1570**^{AB}	860	**1580**^{B}	770

Caption: see Table 2

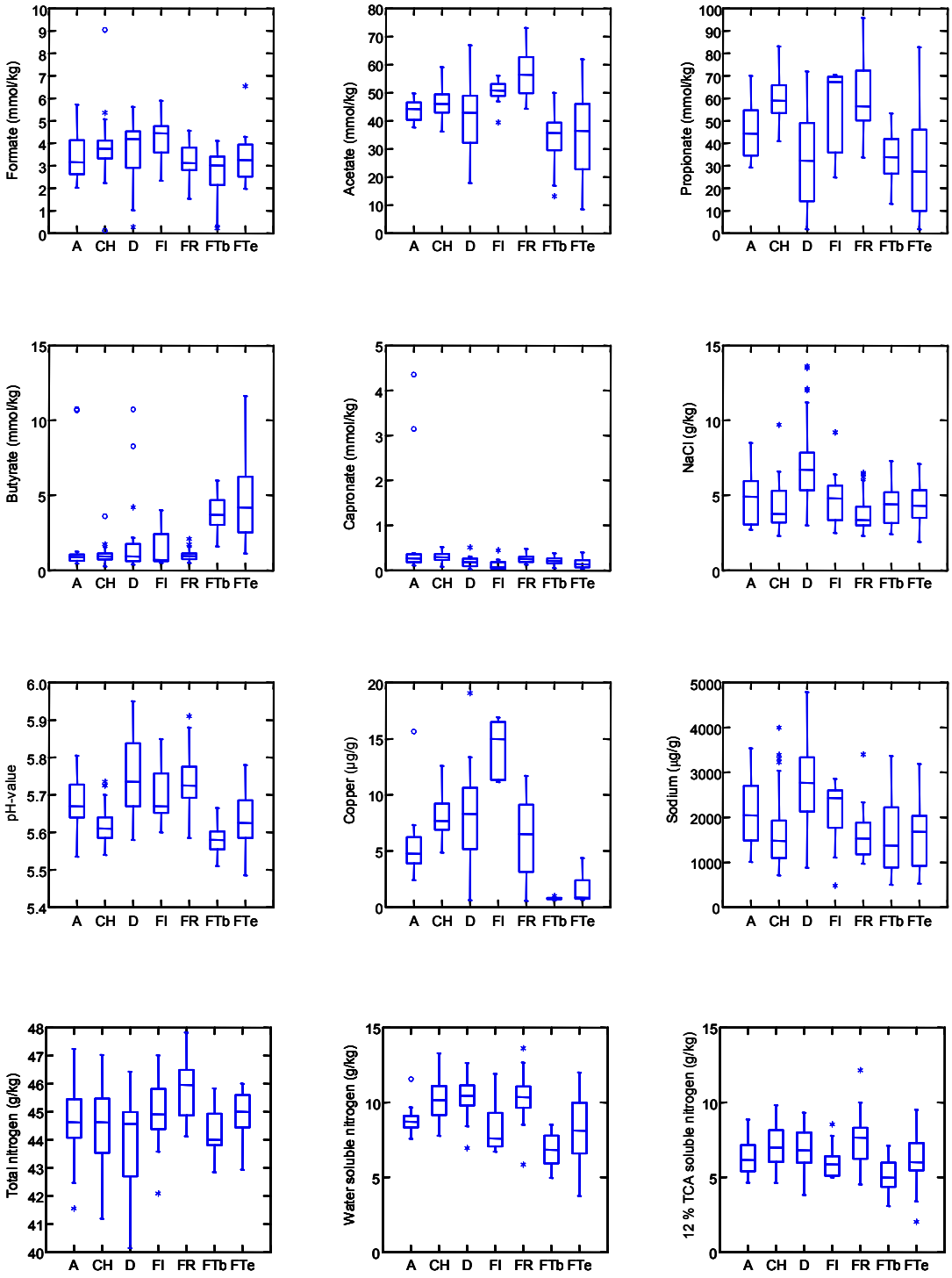

Figure 3 Box plots of the secondary chemical indicators of origin.
Caption: the regions A to FT_e are defined in Figure 1

3.3 Secondary biological indicators

Results are shown in Table 4 and Figure 4. Enterococci and obligate heterofermentative Lb. were counted on plates. These microorganisms are not desirable in cheese because they increase the risk of secondary fermentation. The lowest enterococci concentration was found in CH, FT_b and FI. The OHL concentration was lowest in CH and FI. In Switzerland and Finland, both species are kept at low level thanks to selected starter cultures and strict production hygiene.

Table 4 Secondary biological indicators of origin measured in the 183 Emmental samples

	A		CH		D		FI		FR		FT_b		FT_e	
	x̄	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x	**x̄**	s_x
Enterococci (cfu/g)	**4.9**^{A}	1.0	**2.4**^{B}	1.4	**4.9**^{A}	1.5	**2.2**^{B}	1.8	**4.9**^{A}	1.2	**2.9**^{B}	1.5	**4.8**^{A}	1.4
Obligate het. Lb. (cfu/g)	**6.4**^{A}	0.6	**3.1**^{B}	1.9	**5.6**^{A}	2.0	**3.6**^{B}	2.3	**6.0**^{A}	1.0	**6.0**^{A}	2.0	**6.4**^{A}	1.4
L-lactate (mmol/kg)	**39**^{AB}	11	**27**^{B}	10	**46**^{A}	17	**3**^{C}	8	**28**^{B}	15	**43**^{A}	19	**51**^{A}	25
D-lactate (mmol/kg)	**30**^{B}	10	**25**^{B}	10	**46**^{A}	16	**1**^{C}	3	**24**^{B}	13	**49**^{A}	10	**41**^{AB}	18
Succinate (mmol/kg)	**8.7**^{BC}	3.5	**5.1**^{D}	3.4	**8.6**^{BC}	4.6	**9.8**^{AB}	1.9	**12.4**^{A}	3.3	**8.1**^{BC}	3.2	**6.8**^{CD}	4.0
Pyruvate (mmol/kg)	**5.3**^{AB}	2.6	**4.1**^{B}	1.7	**3.6**^{B}	2.9	**0.6**^{C}	0.7	**5.9**^{A}	3.1	**5.9**^{AB}	3.1	**4.1**^{AB}	3.1
LAP (IU)[1]	**2.5**^{CD}	1.6	**1.6**^{D}	0.7	**9.8**^{C}	9.1	**22.7**^{AB}	8.7	**9.6**^{C}	12.3	**31.0**^{A}	18.5	**21.1**^{AB}	15.4
Lb. helveticus [2]	10 / 5		70 / 0		9 / 14		0 / 12		1 / 31		5 / 14		1 / 11	

Caption: see Table 2

[1] L-Leucine-Aminopeptidase (µmol/min substrate)

[2] Number of sample with: < 3 ng/µL / ≥ 3 ng/µL

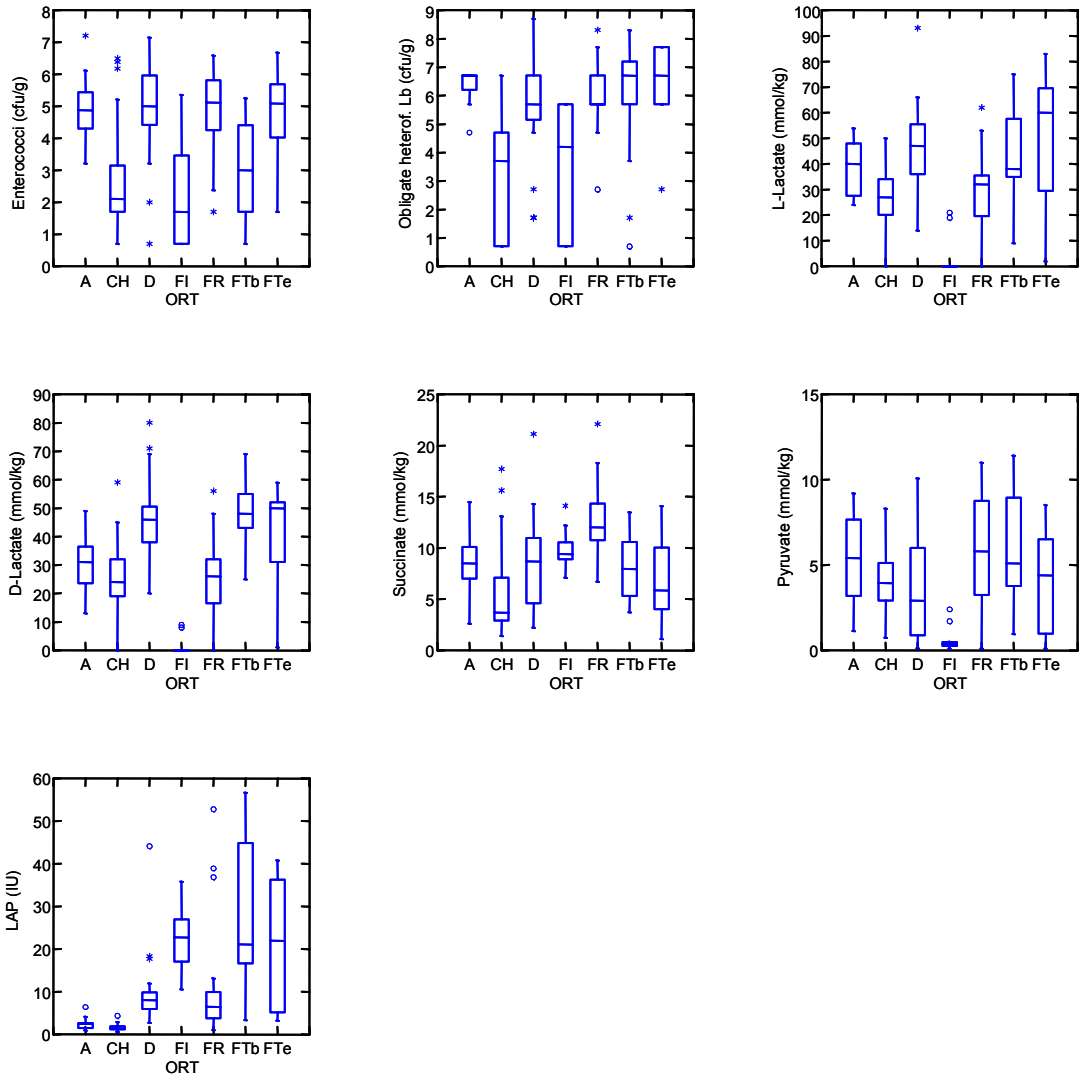

Figure 4 Box plots of the secondary biological indicators of origin.
Caption: the regions A to FT_e are defined in Figure 1

Lactate, which is metabolised to propionic acid, was negatively correlated with both acetate (r = -0.60) and propionate (r = -0.73). Except for two samples, FI no longer contained lactate, mainly due to a high level of curd washing (personal communication). The values for pyruvate were also extremely low in FI. The ratio L-/D-lactate (data not shown) did not produce any additional information. Succinate is produced by certain propionic acid bacteria with strong aspartase activity (Fröhlich-Wyder, Bachmann, Casey, 2002). A moderate aspartase activity may positively influence the quality of Emmental cheeses e.g. openness and flavour intensity. CH showed significantly lower values for succinate than all other regions except FT_e. FR had the highest concentration. It is difficult to interpret these results because no convincing correlation could be found between aspartate and succinate in the preliminary study (Pillonel *et al.*, 2002).
The presence of *Lb. helveticus* was determined semi-quantitatively by specifically amplifying a fragment of the 16S rDNA gene. The information obtained was more or less limited to a presence/absence response. In Switzerland, *Lb. helveticus* is never used in Emmental cheese starters because of its strong proteolytic activity. The amount of *Lb. helveticus* DNA measured in CH was below 1.2 ng/µL in all samples, most of them below the detection limit

of 0.1 ng/µL. The values close to one, indicated a natural contamination of the milk from the environment which most probably occurred during milking. In other regions, Emmental is also sometimes manufactured without use of *Lb. helveticus*. In some samples from FT_b, D or A, values below 1 ng/µL were found. In Table 4, the results were classified into two groups, i.e. < 3 ng/µL and ≥ 3 ng/µL. In the second group, the direct addition of *Lb. helveticus* can be assumed. The samples in this group can therefore not be of Swiss origin. A limit of 3 ng/µL was chosen to avoid any risk of misinterpretation.

An indirect way of obtaining information on the presence of *Lb. helveticus* is the measurement of the L-leucine-aminopeptidase (LAP) activity. This enzyme is produced in significantly higher amounts by *Lb. helveticus* than by *Lb. delbrueckii* (Bouton, Guyot, Dasen & Grappin, 1993). CH and A showed significantly lower values than the other groups. In CH, all values except for one at 4.5 were under 3. 75% of the cheese samples from the other regions showed values higher than 4.

Mostly the correlation between the LAP- and the *Lb. helveticus*-values for each region separately were poor. For instance in CH, the LAP values varied from 0.7 to 4.3 even when no *Lb. helveticus* was detected. The opposite phenomenon was also often observed, i.e. high concentration of bacteria for relatively low LAP-values. The aminopeptidase activity of other microorganisms such as Streptococcus thermophilus and the difference in activity between clones of *Lb helveticus* might explain the discrepancies found (Prost & Chamba, 1994).

3.4 Influence of the season

Most of the indicators (both primary and secondary) are influenced by the season because the composition of the forage changes depending on the time of the year. Specially where silage feeding is not allowed, the change from green fodder to hay could have important consequences on the results. Tables 5 shows for each separate region the parameters which change significantly with the season. Only the parameters dealt with in Tables 2-4 were investigated. Interpretation of these results is made difficult because of the lack of precise information on both manufacture and herd feeding.

Switzerland was the only region where no significant differences were observed. No differences within the secondary indicators were found in both French regions where thermisation is applied. The raw milk French Emmental (FR) had higher level of pyruvate, TCA-SN and zinc during summer. A and D showed similar trends with higher contents of acetate, propionate and zinc during the summer. Moreover the TCA-SN value was higher and the formate concentration lower in summer in A. In FI, three compounds showed significant differences. Propionate was higher in winter whereas caproate and WSN were higher in summer. The elevation of the $\delta^{13}C$ in winter in FT_b and FT_e was not surprising due to the increased proportion of maize during the cold season. The significant $\delta^{13}C$-change observed in FR and A was difficult to explain. However in both seasons the values remained below those typical of maize feeding regions (> -22‰).

Table 5 Parameters significantly influenced by the season of manufacture (P ≤ 0.01)

	A	CH	D	FI	FR	FT_b	FT_e
Formate	W	-	-	-	-	-	-
Acetate	S	-	S	-	-	-	-
Propionate	S	-	S	W	-	-	-
Caproate	-	-	-	S	-	-	-
WSN	-	-	-	S	-	-	-
TCA-SN	S	-	-	-	S	-	-
Pyruvate	-	-	-	-	S	-	-
OHL	-	-	-	-	-	-	-
Zn	S	-	S	-	S	-	-
δ13C	W	-	-	-	W	W	W

S = content significantly higher for the summer production (15 April-30 September)
W = content significantly higher for the winter production (15 October-30 March)

4. Conclusions

The objective of the current work was to statistically evaluate previously selected parameters likely to make it possible to discriminate between various geographic origins of Emmental cheese. The following seven regions were defined: Austria, Finland, France Savoie, France Bretagne, France East-Central, Germany and Switzerland. Some of these showed very characteristic properties. For instance, samples from Finland contained almost no lactate and pyruvate, and more copper than the other samples. In Swiss samples significantly less *Lb. helveticus* and a lower LAP activity were found. However, in all cases, multivariate statistical analysis will be required to reach, if this is ever to be possible, 100% recognition or classification. The latter is subject of a further publication (Pillonel *et al*., 2004).
The influence of the season on the parameters and for each region was also investigated. Regions with a high level of standardisation such as France East-Central and France Bretagne did not show any differences for secondary indicators. In Switzerland a single secondary indicator, in Austria, Germany and France Savoie, two and in Finland four were significantly influenced by the season. As far as the primary indicators are concerned, the zinc values were higher in summer in Germany and France Savoie and the $\delta^{13}C$ were higher in winter in the region with maize silage feed

Acknowledgements
The authors thank the Swiss Federal Office of Public Health for its financial and logistical support, G. Urbach and G. Gremaud for their reviewing of the publication as well as J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland™) and H. Jatila (Valio Ltd, Finland) for collecting the samples.

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CHAPTER 13

Geographic origin of European Emmental. 2. Use of discriminant analysis and artificial neural network for classification purposes.

L. Pillonel, U. Bütikofer, J. O. Bosset
Federal Dairy Research Institute, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel, Switzerland

International Dairy Journal, (submitted)

Abstract

True declaration of the geographic origin is an important issue for food products with international markets such as Emmental cheese. The goal of this work was to classify European Emmental cheeses according to their geographic origin using analytical means. 25 analytical parameters (factors) were measured in 183 samples. The results were combined by multivariate statistical analysis. Discriminant analysis (DA) and artificial neural network (ANN) delivered similar results when all regions and all factors were included. 95% and 91% respectively of the samples were correctly classified in the validation procedure. To reduce the analytical costs and the risk of overfitting, a DA based on a selection of only 11 factors was calculated. In this case, the Jackknifed validation delivered 95% correct assignments. Finally, a system was optimised to discriminate between the Swiss samples and the others. Building a new model for each of the 6 pairs, Switzerland *vs*. another region, 100% correct classification could be achieved for the Swiss samples.

Keywords: artificial neural network, discriminant analysis, Emmental cheese, authenticity, chemometric

1. Introduction

Fraud detection in foods often requires a highly accurate characterisation of the product including the use of many different analytical tools. Interesting reviews on food authenticity have been published either in technique-oriented form (Cordella, Moussa, Martel, Sbirrazzuoli & Lizzani-Cuvelier, 2002, Gremaud, Karlen, Hulliger, 2002) or in food matrix presentation (Dennis, 1998). The determination of the geographic origin of a foodstuff is a difficult task, especially in "living" foods such as cheese which undergo changes during ripening. As a consequence, the results of the selected analytical techniques must often be combined by multivariate analysis, also known as chemometric by chemists.
Chemometrics can be defined as the application of mathematical and statistical methods to maximise the chemical information extracted from data. Chemometrics are powerful tools finding applications in various domains covered by regularly published reviews (e.g. Lavine, 1998, 2000; Lavine & Workman, 2002). Pattern recognition is a specific application of chemometrics which occupies the attention of chemists involved in the fight against food fraud. Two comprehensive review articles focusing on chemometrics for authentication and classification of food products were published recently (Arvanitoyannis & Houwelingen-

Koukaliaroglou, 2003; Tzouros & Arvanitoyannis, 2001). Techniques such as Principal Component Analysis (PCA), Discriminant Analysis (DA), Principal Component Regression (PCReg), Partial Least Square (PLS), Artificial Neural Network (ANN) are commonly used for authentication purposes.

A crucial point of pattern recognition is the validation of the model. In an overfitted model, the classification into categories may superficially appear satisfactory, but is in fact not statistically significant. The following simple rule-of-thumb should therefore be applied before each classification: the number of factors (variates) included in the model should not exceed (n-g)/3, where n is the number of observations and g the number of categories fixed (Defernez & Kemsley, 1997). Moreover no classification should be carried out without cross-validation. A first possible procedure of cross-validation is to assign each observation at random to either a *training* or a *validation* set. The training set is only used to obtain the model, which is then applied in a second step to the validation set. Typically, training and validation set may contain 2/3 and 1/3 of the available observations respectively. If the results between training and validation differ strongly, the model is overfitted or insufficiently adapted. An alternative procedure is the *leave-one-out* or *Jackknifed* validation. It consists of omitting one observation at a time from the data set and using the remaining data set to obtain a model, which is then applied to the omitted observation. This is repeated n times, excluding each observation in turn and reintroducing the previously omitted observation. The results for the excluded observations only are then assessed. Once again, if normal set and Jackknifed validation diverge, the model is overfitted. Both validation procedures seemed to deliver comparable results (Defernez & Kemsley, 1997).

The present paper deals with the use of chemometrics to determine the geographic origin of Emmental cheese. Pattern recognition methods such as DA and ANN are applied to check a possible classification by region of the cheese samples. During a 3-year project, promising analytical techniques were selected and applied to 183 Emmental cheese samples from Europe. A more detailed description of the project as well as the corresponding individual results and univariate statistics have been presented elsewhere (Pillonel *et al*., 2004).

2. Materials and methods

Origin of samples and analytical methods

Sample selection, treatment and characteristics as well as analytical methods used have already been described by Pillonel *et al*. (2004). In short, the 183 samples originated from the seven following regions in Europe: Western Austria (=A), Switzerland (=CH), South Germany (=D), Finland (=FI), France Savoie raw milk (=FR), France Bretagne thermized milk (=FT_b) and France East-Central thermized milk (=FT_e). The 25 investigated factors finally retained as significant were the following: volatile organic acids formic (C1), acetic (C2), propionic (C3), n-butyric (C4) and n-caproic (C6), total nitrogen (TN), water-soluble nitrogen (WSN) and 12%-TCA soluble nitrogen (TCA-SN), sodium chloride, L- and D-lactate, succinate and pyruvate, L-leucine-aminopeptidase activity (LAP), pH-value, enterococci (ECOC) and obligate heterofermentative lactobacilli (OHL), sodium (Na), copper (Cu), magnesium (Mg), zinc (Zn) as well as the stable isotope ratios δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. *Lb. helveticus* was not retained for modelling due to the non-quantitative results, but it still delivered most interesting results.

The 20 samples analysed in the preliminary study (Pillonel *et al*., 2004) were also integrated in the current work. LAP activity and $^{34}S/^{42}S$ isotope ratio had however not yet been investigated in the preliminary study. To allow the software to work correctly, missing values were replaced by the average values of the corresponding category.

Discriminant analyses
Discriminant analysis on the correlation matrix was performed with the software Systat for Windows version 9.0 (SPSS Inc., Chicago, USA). A stepwise backward elimination was carried out to select the best variates. In this way only the most significant factors out of the 25 were used in the corresponding models. Results were validated using the leave-one-out cross validation (Jackknifed classification matrix).

Artificial neural network
A feed forward neural network trained by back-propagation was calculated using the software S-Plus (Insightful, Seattle, USA). The working principle is explained in short. Neurons are sorted into three layers: input, hidden and output layer. There are as many input neurons as factors and as many output neurons as categories. Neurons are connected to all of the previous layers by weighted connections. In each neuron the sum of the weighted signals is calculated and when it exceeds a certain threshold, it is processed by a so called transfer function and sent to all neurons in the next layer. In the training sequence, the output of the network is compared to known values and errors are back-propagated to the hidden and input layers to adjust the weights and minimise the error. The procedure is repeated until the errors between the output and known values are minimised. A training set was built with 2/3 of the observations randomly selected (n = 127). The remaining data (n = 56) were used for validation.

3. Results

As the group assignment for the collected data is known, only trained (or supervised) classification techniques were considered. Discriminant analysis is a simple and well understood way of achieving a group assignment (Kaufmann, 1997). The classification is based on Mahalanobis distances and a confidence level is available for each observation. Three approaches with different group assignments were first compared using discriminant analysis. In the first approach, all regions were included in the model in a single step. In the second approach, only two categories were considered, i.e. a selected region *vs* the remaining regions pooled in a second category. In the last approach, one region was selected and compared with the other regions considered one by one.
Among the numerous available statistical methods for pattern recognition, artificial neural network (ANN) was also tested for classifying the samples in a single step. ANN has proven to be a powerful tool for large data sets. The most popular ANN configuration is the back-propagation network (BPN). BPN may give better results than PLS or PCR (Horimoto, Lee & Nakai, 1997).

Model 1: All regions considered simultaneously using DA
An excellent classification leading to only two CH samples misclassified was obtained including 18 factors in the model. In the Jackknifed classification, nine samples were misclassified, indicating a certain degree of overfitting. Moreover the analysis of so many factors would cost approx. 850 € / sample. However, reducing the model to 11 factors (C3, pH, WSN, D-lactate, succinate, LAP, Cu, δ^2H, δ^{13}C, δ^{15}N and δ^{34}S), the costs could be reduced to 650 €, 96% (95% in the Jackknifed validation) overall correct assignment still being achieved. One Austrian, five Swiss, two German and one French (FR) samples were misclassified in the Jackknifed validation (Table 1). The probabilities for Swiss membership of the misclassified Swiss samples were all under 0.23. Values equal to or greater than 0.50 ensure a classification as Swiss samples. This highlights the fact that the five misclassified samples showed unusual properties for Swiss Emmental. A visualisation of the results is

hardly possible due to their multidimensional character. Figure 1 shows the canonical scores of the first four dimensions. Even so, the discrimination is optically not evident.

Table 1 Jackknifed classification matrix of all observation using 11 factors*

	A	CH	D	FI	FR	FTb	FTe	%correct
A	14	0	1	0	0	0	0	**93**
CH	3	65	2	0	0	0	0	**93**
D	0	0	21	0	1	0	1	**91**
FI	0	0	0	12	0	0	0	**100**
FR	1	0	0	0	30	0	0	**97**
FT_b	0	0	0	0	0	19	0	**100**
FT_e	0	0	0	0	0	0	12	**100**
Total	18	65	24	12	31	19	13	**95**

* C3, pH, WSN, D-lactate, succinate, LAP, Cu, δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$

By a further reduction to the factors D-lactate, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, only the regions distant from one another were correctly separated (FT_b, FT_e, FI). This illustrates the effectiveness of stable isotope ratios for differentiating complex food products with distant geographic origins.

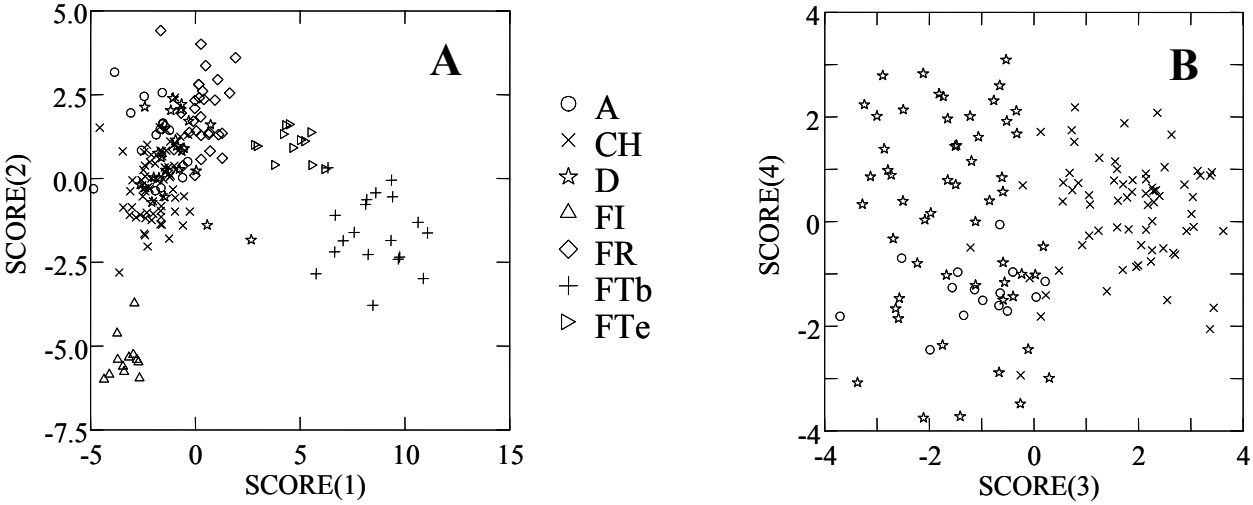

Figure 1 Canonical scores of the DA using 11 factors. (**A**) Discrimination between the categories FI, FT_b and FT_e using the 1st and 2nd dimensions. (**B**) Partially discrimination between these four categories in the 3rd and 4th dimensions. Only the categories A, CH, D, and FR are represented.

Model 2: All regions considered simultaneously using ANN

A backward elimination of less significant factors as done for DA is not possible here. Therefore all 25 factors were used in the model. One difficulty of ANN is the arbitrary choice of the start weights, the weight decay and the number of neurons in the hidden layer. Various models with a number of neurons between 9 and 20, start weights in the ranges [-0.5; 0.5], [-0.7; 0.7] and [-1; 1] as well as a weight decay of 0.01 or 0.001 were compared. Out of the 72 models tested, the one with 19 hidden neurons, weight decay of 0.01 and start weights in the range [-0.5; 0.5] gave the best results. All samples were correctly classified in the training set. In the validation set, 5 samples were misclassified (Table 2). Two A and one FR were put in the D group, two FT_b in the FT_e group.

The distribution of the training/validation sets was sometimes far from 2:1 for the categories with few observations. This explains the very low classification rate in the regions A or FT_b though only two samples each were misclassified (Table 2). Switzerland was the group with the most samples and therefore the most dependable for use in an ANN. For the other categories, the number of samples was somewhat too small. All Swiss samples were correctly classified so that the current model can be said to be quite reliable for determining if an unknown sample originates from Switzerland or not. The actual major drawback of such a discrimination is the price generated by the measurement of the 25 factors.

Table 2 Validation matrix (1/3 observations) using all 25 factors in ANN

	A	CH	D	FI	FR	FTb	FTe	%correct
A	3	0	2	0	0	0	0	**60**
CH	0	24	0	0	0	0	0	**100**
D	0	0	4	0	0	0	0	**100**
FI	0	0	0	6	0	0	0	**100**
FR	0	0	1	0	11	0	0	**92**
FT_b	0	0	0	0	0	1	2	**33**
FT_e	0	0	0	0	0	0	2	**100**
Total	3	24	7	6	11	1	4	**91**

To get a direct comparison with model 1, a DA was carried out with the identical training/validation set as used in ANN. In the training model, only one CH sample was misclassified as D. In the validation set, one A, one CH and one D sample were wrongly classified (Table 3). The results of both ANN and DA were therefore absolutely comparable. The better classification obtained for CH with ANN tend to show that the performances of the technique could be enhanced with a larger database.

Table 3 Validation matrix (1/3 observations) using all 25 factors in DA

	A	CH	D	FI	FR	FTb	FTe	%correct
A	4	0	1	0	0	0	0	**80**
CH	0	23	0	0	1	0	0	**96**
D	1	0	3	0	0	0	0	**75**
FI	0	0	0	6	0	0	0	**100**
FR	0	0	0	0	12	0	0	**100**
FT_b	0	0	0	0	0	3	0	**100**
FT_e	0	0	0	0	0	0	2	**100**
Total	5	23	4	6	13	3	2	**95**

Model 3: one selected region vs the others pooled using DA

Each region taken individually one after the other was compared to all others pooled into one category. For certain regions, this may allow a better discrimination or/and lower costs to be achieved.

For FI the separation was trivial. The factors C3, LAP, L-lactate, pyruvate, zinc, and $\delta^{13}C$ made possible a perfect separation even in the Jackknifed validation.

For FR, 97% correct classification was achieved using the factors C1, C2, TN, Zn, $\delta^{15}N$, $\delta^{34}S$. The FR samples were correctly assigned whereas two samples from each the categories CH, D and FT_e were assigned to the FR category.

Only a small improvement was achieved *for A*. Using 13 factors (C2, C4, C6, NaCl, pH, TCA-SN, WSN, OHL, pyruvate, Cu, Zn, $\delta^{2}H$, $\delta^{34}S$), all Austrian Emmentals were correctly classified and one sample from each the categories CH, FT_e and FR were misclassified. A

great non-homogeneity among the sample from Austria could be observed. This is partly due to the diversity of their origin (Vorarlberg, Salzburg) and manufacture (copper vs. stainless steel vat).
A small improvement was also achieved *for CH*. Using the 12 factors C3, NaCl, pH, TCA-SN, WSN, enterococci, OHL, LAP, succinate, Mg, $\delta^{15}N$ and $\delta^{34}S$, only three Swiss samples were misclassified and no sample was wrongly classified as Swiss.
For the remaining regions *FT_e, FT_b and D*, the 2-categories approach did not improve the discrimination, whether with regard to the number of factors required, nor to the percentage of correct classification.

Model 4: Switzerland vs the others taken one by one using DA
In this approach, a given region is compared to the others considered one by one. An independent model using a specific set of factors is therefore created for each pair. This approach was applied as an example to the region Switzerland to determine if any improvement could be achieved in comparison with both preceding models. A stepwise backward elimination was first carried out for each pair. Then the factors were compared and manually adapted in order to minimise the number of factors needed for all pairs. At the end, 15 analytical variates (C2, C3, pH, TN, OHL, ECOC, D+L-lactate, succinate, pyruvate, LAP, δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) were retained. The factors required for each pair are listed in Table 4. A 100% correct classification was achieved in all pairs, also in the Jackknifed validation. For this, the origin of the foreign samples must of course be known to apply the correct model. For a sample whose origin is absolutely unknown, each of the six models have to be run. If the sample always land in the group Switzerland, it is a Swiss sample. If not, it is foreign, but it is not possible to precisely determine its origin. The one by one approach is therefore complementary to the global approach. The former makes it possible to check with a high confidence level if the sample is of Swiss origin or not and the latter gives reliable general information about the geographic origin.

Table 4 Factors used in model 4

CH *vs*	**A**	**D**	**FI**	**FR**	**FT_b**	**FT_e**
C2				X		X
C3	X			X		X
pH		X				
TN		X				
OHL	X			X		
ECOC		X		X		
D-lactat	X	X	X			
L-lactate	X					
Succinate	X	X				
Pyruvate		X	X			
LAP	X	X	X			
δ^2H	X					
$\delta^{13}C$			X		X	X
$\delta^{15}N$	X			X	X	X
$\delta^{34}S$	X		X		X	

Conclusion

An attempt was made to classify 183 Emmental cheese samples selected from 7 European regions according to their 7 geographic origins. A maximum of 25 factors (analytical parameters) was available for multivariate analyses. Discriminant analysis and artificial neural network delivered comparable results when all factors were used. In the training set, 99 to 100% correct classification was achieved, whereas in the validation set, rates between 91 and 95% were found. The size of the database was however somewhat too small for ANN to develop its whole power. A further drawback of ANN is its black box character. It is not possible to interpret any result or find any relationship between input and output. It is therefore also difficult to reduce the number of factors, the costs and the risk of overfitting by selecting the most appropriate factors.
The latter operation is easily carried out in DA using stepwise backward elimination. A new model was optimised using only 11 factors (C3, pH-value, WSN, D-lactate, succinate, LAP, Cu, δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) and leading to 95% correct classifications in the Jackknifed validations. In a 2-group approach, samples of one category were compared with all others pooled in a second category. Only slight improvements were achieved in this way. A further 2-group approach was tested for optimising the separation of the Swiss Emmental only. For each the six pairs Switzerland *vs* another region, a new model was built. In this way, it was possible to achieve 100% correct identification for the Swiss samples in the Jackknifed validation using 15 factors (C2, C3, pH, TN, OHL, ECOC, D+L-lactate, succinate, pyruvate, LAP, δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$).
The analytical parameters selected over the 3-year project, combined with discriminant analysis were therefore able to assign unknown Emmental samples to their geographic origin with a high confidence level.

Acknowledgements
The authors thank the Swiss Federal Office of Public Health for its financial and logistical support, G. Urbach and G. Gremaud for their reviewing of the publication as well as J.F. Chamba (ITFF, France), M. Carl (MUVA Germany), H. Spring (Emmentaler Switzerland™) and H. Jatila (Valio Ltd, Finland) for collecting the samples.

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CHAPTER 14

Analytical methods for the authentication and traceability of Raclette Suisse® and Fontina PDO cheese

L. Pillonel, U. Bütikofer, J.O. Bosset
Federal Dairy Research Institute, 3003 Berne (Switzerland)
A. Rossmann
Isolab GmbH, 85301 Schweitenkirchen (Germany)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switzerland)

Mitteilungen aus Lebensmitteluntersuchung und Hygiene, accepted.

Introduction

Among the different aspects of food authenticity, the geographic origin of a product is one gaining more importance with the increasing mobility and the low transport costs. The dairy sector may also be victim of fraudulent mislabelling. Mislabelling of butter has already been reported and successfully recognised as such by isotope ratio mass spectrometry (IRMS) (1). Milk transportation for cheese making is also a reality, as for instance Dutch milk being transformed in the North of Italy. Analytical control systems should be developed to ensure the genuineness of manufactured dairy products and their correct labelling Mislabelling of Emmental cheese has already been investigated within a 3-year study (2, 3, and references therein). After the selection of pertinent analytical tools within a preliminary study, it was possible to create and test a mathematical model for the assignment of geographic origin according to a database built on data from more than 180 samples.

A further interesting product in Switzerland is Raclette cheese. As for Emmental, its production area is spread all over Switzerland and is of strong economical importance for the Swiss dairy sector. Importation of Raclette cheese originates largely from France where it is partly produced at lower cost. Mislabelling as Raclette Suisse is therefore conceivable and should be prevented by developing suitable analytical tools.

In the Aosta Valley in Italy, Fontina PDO cheese is produced in a very traditional way. This cheese type is appreciated in the Italian kitchen. However the production in the restricted PDO area is well below the demand. “Fontal” is produced mostly in the North of Italy and is an industrial imitation making up for the shortage of Fontina. Once again it is important to protect the authentic product with high added value against fraudulent substitution with industrial imitations.

The goal of the current work was to investigate some analytical techniques, based on the knowledge acquired during an Emmental study, for differentiating Raclette Suisse from French Raclette and Fontina PDO from Italian Fontal. Both primary (not influenced by the milk transformation) and secondary indicators (depending on the manufacturing technology) were considered. The manufacturing technologz (e.g. use of additives, milk heat treatment) is an expression of a tradition and the latter is bound to a region of origin. Therefore secondary indicators can be useful complement to primary indicators (2).

The study was too small to deliver robust models for origin assignment but was only aimed at pointing to possible ways of doing large scale studies which need to be carried out to ensure good reliability of the models.

Material and methods

Samples

The twenty-eight Swiss Raclette samples were provided by Raclette Suisse® (Berne, Switzerland) from different regions in Switzerland. For the parameters lysozyme, natamycin and sorbic/benzoic acid, only half the Swiss samples were investigated. The fourteen French Raclette cheese samples were purchased directly in four different stores in France. Six were declared from Savoie or Haute-Savoie, one from Massif Central and one from Normandie. The origin of the remaining six samples was not known. All Raclette cheese samples were of type "nature".

The sixteen Fontina PDO samples were provided by the "Consorzio produttori Fontina" (Aosta, Italy). They were all produced during the winter in the valley. Ten Fontal cheeses were purchased in four different stores in the North of Italy (Fontal NI). One further Fontal cheese with Swiss manufacture (Fontal CH) was bought in Tessin.

All samples were kept at –18°C until analysis. Natamycin and sorbic acid were measured in the rind. For the remaining analyses, 2 cm from the rind were discarded. The samples delivered to the various participating laboratories were cut into slices across the whole height of the block. This helped to avoid misinterpretation of results due to gradients in the block.

Analytical methods

A) Raclette cheese

The following standard chemical analyses were carried out: water gravimetrically (4), fat according to Gerber van Gulik (5), 12 g/L TCA soluble nitrogen (TCA-SN) and water soluble nitrogen (WSN) according to Kjeldahl (6), sodium chloride potentiometrically with a silver electrode (7) and volatile organic acids by titration and gas chromatography (8). The pH-value was determined at room temperature using a penetrometric electrode (Mettler-Toledo, article no. 104063123).

Nitrite was determined after fat and protein precipitation. The quantification occurred photometrically at 538 nm after addition of sulfanylic acid amide and N-(1-naphtyl)-ethylendiamine dihydrochloride to the filtrate. Nitrate was determined after reduction to nitrite with cadmium covered with copper (9).

Natamycin was extracted with methanol and quantified using high performance liquid chromatography equipped with a fluorescence detector (HPLC) (10). Sorbic and benzoic acid were derivatised with potassium hexacyanoferrate at pH 8, extracted with methanol and quantified using HPLC and an UV detector (11). Lysozyme was determined in the supernatant of a cheese-sodium chloride-water suspension at pH 4.3 using HPLC and a fluorescence detector (internal ALP method).

For the determination of calcium, approximately 1 g of grated cheese was digested in 5 mL suprapure nitric acid (650 g/kg) at normal pressure. The solutions were analysed with an air-acetylene flame atomic absorption spectrometer (12).

$^{15}N/^{14}N$, $^{13}C/^{12}C$, $^{2}H/^{1}H$ and $^{34}S/^{32}S$ ratios were determined in a protein fraction obtained as follows: the grated cheese samples were freeze-dried and then defatted with petroleum ether (Merck AG, Darmstadt, analytical grade) in a soxhlet apparatus. C, N and S were measured by Elementar elemental analyser Vario EL III coupled to an isotope ratio mass spectrometer (IRMS) AP 2003 (Elementar Analysensysteme GmbH, Hanau, Germany, and GVI Instruments Ltd. Manchester, UK). D/H ratios were measured on a Thermo Instruments delta XL plus IRMS coupled with a Thermo Instruments high temperature pyrolysis unit.

The following standards with known ratios were used: a standard casein (Sigma-Aldrich, analytical grade) which had been calibrated in a European research project (SMT4-CT2236-1998) for $^{13}C/^{12}C$ and $^{15}N/^{14}N$, and later on for $^{2}H/^{1}H$ and $^{34}S/^{32}S$ against official reference materials (PEF-1 and NIST-22, V-CDT and silver sulfide, respectively). The values were reported in the δ-scale (‰) according to the corresponding international standards (PDB,

NBS-22, V-CDT, air N_2). To check the reliability of the analyses, additional IHRM (in house reference materials) of known isotopic composition were used (wheat flour, lactose, sucrose).

B) Fontina/Fontal cheese
Volatile organic acids and stable isotope ratios were investigated using the same methods as for Raclette cheese. The activity of the alkaline phosphatase (ALP) was measured photometrically with p-nitrophenylphosphate as substrate (internal method FAM ME 10202O.211). For the latter, the material for analysis was, in this case only, sampled directly under the rind.
The samples were also investigated using near infrared spectroscopy (NIR). Approx. 150 g grated cheese were placed in a glass Petri dish and measured by diffuse reflection on a Büchi NIRLab N-200 spectrometer (Flawil, Switzerland). The Petri dish rotates around its centre during the measurement. For each sample 64 scans were recorded from 4000 cm^{-1} to 10000 cm^{-1} with a spectral resolution of 2 cm^{-1}.
Lastly the volatile compounds were considered using an MS-based electronic nose of type SMart Nose (LDZ, CH-2074 Marin) equipped with a Combi Pal autosampler (CTC Analytics, CH-4222 Zwingen). The headspace volatiles were preconcentrated using an INDEx syringe (LDZ, CH-2074 Marin). Analytical conditions and data treatment were described elsewhere (13).

Statistical analyses
The NIR spectra were normalised between 0 and 1. Principal component scores from the correlation matrix were computed with PLSPlus/IQ Vs. 5.07 (Thermo Galactic, Salem, NH). In all cases, Principal Component Analysis (PCA) and Discriminant Analysis (DA) were performed using Systat Vs. 9.0 (SPSS Inc., Chicago, IL).

Results and discussion

Raclette cheese
As the regional origin of four French samples was not known, only the categories Swiss and France were considered in a first step. From the 22 parameters measured (Table 1), only the following ten showed significant differences between the countries of origin: chloride, calcium, benzoic acid, lysozyme, natamycin, nitrite, δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$.
The calcium content was significantly lower in Switzerland. Calcium chloride was most probably added to the milk for processing in France. Sorbic acid, which is allowed as additive for surface treatment, was not found in any sample. The benzoic acid concentration was higher in Raclette Swiss. Benzoic acid in cheese can be produced by three different natural pathways: reversible conversion from hippuric acid in the whole cheese body, breakdown of phenylalanine and autoxidation of benzaldehyde on the surface of smear-ripened cheeses (14). The concentration of this acid depends on several factors such as the composition of the microbial flora, the kind of cheese curing and ripening conditions. The current findings should therefore be confirmed with a higher number of observations. Lysozyme, natamycin and nitrite are additives whose use is authorised by EU and Swiss legislations. However the organisation “Raclette Suisse” voluntarily renounced their use to offer a 100% natural product to consumers[1]. Lyzozyme and nitrate/nitrite are often added to milk to prevent butyric acid fermentation. The lysozyme concentrations were below the detection limit of 15 mg/kg for all Swiss and for six French samples. The remaining French samples showed concentrations between 56 and 376 mg/kg.

[1] From 2004, colouring additives are also forbidden for the manufacture of Raclette Suisse®. The presence of such additives may be used as further indicator.

Table 1 Parameters measured in the investigated Swiss and French Raclette cheeses

	ANOVA	Raclette Suisse (n = 28)				French raclette (n = 14)			
Parameters		**x**	s_x	Min.	Max.	**x**	s_x	Min.	Max.
Water (g/kg)	-	**418**	15	388	447	**419**	20	388	457
Fat (g/kg)	-	**285**	14	257	317	**286**	18	265	338
Chloride (g/kg)	**	**22^{A}**	3.0	16.6	30	**19^{B}**	3.8	11.3	24.6
Calcium (g/kg)	***	**6.41^{B}**	0.56	5.13	7.28	**7.00^{A}**	0.43	6.42	7.65
pH-value	-	**5.65**	0.21	5.38	6.19	**5.57**	0.23	5.16	6.04
TCA-SN (g/kg)	-	**6.1**	1.1	3.9	7.9	**6.2**	1.4	3.69	8.36
WSN (g/kg)	-	**17.5**	6.5	9.8	35.8	**15.1**	5.2	7.68	26.1
Formic acid (mmol/kg)	-	**1.02**	0.93	0.22	5.14	**0.86**	0.33	0.25	1.62
Acetic acid (mmol/kg)	-	**12.5**	6.3	3.0	25.3	**16**	12	2.96	53.5
Propionic acid (mmol/kg)	-	**0.94**	3.2	0	16.8	**1.0**	3.0	0.01	11.4
n-Butyric acid (mmol/kg)	-	**0.72**	0.57	0.27	2.95	**0.51**	0.31	0.16	1.22
n-Hexanoic acid (mmol/kg)	-	**0.09**	0.06	0	0.24	**0.08**	0.06	0.02	0.25
Benzoic acid (mg/kg)	***	**65^{A}**	48	7.05	157	**15^{B}**	13	7.9	60
Sorbic acid (mg/kg)	-	**< 1**	-	0.5	0.5	**< 1**	-	0.5	0.5
Lysozyme (mg/kg)	***	**< 15^{B}**	-	10	10	**121^{A}**	131	10	376
Natamycin (mg/kg)	***	**< 0.1^{B}**	-	0.05	0.05	**7.0^{A}**	6.3	0.05	19.3
Nitrate (mg/kg)	-	**1.8**	1.0	1	4.3	**2.3**	1.2	1	4.1
Nitrite (mg/kg)	*	**0.18^{B}**	0.11	0.1	0.42	**0.33^{A}**	0.26	0.1	1.0
$\delta^{2}H$ (‰)	***	**-123.5^{B}**	4.0	-133.1	-116.0	**-102.8^{A}**	8.0	-115.4	-90.1
$\delta^{13}C$ (‰)	***	**-24.0^{B}**	1.0	-25.9	-22.2	**-19.6^{A}**	2.9	-25.3	-16.3
$\delta^{15}N$ (‰)	***	**5.7^{A}**	0.8	4.5	7.4	**4.7^{B}**	0.7	3.3	5.8
$\delta^{34}S$ (‰)	***	**3.7^{B}**	1.9	-1.8	6.2	**5.9^{A}**	0.9	4.7	7.3

Caption: x = mean value; s_x = standard deviation; Min. = minimum; Max. = maximum
ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or AB = A and B overlap by using an univariate discriminant analysis

The nitrite values were slightly lower in Switzerland but the broad variation ranges within one category rendered this potential indicator less significant. Natamycin is used as fungicide to protect the smear against moulds. Its concentration was below the detection limit of 0.1 mg/kg in all Swiss samples and in a single French one. The remaining French samples showed values between 0.7 and 19.3 mg/kg. Therefore samples with lysozyme or natamycin value over the detection limit can definitively be excluded from the Swiss category but the opposite is not true. Furthermore a Swiss manufacturer might illegally use additives. To avoid any confusion with foreign cheeses, indicators not depending on the manufacturing process are needed.

The last four parameters (δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) belonged to the group of primary indicators. They were already successfully used in dairy products (1, 15, 16). In cheese making, these are only function of the composition of the milk used, and not of the manufacturing technology. The values for the four stable isotope ratios in Raclette Suisse were comparable to those found in Emmentaler Switzerland cheese (17). Even the $\delta^{13}C$ values were all typical for maize silage-free zone (< -22‰). In France, two sub-categories could clearly be distinguished with the samples originating from Northwest (NW) and those from East-Central, Savoie and Haute-Savoie (EC). Figure 1 shows the correlation between δ^2H and $\delta^{34}S$ in French Raclette and highlights the obvious groups. The parameters with significantly different content between both French regions are listed in Table 2. The values in the category NW were comparable to those found in the Emmental cheese FT_b (Bretagne) and the ones in the category EC were in the range of the categories Emmental cheese FR (Savoie) and FT_e (East-Central) (17). The absolute clear differentiation between both regions brought to light the first cases of fraud. Indeed, two samples labelled with "Fabriqué en Haute-Savoie" were, according to the analytical results, manufactured in a coastal region, or at least the milk used was not from Haute-Savoie. For more details on the interpretation of stable isotope ratios, see in (17).

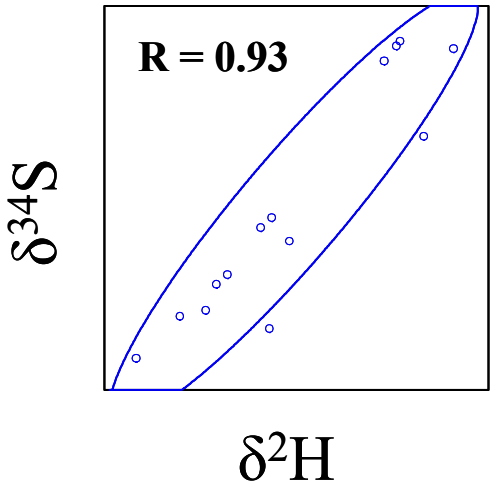

Figure 1 Correlation between the stable isotope ratios δ^2H and $\delta^{34}S$ in French Raclette cheese.

A PCA using the factors δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and calcium was carried out. The scores of the first two principal component allowed a good separation of the regions of (Figure 2).

Table 2 Parameters with significant differences between France Northwest and France Centre/East

	Raclette Suisse (n = 28)		Raclette Centre/East (n = 9)		Raclette Northwest (n = 5)	
Parameters	**x**	s_x	**x**	s_x		
Acetic acid (mmol/kg)	**12.5**^{B}	6.3	**21**^{A}	13	**5.6**^{B}	1.9
Calcium (g/kg)	**6.41**^{B}	0.56	**7.20**^{A}	0.40	**6.64**^{AB}	0.20
$\delta^{2}H$ (‰)	**-123.5**^{C}	4.0	**-108.0**^{B}	4.0	**-93.4**^{A}	2.2
$\delta^{13}C$ (‰)	**-24.0**^{C}	1.0	**-21.2**^{B}	2.2	**-16.72**^{A}	0.33
$\delta^{15}N$ (‰)	**5.7**^{A}	0.8	**4.41**^{B}	0.39	**5.12**^{AB}	1.04
$\delta^{34}S$ (‰)	**3.7**^{C}	1.9	**5.29**^{AB}	0.39	**7.05**^{A}	0.32

Caption: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or
AB = A and B overlap by using an univariate discriminant analysis

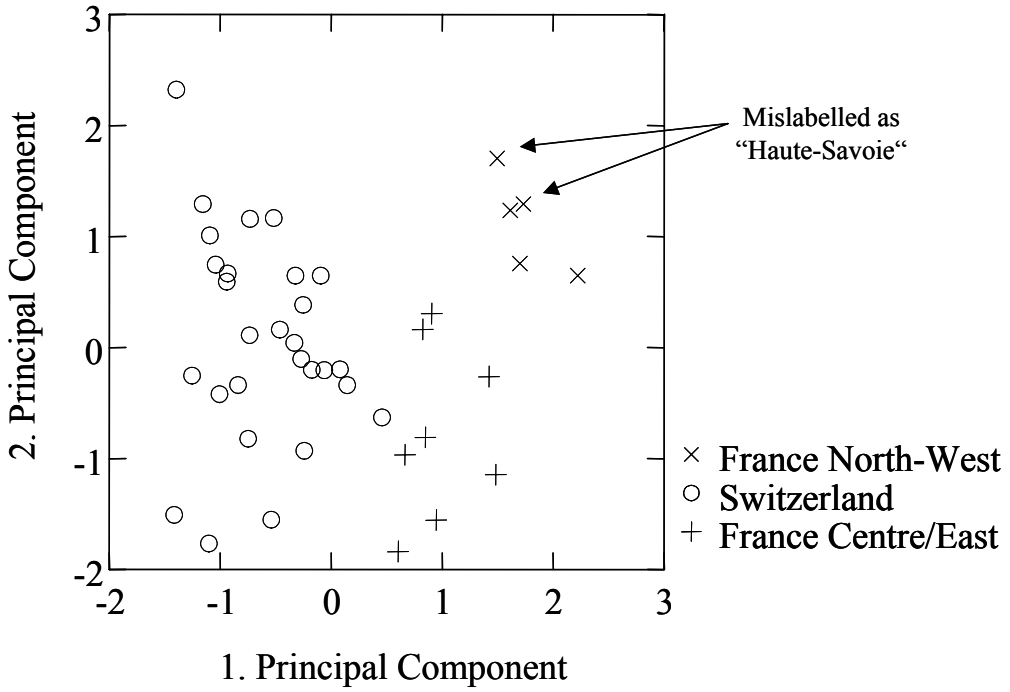

Figure 2 Scores of principal component analysis using the factors δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and calcium. Discrimination between Raclette cheese originating from Switzerland, France North-West and France East/Centre. Evidence of mislabelling of two samples.

Fontina and Fontal cheese

The results of the various parameters measured are listed in Table 3. To be recognised as PDO, Fontina cheese must be made from raw milk. Usually, the milk used for the manufacture of industrial semi-hard cheese is pasteurised. Indeed the results of the ALP activity indicated clearly that pasteurisation was carried out in the regions outside the Aosta Valley. For Fontina PDO, the ALP values varied between 1799 and 5046 IU/kg. In Fontal cheese, all values were 0 expect one at 56 IU/kg. ALP is therefore a perfect indicator for recognising the authenticity of Fontina PDO among the samples investigated.

The parameters δ^2H, $\delta^{13}C$ and $\delta^{15}N$ also showed significant difference. The higher δ^2H values encountered in Fontal NI may be explained by the proximity to the sea compared to the Aosta Valley and the region where Fontal CH was manufactured. The reason for the higher $\delta^{13}C$ value in Fontal NI is clearly the use of maize silage. Figure 3 shows the scores of a principal component analysis using the parameters ALP, δ^2H and $\delta^{13}C$. The separation between the regions of origin is perfect. The significantly lower $\delta^{15}N$ values in Fontina can be explained by the very extensive agriculture encountered in this alpine valley. The volatile short-chain acids and $\delta^{34}S$ did not deliver useful results.

An interesting discrimination could also be achieved using the electronic nose. A PCA combining the signal of the mass-to-charge ratios 45, 83 and 86 showed a good separation between the two groups Fontina PDO and Fontal NI/CH (Figure 4). As volatile compounds are very sensitive to variations in manufacture, these results must be confirmed with more samples. The use of volatile compounds for geographic origin assignment will be specially delicate during the summer months when Fontina is produced on different pastures in highland regions. The diversity among the PDO sample will strongly increase and might render these indicators less significant.

Table 3 Parameters measured in the investigated Fontina and Fontal cheeses

Parameters	ANOVA	Fontina PDO (n = 16)		Fontal NI (n = 10)		Fontal CH (n = 1)	
		x	s_x	**x**	s_x	**x**	s_x
AP (IU/kg)	***	**2752^{A}**	1011	**6^{B}**	18	**0^{B}**	-
δ^2H (‰)	***	**-127.66^{B}**	5.3	**-110.7^{A}**	5.1	**-123.8^{AB}**	-
δ^{13}C (‰)	***	**-23.49^{B}**	0.90	**-19.80^{A}**	2.2	**-24.68^{B}**	-
δ^{15}N (‰)	***	**4.51^{B}**	0.49	**5.72^{A}**	0.77	**6.29^{A}**	-
δ^{34}S (‰)	-	**4.52**	1.20	**5.26**	0.75	**3.76**	-
C1 (mmol/kg)	-	**1.43**	0.34	**1.8**	0.85	**2.12**	-
C2 (mmol/kg)	-	**10.57**	3.95	**10.6**	5.02	**16.3**	-
C3 (mmol/kg)	-	**0.41**	0.66	**0.2**	0.35	**0.08**	-
n-C4 (mmol/kg)	-	**0.48**	0.47	**1.1**	0.78	**0.78**	-
n-C6 (mmol/kg)	-	**0.08**	0.03	**0.2**	0.25	**0.19**	-

Caption: x = mean value; s_x = standard deviation; ANOVA: ns = not significant, *) $p \leq 0.05$, **) $p \leq 0.01$, ***) $p \leq 0.001$
Production sites: A>B>C>D (=significantly different contents $p \leq 0.01$) or
AB = A and B overlap by using an univariate discriminant analysis

Principal component (PC) scores were extracted from the NIR data set and a discriminant analysis was applied on the first 10 PCs. In the Jackknifed classification, only 50% correct assignment was achieved, which is not sufficient.

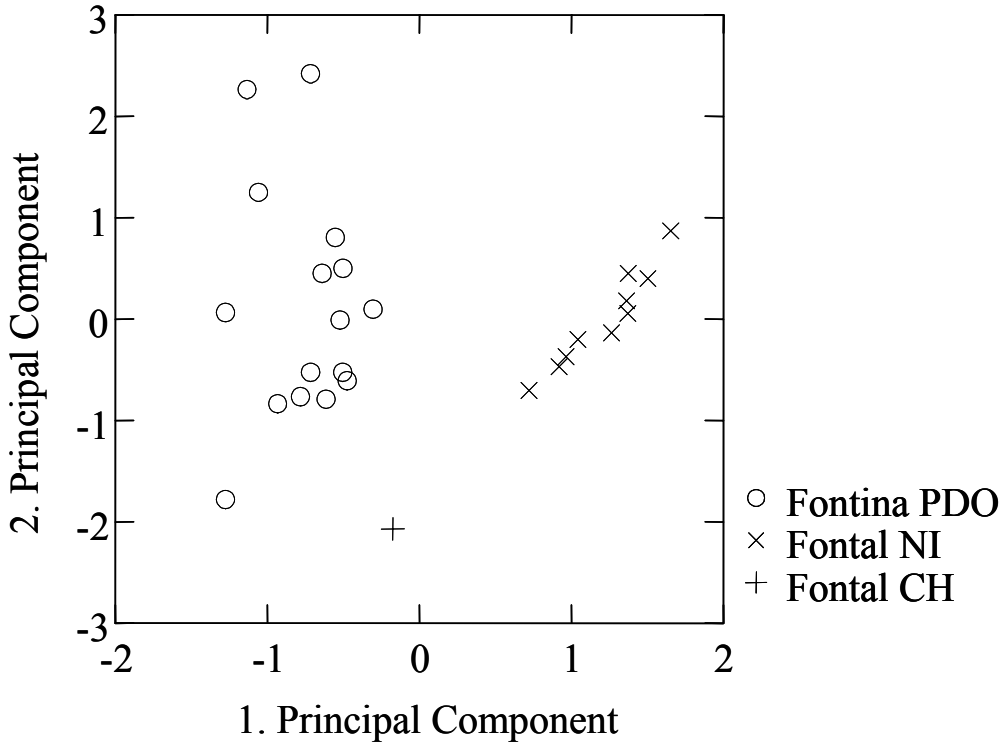

Figure 3 Scores of principal component analysis using the factors ALP, δ^2H and δ^{13}C. Discrimination between the true Fontina PDO, Fontal cheese produced in the North of Italy (NI) and in Switzerland (CH).

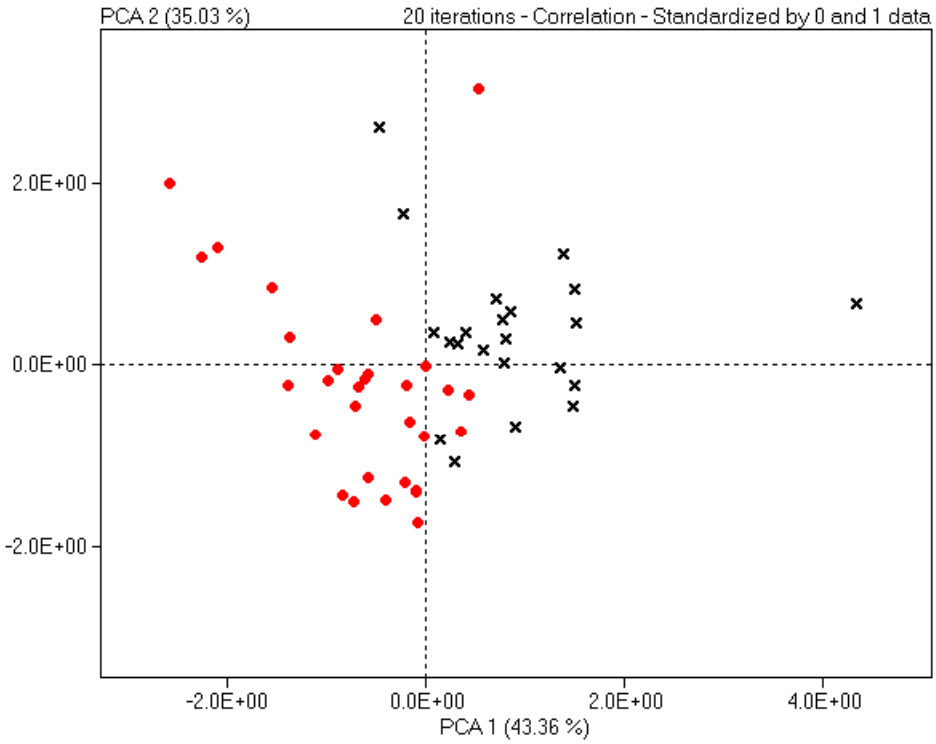

Figure 4 Scores of principal component analysis using the factors m/z= 45, 83, 86 from the electronic nose. Discrimination between the true Fontina PDO and Fontal cheese produced in the North of Italy (NI) and Switzerland (CH).

Conclusion

Apart from Emmentaler Switzerland, Raclette Suisse is a cheese typically made in Switzerland which could be exposed to fraudulent substitution with foreign imitations. The presence of lysozyme or natamycin, both additives allowed by EU and Swiss legislations but not by the organisation "Raclette Suisse", can be used to exclude a Swiss origin. Both are secondary indicators which are not directly related to the geographic origin. Primary indicators such as stable isotope ratios are very useful in food traceability. By measuring the stable isotope ratios δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, two subcategories were clearly formed within the French samples, i.e. northwest and centre/east France. A principal component analysis (PCA) using the four stable isotope ratios and the calcium content resulted in a good separation in the three categories. These analyses brought to light two likely cases of mislabelling.

Fontina PDO is a cheese produced exclusively in the Aosta Valley. Imitations are manufactured in the North of Italy and in Switzerland and sold under the name Fontal. It was possible to discriminate between the true Fontina PDO and its imitations thanks to the alkaline phosphatase (ALP) activity. The former is a raw milk cheese with enzyme activity higher than 1700 IU/kg whereas the corresponding imitations are made from pasteurised milk with ALP activity lower than 56 IU/kg, mostly of 0 IU/kg. A PCA using the parameters ALP, δ^2H and $\delta^{13}C$ allowed a perfect separation between Fontal north Italy, Fontal Switzerland and Fontina PDO. NIR analyses did not produce any significant discrimination

Acknowledgement

The authors thank the Swiss Federal Office of Public Health for its financial and logistical support to the project, G. Urbach and G. Gremaud for their reviewing of the publication as well as A. Chatel, and J.F. Chamba for their help in collecting the samples.

Summary
The following analytical parameters were considered as potential markers of origin for Raclette Suisse: fat, water, 12%-TCA soluble nitrogen, water soluble nitrogen, chloride, volatile short-chain acids, pH-value, nitrite/nitrate, natamycin, sorbic and benzoic acids, lysozyme, calcium, $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ ratios. A principal component analysis using these four stable isotope ratios as well as the calcium content showed a good separation of the three regions of origin, i.e. Switzerland, France Northwest and France Centre/East. The additives natamycin and lysozyme were not detected in the Swiss samples. For determining the authenticity of Fontina PDO, the parameters alkaline phosphatase, volatile short-chain acids, $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ were measured. The genuine Fontina cheese was clearly differentiated from imitations (Fontal cheese) by the alkaline phosphatase activity due to different heat treatments of the milk. Further measurements were carried out using a NIR spectrometer and a MS-based electronic nose. The former equipment did not deliver useful results whereas a good separation was achieved using the latter equipment.

Zusammenfassung
Die folgenden analytischen Parameter wurden als potenzielle Herkunftsindikatoren für Raclette Suisse betrachtet: Fett, Wasser, 12%-TCA löslicher Stickstoff, wasserlöslicher Stickstoff, Chlorid, flüchtige kurzkettige Säuren, pH-Wert, Nitrit/Nitrat, Natamycin, Sorbin- und Benzoesäure, Lysozym, Kalzium und das Verhältnis verschiedener Isotope ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ und $^{34}S/^{32}S$). Eine Hauptkomponentenanalyse mit diesen vier Isotopenverhältnissen sowie dem Kalziumgehalt zeigte eine Trennung der Herkunftsregionen, d.h. Schweiz, Frankreich Nordwesten und Frankreich Zentrum/Osten. Die Zusatzstoffe Natamycin und Lysozym konnten in den schweizerischen Proben nicht nachgewiesen werden. Für die Bestimmung der Authentizität von Fontina GUB wurden die Parameter alkalische Phosphatase, flüchtige kurzkettige Säuren, $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ und $^{34}S/^{32}S$ herbeigezogen. Die echte Fontina GUB unterschied sich klar von Nachahmungen (Fontalkäse) dank der alkalischen Phosphatase-Aktivität auf Grund unterschiedlicher Hitzebehandlungen der Kessimilch. Ausserdem wurden noch Messungen mit einem NIR-Spektrometer und einer auf Massenspektrometrie basierenden elektronischen Nase durchgeführt. Das erste Gerät lieferte keine eindeutige Resultate, während mit dem zweiten Gerät eine gute Trennung erreicht werden konnte.

Résumé
Les paramètres analytiques suivants ont été considérés comme marqueurs potentiels d'origine géographique pour la Raclette Suisse : teneurs en matière grasse, eau, azote soluble dans le TCA à 12%, azote soluble dans l'eau, chlorure, acides volatils à courte chaîne, valeur de pH, nitrite/nitrate, natamycine, lysozyme, calcium, rapports $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ et $^{34}S/^{32}S$. Une analyse par composantes principales incluant ces quatre rapports isotopiques ainsi que la teneur en calcium montre une séparation des échantillons de fromage à raclette selon leurs trois régions d'origine, à savoir la Suisse, le Nord-Ouest et le Centre/Est de la France. La concentration des additifs natamycine et lysozyme se situait en-dessous du seuil de détection dans les échantillons suisses. En vue de l'authentification de la Fontina AOC, les teneurs en phosphatase alcaline, acides volatils à courte chaîne, rapports $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ et $^{34}S/^{32}S$ ont été examinés. La véritable Fontina AOC se différenciait clairement des imitations (fromage de Fontal) par l'activité de la phosphatase alcaline après les différents traitements thermiques appliqués au lait. Des mesures ont également été effectuées à l'aide d'un spectromètre NIR et d'un nez électronique basé sur la spectrométrie de masse. Le premier cité n'a donné aucun résultat significatif alors que le second a permis une bonne séparation.

Key words
Authenticity, Raclette, Fontina, Fontal, PDO, Cheese, Lysozyme, Natamycin, Stable isotope ratio, Calcium, Alkaline phosphatase

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Corresponding author: Dr. Jacques-Olivier Bosset, Chemistry & Physics unit, Agroscope Liebefeld-Posieux (ALP), CH-3003 Berne, E-mail: jacques-olivier.bosset@alp.admin.ch

CHAPTER 15

Authenticity of provenance of Swiss cheeses: conclusion of the project, recommendation to food control laboratories and perspective for the future

L. Pillonel, J.O. Bosset
Federal Dairy Research Institute, 3003 Berne (Switzerland)
R. Tabacchi
University of Neuchâtel, 2007 Neuchâtel (Switerland)

Mitteilungen aus Lebensmitteluntersuchung und Hygiene, submitted.

Introduction

Genuineness of foods in regard to their geographic origin is a well-known problem for products such as wine, meat, olive oil or honey. All these products are manufactured and consumed in many countries and their sell price vary strongly depending on their origin. Both food properties, international market and price differences, make fraud possible and interesting. Several European cheeses meet these conditions too, e.g. Camembert, Emmental, Parmesan, Feta, Gouda, Cheddar. For many consumers, it does not matter where the milk or the cheese comes from, as long as the product satisfies the quality requirements. This explains maybe the scarce literature dedicated to the geographic origin of cheeses. However in certain regions, especially in Latin and Alpine countries, much more importance is given to the origin of foods manufactured according to a long tradition and whose image is bounded to a regional or national identity. Feta, Camembert, Parmiggiano Reggiano, Emmental, Raclette are some examples of cheeses defended by understandable patriotism where analytical tools for ensuring their authenticity correspond to a real demand. A further cheese category of interest, often with smaller production scale and rather regional significance, is represented by the PDO cheeses. In this case, the limits are not given by national boundaries but by local border lines strictly defined in the schedule of conditions according to the PDO registration. In this case, fraud often comes from regions close to the PDO area and not necessarily from abroad. PDO labels confer an additional affective value to the product. Consumers show their will to support handcraft and traditional activities by accepting prices up to 20% higher than for standard products.

The goal of the current article is to summarise the results already published in a series of paper (1-10) within a 3-year project on the authenticity of Emmentaler Switzerland™. These results are presented in such a way that they should be useful and usable for canton chemists in a real case of fraud suspicion. Some preliminary results obtained for Raclette Suisse® are also shortly reminded (11). Finally some attention is dedicated to the potential and perspectives of the analytical approach for the authentication of other cheeses manufactured in Switzerland.

Authenticity of Emmentaler Switzerland™: a case study

Emmentaler Switzerland™ is manufactured in a large arc in middle Switzerland, from west (Fribourg) to east (Chur). The technology of milk transformation is however almost the same in all cheese dairies. Around 99% of the cheese makers use cultures from the FAM and the fabrication process is very similar. This makes it possible to use secondary indicators, i.e.

those depending on the fabrication technology, for authentication purposes. In opposition, primary indicators depend only on the milk used. The analytical methods and parameters used in this project are presented in Table 1. Sampling procedure, samples' origin, ripening time and analytical methods are described in detail elsewhere (9). Discriminant analysis and artificial neural network were used to classify the 183 samples into the seven corresponding European regions of origin (10). Emmental is of course also manufactured outside Europe. North America, Australia, New Zealand and Eastern Europe are also important Emmental manufacturers. These regions were not included in the current study for logistical reasons but discriminating the corresponding cheese samples from the Swiss ones should be possible using for instance stable isotope ratios.

Table 1 Analytical methods used for the authentication of Emmentaler Switzerland™ and their corresponding price

Method/Parameter	Included parameters	Price (CHF)
Volatile short chain acids	Formic acid (C1)	110
	Acetic acid (C2)	
	Propionic acid (C3)	
	Butyric acid (C4)	
	Pentanoic acid (C5)	
	Hexanoic acid (C6)	
Chloride	Cl	33
pH-value	pH	22
Total nitrogen	TN	68
Water soluble nitrogen	WSN	79
12%-TCA soluble nitrogen	TCA-SN	79
Enterococci	ENT	30
Obligate heteroferm. Lb.	OHL	45
Lb helveticus	*Lb helveticus*	100
Lactate	L-Lact	99
	D-Lact	
Pyruvate	Pyru	65
Succinate	Succ	65
L-Leucin-aminopeptidase	LAP	56
Elements (AAS)[1]	Cu, Mg, Na, Zn	150
Stable isotope ratio (IRMS)[2]	δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$	500

[1] The price is about 50.- for a single element.
[2] The hydrogen ratio is not measured on a the same instrument as the three others. Price correction of ca. 100.- if only one instrument is used.

Using eleven parameters (C3, pH, WSN, D-Lact, Succ, LAP, Cu, δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$; see abbreviations in Table 1) in a discriminant analysis (DA), a 95% correct classification was achieved in the Jackknifed validation for all 7 categories (*global model*). Five Swiss Emmental out of 70 were misclassified as German or Austrian cheeses. Inversely no foreign samples were classifieded in the Swiss category.
A second approach was tested to optimise the discrimination of Emmentaler Switzerland. In the latter, the Swiss samples were confronted pairwise to the samples of the other regions considered separately. In other words, a new DA was carried out for each the six pairs Switzerland *vs.* another region (*pairwise model*). In each pair, 100% correct classification was achieved using the corresponding groups of parameters presented in Table 2. In a concrete

case, all six models must be calculated. If an unknown sample is then assigned to the Swiss category in all six models, it can be proclaimed Emmentaler Switzerland with a very high reliability. The drawback of both approaches is their price. The measurement of the 15 parameters from the *pairwise model* will cost approximately 1050 CHF. The missing parameters to complete the *global model*, WSN and Cu, will cause additional costs of approximately 130 CHF.

Table 2 Parameters used in the *pairwise models*

CH *vs*	**A**	**D**	**FI**	**FR**	**FT_b**	**FT_e**
C2				X		X
C3	X			X		X
pH		X				
TN		X				
OHL	X			X		
ENT		X		X		
D-lactate	X	X	X			
L-lactate	X					
Succinate	X	X				
Pyruvate		X	X			
LAP	X	X	X			
δ^2H	X					
$\delta^{13}C$			X		X	X
$\delta^{15}N$	X			X	X	X
$\delta^{34}S$	X		X		X	

Moreover there are some robust parameters, not influenced by the season, which are typical criteria for Switzerland. The content in *Lb helveticus* and LAP are the most interesting ones. *Lb helveticus* is no more used in the cheese starters in Switzerland at present, whereas it is frequently used abroad to shorten the ripening time. Its presence was determined by DNA analysis after polymerase chain reaction. This analysis was not quantitative and the results were therefore separated into two groups with an arbitrary upper limit of 3 ng DNA/g. This threshold was chosen in such a way that the samples belonging to the group with highest values were certainly not originating from Switzerland (Table 3). 77% of the foreign samples could in this way be excluded from the Swiss category. This indicator will of course only be valid as long as this species will not be reintroduced in the manufacturing process of Emmentaler Switzerland. LAP is an enzyme which exhibits very low activities in the Emmentaler Switzerland. The highest value found in a Swiss sample was 4.3 IU/kg. All other values were below 3. A threshold value of 10 IU/kg was set to ensure that all samples with higher activity were not Swiss. 50% of the foreign samples could be excluded in this manner. Combining both LAP and *Lb helveticus* results, only 15% out of the 113 investigated foreign samples could still potentially be misclassified as Swiss ones. Figure 1 summarises a possible way of resolving efficiently the problem of the geographic origin of European Emmental cheese. Samples manufactured oversee will not be assigned correctly in the *global model* because the corresponding regions were not considered for the sampling but it will be at least possible to exclude them from the Swiss category using the *pairwise model*. The database for carrying out the DA is available by the authors.

Table 3 *Lb helveticus* content and LAP activity in the investigated 183 Emmental samples

	Switzerland	Austria	Finland	France Bretagne	France Savoie	France East-central	Germany
	CH	A	FI	FT_b	FR	FT_e	D
Lb helveticus							
≤ 3 ng/g DNA	70	10	0	5	1	1	9
> 3 ng/g DNA	0	5	12	14	31	11	14
LAP (IU)							
≤ 10	70	15	0	2	21	5	14
> 10	0	0	12	17	11	7	9

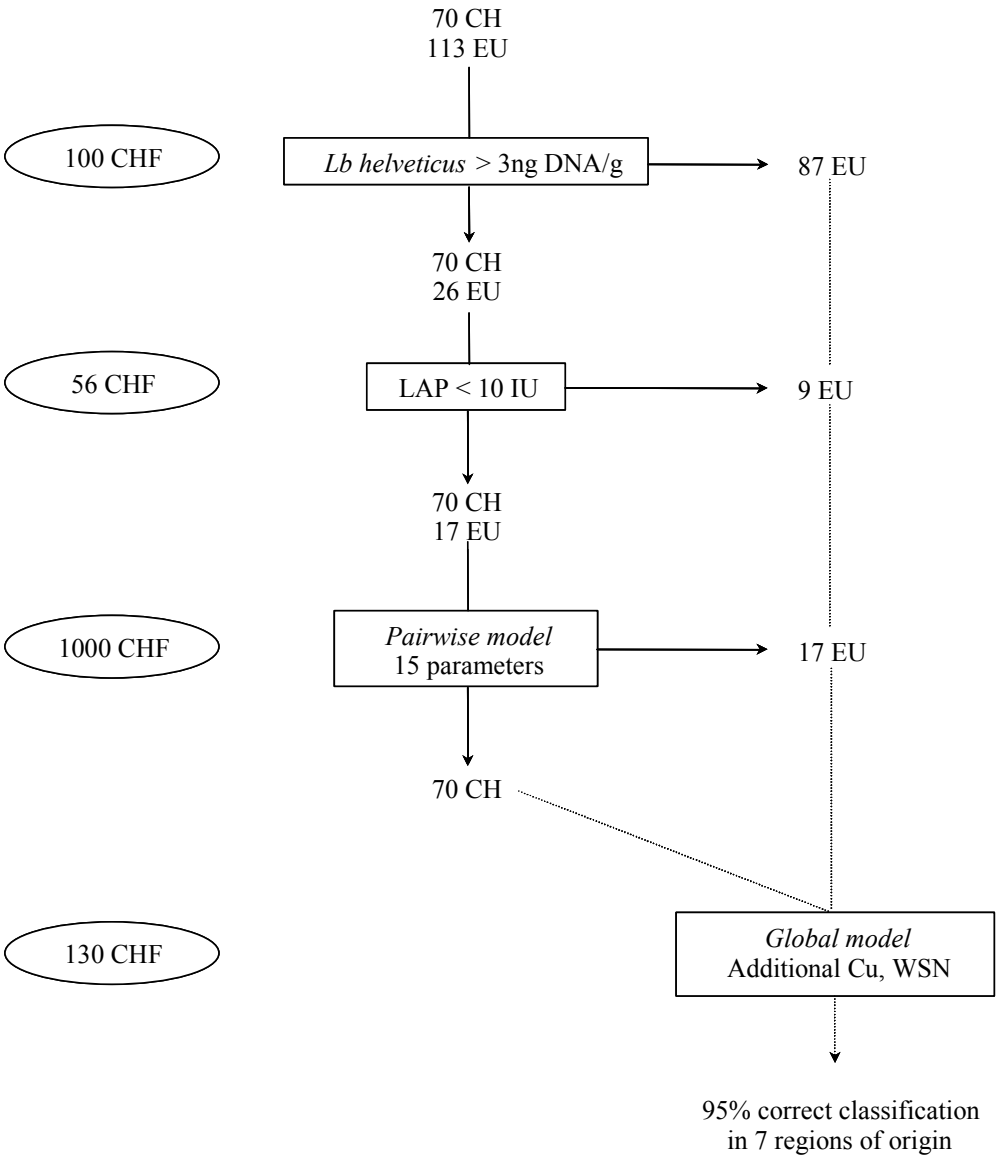

Figure 1 Flow chart for the determination of the geographic origin of European Emmental cheese. The analysis of *Lb helveticus* is not required for performing the *pairwise* and *global models* but provide complementary information.

Finally fluorescence and mid-infrared spectra were also recorded on the cheese samples (12-14). 77% correct classification was obtained in the validation set combining 80 principal component (PC) scores from both spectroscopic methods (14). The introduction of some of these PC scores in the discriminant analyses mentioned above, did not significantly improve the classification power of the model (data not shown). These spectroscopic methods were therefore not retained for application in control procedure.
Emmentaler Switzerland™ in block is already well protected against fraud by its guarantee mark in the rind. The analytical investigations are of interest for pre-packed and grated cheese.

Authenticity of Raclette Suisse: a preliminary study
Raclette Suisse® is surely the second most probable cheese type in Switzerland likely to be substituted with cheaper imitations. Germany, Austria and specially France manufacture Raclette cheese partly at lower costs and export it into Switzerland. Unlike Emmental, the manufacturing conditions of Raclette vary strongly, also within Switzerland. Starter cultures, milk heat treatment (raw *vs* pasteurised) and smear culture are some of the technological parameters which may change from one cheese dairy to the other. The use of secondary indicators was therefore less indicated. However the presence of some additives such as natamycin and lysozyme delivered highly interesting information for discriminating between Swiss and foreign Raclette cheeses. The organisation "Raclette Suisse" voluntarily renounced the use of such additives to offer a 100% natural product to consumers. In all investigated French samples, at least either natamycin or lysozyme were detected. But it is not possible to exclude the existence of foreign samples free from additives. Primary indicators such as the stable isotope ratios $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ were also investigated. They made it possible, in combination with the calcium content (a secondary indicator), a perfect discrimination into the three regions of origin Switzerland, France North-West and France East/Centre (Figure 2). More details on this preliminary study are given elsewhere (11). The organisation Raclette Suisse® only just decided to ban also smear colorant in their products. The presence of colorant will therefore be a further indicator for the non-Swiss origin.

Perspectives for other cheese types from Switzerland
The most important Swiss cheeses with their corresponding tonnage are listed in Table 4. The risk of mislabelling in Switzerland and abroad, the technical feasibility and the economic viability of the analytical approach were arbitrarily evaluated for each cheese type (subjective estimation).
Even if they do not carry a label with an independent control authority such as Protected Denomination of Origin (PDO) or Protected Geographical Indication (PGI), most of the hard and semi-hard cheese type made in Switzerland undergo a schedule of conditions defining a restricted production area. It is therefore theoretically possible to find illegal Swiss imitations of such products. The probability of finding foreign imitations depends on the cheese type considered. The risk is very low for special products such as Appenzeller cheese and very high for more common types such as Raclette cheese. Only in the latter case it makes sense to invest in research for differentiating Swiss samples from foreign ones. A special group of cheeses without any label but of regional or even national importance are the Alp (highland) cheeses. Cheeses of this group may suffer disloyal local concurrence because their manufacturing is only allowed during the summering of cattle on mountain pastures.

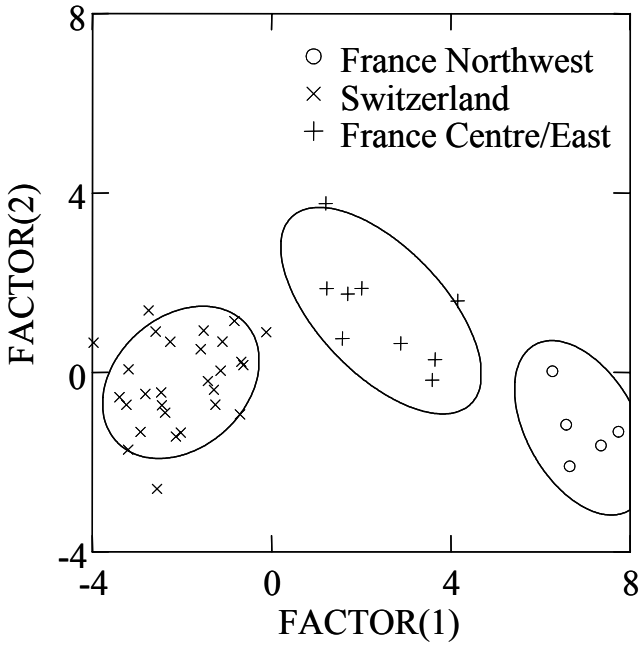

Figure 2 Canonical scores of a discriminant analysis using the factors δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$ and calcium. Discrimination between Raclette cheese originating from Switzerland, France North-West and France East/Centre.

Imitations made from milk obtained in the valley represent therefore a high potential of fraud. Alp cheeses are very appreciated in Switzerland. Moreover the prices for these products bounded to a good deal handwork are of course higher. As these cheeses are sold almost exclusively from private or small local stores, it is also difficult to survey the genuineness of large amounts. Chemical indicators are already known for differentiating cheeses manufactured in high- or lowlands. Terpenes (15-17) and conjugated linoleic acids (18-19) are good indicators of the diversity of the botanical flora which increases from lowland to mountain pastures. Local or regional differences occur in function of the geomorphology, geology and climatology from one zone to the other. These indicators are therefore limited to cases with important altitude differences. Recently a method based on fluorescence delivered excellent results for differentiating the three cheese types L'Etivaz, highland and lowland Gruyere (20). Moreover some highland cheeses such as L'Etivaz must be made on an open fire with wood. Polycyclic aromatic hydrocarbons contained in the dissolved smoke are sensitive and specific indicators for such cheeses (21).

Acknowledgement
The authors thank the Swiss Federal Office of Public Health for its financial and logistical support to the project as well as G. Urbach and G. Gremaud for their reviewing of the publication.

Table 4 Main cheese types from Switzerland and their risk toward mislabelling of origin

Cheese type	Tons	At home			Abroad		
		Risk	Feasibility[1]	Needs/cost[1]	Risk	Feasibility[1]	Needs/cost[1]
No label[3]							
Alp cheese	3`000	High	++/0[2]	+	Low	++/0	0
Tilsiter	5`977	Middle	0	0	High	+	+
Appenzeller	7`912	Middle	0	0	No	-	-
Raclette Suisse	12`125	Middle	0	0	High	++	++
PDO							
L'Etivaz	320	High	++/0[2]	+	No	-	-
Formaggio d'alpe ticinese	400	Middle	++	+	Middle	+	0
Vacherin Mont d'Or	534	Middle	0	0	Middle	0	0
Tête de Moine	1`461	Middle	0	0	No	-	-
Sbrinz	2`475	Middle	0	0	High	++	++
Gruyère	24`965	High	0	+	Low	+	0
PDO candidates							
Walliser Raclette cheese	2`013	High	+	+	High	++	++
Emmental	35`533	Low	0	0	High	++	++
PGI [1]-candidate							
Vacherin Fribourgeois[1]	2`106	Middle	0	0	No	-	-

[1] "++"= good; "+"= fair; "0"= poor; "-"= not to be considered

[2] "++" if produced in lowland, "0" if produced in highland but not according to the requirements

[3] These cheese types have product specific labels which are not controlled by an independent organism

Summary
The main conclusions of a 3-year project on the authenticity of Emmental cheese are presented. The discrimination between the 183 Emmental samples originating from seven European regions (Switzerland, France Savoie, France Bretagne, France East-Central, South of Germany, Austria and Finland) was correct at a rate of 95% using a *global model*. The Emmentaler Switzerland™ could even be recognised to 100% in a *pairwise model*. The main drawback of these models is their elevated costs (approx. 1050 CHF / sample). Complementary, a preliminary study on the authenticity of Raclette Suisse® pointed out the potential of stable isotope ratios (δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) and calcium content to discriminate between Raclette Suisse® and Raclette cheese manufactured in France. Finally a subjective analysis of the risks of mislabelling, the analytical feasibility and the cost/benefit ratios for the main cheese types made in Switzerland is presented. It shows that only a few cheese types such as Sbrinz and Walliser Raclette would present cost/benefit ratios really interesting enough to justify laboratory research.

Zusammenfassung
Die wichtigsten Schlussfolgerungen eines dreijährigen Projektes über die Authentizität von Emmentalerkäse werden vorgestellt. Die 183 Emmentalerproben aus sieben europäischen Regionen (Schweiz, Frankreich Savoyen, Frankreich Bretagne, Frankreich Ost-Zentrum, Süden von Deutschland, Österreich und Finnland) konnte zu 95% korrekt mit einem *globalen Modell* diskriminiert werden. Der Emmentaler Switzerland™ wurde mit einem *paarweise Modell* sogar zu 100% richtig erkannt. Der Hauptnachteil dieser Modelle ist dessen hoher Preis (1050 CHF / Probe). Daneben zeigte eine Vorstudie über die Echtheit von Raclette Suisse® das Potenzial der Verhältnisse von stabilen Isotopen (δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) und des Kalziumgehaltes, um zwischen Raclette Suisse® und in Frankreich hergestellten Raclette zu diskriminieren. Schliesslich wird eine subjektive Analyse der Risiken einer falschen Deklaration, die analytische Machbarkeit und das Kosten/Nutzen-Verhältnis für die in der Schweiz hergestellten wichtigsten Käsesorten vorgestellt. Es zeigt sich, dass nur wenige Käsesorten wie Sbrinz oder Walliser Raclettekäse ein interessantes Kosten/Nutzen-Verhältnis aufweisen würden, um weitere Forschung zu rechtfertigen.

Résumé
Les principales conclusions d'un projet de trois ans sur l'authenticité du fromage Emmental sont présentées. La discrimination de 183 échantillons d'Emmental provenant de sept régions européennes (Suisse, France Savoie, France Bretagne, France est-central, sud de l'Allemagne, Autriche et Finlande) a été possible à 95% avec un *modèle global*. L'Emmentaler Switzerland™ a même pu être reconnu à 100% dans un *modèle par paires*. Le principal inconvénient de ces modèles est leur prix élevé (env. 1050 CHF / échantillon). En complément, une étude préliminaire sur l'authenticité de la Raclette Suisse® a mis en évidence le potentiel des rapports d'isotopes stables (δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) et de la teneur en calcium pour discriminer la Raclette Suisse® et la raclette produite en France. Finalement une analyse subjective des risques de fausse déclaration, de la faisabilité analytique et des rapports coûts/profit pour les principaux fromages suisses est présentée. Elle montre que seules quelques sortes de fromage comme le Sbrinz ou la Raclette du Valais présenteraient des rapports coûts/profit suffisamment bas pour justifier une investigation analytique.

Key words
Authenticity, Traceability, Emmental cheese, Raclette, PDO, Geographic origin

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Corresponding author: Dr. Jacques-Olivier Bosset, Chemistry & Physic unit, Agroscope Liebefeld-Posieux (ALP), CH-3003 Berne, E-mail: jacques-olivier.bosset@alp.admin.ch

Annex A
Assay for the geographic authentication of Emmental cheese using INDEx coupled to an MS-based electronic nose

Introduction
In a preliminary study, a MS-based electronic nose was tested in static headspace mode to discriminate between the geographic origins of the 20 investigated Emmental cheeses (Chapter 9). Though no complete discrimination could be achieved, the results showed a good trend towards grouping. It is known that preconcentration of the volatiles prior to injection enhances the discriminating ability when medium- and high boiling compounds are of interest. For instance, preconcentration systems such as Solid Phase Micro-Extraction (SPME) and Inside Needle Dynamic Extraction (INDEx) significantly improved the differentiation between three processed cheese types (Chapter 4). Though still at a developmental stage, INDEx had important advantages over SPME with respect to the longevity of the sorbent phase and the speed of the analysis.
The aim of the current investigation was to evaluate the capacity of an MS-based electronic nose of type SMart Nose, combined with the INDEx preconcentration system, to differentiate Emmental samples according to their geographic origin. Due to several technical difficulties, the experiment had to be suspended before all available cheese samples had been analysed.

Material and Methods
Cheese samples
Only 102 out of the 183 Emmental samples described in Chapter 12 could be analysed. The major problem occurred with the INDEx needle while a single specimen was available. It was irreversibly damaged by inadequate handling and the work could not be continued with the remaining samples.

SMart Nose analysis
Sample preparation, analytical equipment, working conditions and data acquisition are already described in Chapter 4 for INDEx. The samples were analysed in six series on different days according to Table 1. A processed cheese of type "1/4 fett" was also analysed at the same time and served as control material together with blanks for the standardisation of the other values (see Chapter 3). Principal component analysis (PCA) and discriminant analysis (DA) treatments were performed on the selected most discriminating masses.

Table 1 Scheme of analyses of Emmental

	A	CH	D	FI	FR	FTb	FTe
Day 0	2	1	1	-	1	-	1
Day 1	3	4	2	1	2	-	-
Day 3	1	8	6	1	5	3	3
Day 4	1	5	2	3	2	1	1
Day 5	1	9	3	2	2	2	2
Day 7	1	10	3	-	4	4	-
Total	**9**	**36**	**17**	**7**	**16**	**10**	**7**

Caption: the numbers indicate how many samples from a given region were analysed within the corresponding day

Results and discussion

In a first approach, all regions were considered simultaneously. After a PCA including six ion masses (31, 100, 58, 66, 102, 44), only 46% of the samples were correctly assigned. A discriminant analysis (DA) including 13 ion masses slightly improved the results reaching 57% correct classification.

In a second approach, the Swiss samples were compared one by one with the other regions considered (Model 4 in chapter 13). For all pairs, the overall correct classification rate was between 71% and 93% in the PCA using an optimised set of masses for each pair. The number of observations available compared to the number of factors required in the model was too small to allow DA to work adequately. taking into account the poor results obtained in the first approach, it is improbable that any good classification could be achieved, even with a supervised model.

Conclusion

A new preconcentration technique, called INDEx, coupled to an MS-based electronic nose was tested for its ability to distinguish Emmental cheese according to its geographic origin. The INDEx needle was irreversibly damaged by inappropriate handling when only about half the samples had been measured. The data obtained were however analysed. In a discriminant analysis containing groups from all the origins, only 57% of the samples could be correctly assigned. One possible explanation for these poor results is that the MS-based electronic nose may not be adequate for resolving the problem of geographic origin of Emmental. The second possible explanation is that the volatile compounds themselves might not be adapted to this task. The variability that occurs in the volatile profile of cheese manufactured in a same region might be too large to use volatiles as indicators. For example, forage and milk microbial activity, both of which strongly influence the composition of volatiles, can vary from farm to farm and from day to day. The positive results obtained in Chapter 9 were based on only 20 samples and were therefore not representative. A complete GC-MS analysis of all 183 samples would be necessary to find out whether volatiles would or would not be useful for this task. Such an investigation would be extremely time-consuming if a standard Purge & Trap technique were to be used. Automated systems using a thermal desorption unit with offline loaded traps or SPME should be investigated.

Annex B
Use of a thermal desorption unit for the analysis of volatile compounds in food: preliminary experiments

Introduction
In the preliminary study, volatile compounds from Emmental cheese were determined using Purge & Trap/GC-MS (Chapter 9). In this small scale study containing only 20 samples, volatiles were shown to be very interesting indicators of origin, though most of the key components were close to the MS detection limit. Moreover the extraction and injection technique used for the analysis was extremely time-consuming because several manual operations were required. The analysis of 183 samples would have been very time-consuming. A newly acquired instruments made possible an automated desorption and analysis of a series of traps which had previously been loaded offline.
The initial idea was to develop a method for the determination of volatiles in Emmental using an instrument with offline loaded traps and automated thermal desorption. However, due to a delivery delay of three months of the instrument and problems of contaminations in the first weeks of operation, only some aspects of the loading conditions and the problem of water were investigated. The amount of water in the gaseous phase of water-rich matrices may lead to a series of more or less serious problems. A very complete description of the latter was presented in the thesis of Canac-Arteaga[1]. The major difficulties encountered were the formation of artefacts, the obstruction of the cryotrap, the degradation of polar columns, the shift in the retention times, the worsening of the separation and the creation of double peaks. The goal of the current work was to gather information in order to optimise the loading conditions to limit or even prevent such problems. The improvement of the chromatographic resolution was only of secondary importance.

Material and Methods
Description and preparation of the samples
In some of the experiments, a processed cheese of type "Salami" as described in Chapter 2 was used as control material. For the other experiments, Swiss Emmental cheese was used. 200 g Emmental cheese were grated, mixed and kept at –20°C and used as standard cheese. Two grams grated cheese or 7 g suspension (10 g cheese in 40 g degased Millipore water, homogenised for 1 min at 8000 rpm) were placed in the loading cell described below.

Loading cell
A special glass apparatus was designed for loading the traps. (Figure 1). The lid had two openings, one for the gas inlet and one for the cartridge containing the absorption material. The Teflon gas inlet tube could be easily moved up and down to allow both a purge at the surface or into the bulk of the sample. The cartridge was normally set directly on the lid as shown in Figure 1. To investigate some effects of water condensation, the cartridge was sometimes connected to the loading cell by a 80 cm long Teflon tube. If not specified, the traps were loaded for 8 min with a purge of 30 mL/min nitrogen (> 99.999%). A trial with two traps connected one after the other confirmed that the trap was not overloaded by these conditions (data not shown).

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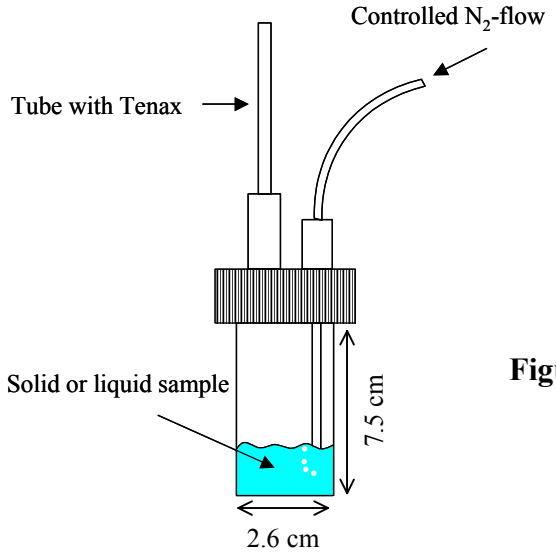

Figure 1 Scheme of the loading cell

TDS-GC system

A GC-MS Agilent 5973N (Agilent, Palo Alto, USA) was equipped with a thermodesorption unit TDSA2 with programmable temperature vaporization inlet (PTV) (Gerstel, Mühlheim, Germany). Tenax TA Thermal Desorption Tubes were used (Gerstel, art. 012260-005-00). 20 cartridges can be placed on the rack and undergo a fully automated thermal desorption and analysis. Figure 2 shows a diagram of the system pneumatics. A working cycle of the TDS unit can be divided into three steps: dry purge, desorption and cryotrap desorption. In the Purge&Trap system of Tekmar, the three steps are carried out within three independent circuits connected by a 6-port valve. In the TDS system, one single circuit manages all three consecutive steps by splitting the flow at the outlet of the oven and in the PTV inlet (Figure 2).

The following parameters were set in the Agilent program for the GC-MS: purge gas, nitrogen; solvent vent mode; pressure, 98 kPa; total flow at 54 mL/min; vent flow at 50 mL/min; vent pressure at 98 kPa for 0.10 min; purge flow to split vent at 50 mL/min for1.00 min. The temperature for the cryotrap is controlled from the Gerstel program: hold at –140 °C; heated at 12 °C/s to 260 °C, hold for 5 min.

Separations were performed on a 60m x 0.32 mm x 1 µm DB-1 column (Agilent). This may not be the ideal column for the separation of Emmental volatile compounds, but it has already been used in other investigations (Chapter 9), making comparison possible. Temperature program was as follows: hold 10 min at 35°C, heated at 3.5 °C/min to 140°C, heated at 5 °C/min to 170 °C, heated at 40 °C/min to 250 °C and hold for 5 min.

Two detectors, a FID and a MSD, were mounted in parallel by splitting the flow at the end of the capillary column. The MSD operated in the scan mode from 18 to 250 amu at 3.15 scan/s, ionisation was by EI at 70 eV.

The MS signals (peak height) were used to compare the results obtained. Within a series of experiments, a single autotuning was carried out.

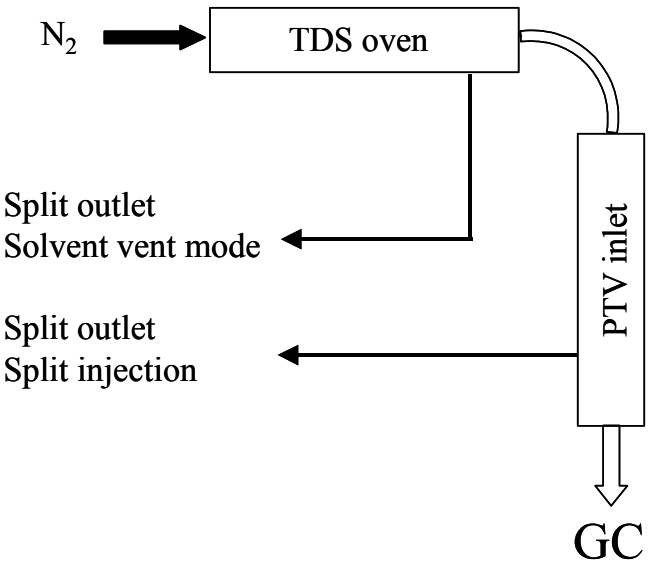

Figure 2 Schema of the TDS unit pneumatic

Results and discussion

Water peak in blanks

Empty dry tubes were desorbed to check the minimal water amount coming into the system. The water signal was measured using the molecular ion m/z=18. The TDS worked first in the *sample remove mode*. After each desorption, the sample tube was removed and replaced with the zero-tube (empty tube always in position zero) to avoid any further contamination during the analysis. The signal intensity of the water peak was $2.5 \cdot 10^8$. This value is abnormally high. There is probably a leak in the transfer line between the TDS oven and the cryofocussing unit. In the *standard mode*, the sample tube remains in the oven during the whole analysis. The oven is opened once instead of twice during the analysis. The water peak intensity was reduced to half the value found in the *sample remove mode*. This might be explained by the pneumatic concept of the system. By opening the door of the oven, ambient air and humidity reach the cryotrap where water freezes. This problem does not exist in instruments with separated circuits connected by a 6-port valve. For the following experiments, the *standard mode* was used.

Dry purge of the traps

Dry purge is the most common technique used in Purge&Trap extraction to reduce the amount of water transferred onto the capillary column during thermodesorption of the trap. Two grams processed cheese of type "salami" was used as control material for the following experiments. First the uptake of water from the trap was checked by loading it with two different purge volumes without applying any dry purge. A water peak area of $12 \cdot 10^8$ was observed after 25 min purge at 48mL/min (= 1200 mL). The peak surface was only of $5.5 \cdot 10^8$ after 8 min purge at 34 mL/min (= 272 mL). Using the latter loading conditions, a dry purge step of 3 min at 30°C in the TDS-oven was inserted (*solvent vent mode*). The water peak still showed an area of $5.5 \cdot 10^8$. The reason for this fact was likely to be the following: the *solvent vent mode* does not close the way to the cold trap. It only splits the flow at the outlet of the oven. A part of the flow still goes through the cold trap where water freezes. Theoretically, the water amount should have diminished drastically in this mode of operation, but for unexplained reasons, this was not the case. Some leak in the system might be responsible for this unexpected finding. A further experiment was carried out where a dry purge was carried

out outside the TDS-oven using a Teflon tube to connect the cartridge with the gas supply. The trap was purged with nitrogen (3 min, 30 mL/min) before placing it in the TDS for desorption. A water peak area of $1.7 \bullet 10^8$ was observed, corresponding to the minimum amount obtained for blanks. The additional water trapped from the sample could therefore be eliminated quantitatively on this way. For the following experiments, the cartridge was dried outside the oven prior to desorption.

Effect of liner type
The performances of a liner with swirl holes (Gerstel, GC 007001-010-00) and a liner filled with Tenax (Gerstel, GC 008968-005-00) were compared. The Emmental suspension was used as control material. The two corresponding chromatograms are presented in Figure 3. The main difference observed was the peak width. The peaks were much narrower using the Tenax liner. The retention capacity was also slightly increased. The drawbacks of this liner were the occurrence of some Tenax break down products (e.g. by 37 min) and the slightly increased noise of the base line. However, the Tenax liner was preferred for the following experiments.

Effects of various loading conditions of the traps
Six experiments under various conditions were carried out to compare the effect of water condensation. The loading conditions as well as the results are presented in Table 1. Grated Emmental was used as control material. The peak identification was completed using the MS data. Therefore only selected compounds whose mass spectra closely matched standards were considered. Furthermore, only a few compounds from each chemical group were selected to get a representative pattern. The comments and recommendations made in the text are naturally only valid if a DB1 chromatographic column is used. The problems with acid compounds would be totally different with a polar column. One should however keep in mind the final intention of the authors which is to compare the volatile profile of various Emmental cheeses. It is therefore irrelevant if the method applied does or does not reproduce a true image of the volatile compounds.
The effect of water addition to the sample was highlighted by comparing the group **A** (suspension) and **B** (grated). With exception of the high boiling aldehydes heptanal and octanal, a higher signal was found for all compounds using the grated form. Specially the acid recovery was greatly enhanced. This is however a drawback rather than an advantage. In screening approaches using a relatively apolar column such as in the current work, the broad acid peaks may overlap other important peaks. In Table 1, 2-butanol and butanal are some examples, but there are other compounds hidden by the huge acid peaks. Similar findings were reported by Larrayoz *et al*.[2]. Acids are generally quantified by other methods (Chapter 5). The use of grated cheese can therefore only partially be recommended using a DB1 column.

[2] Larrayoz P., Addis M., Gauch R., Bosset J.O. (2001). Comparison of dynamic headspace and simultaneous distillation extraction techniques used for the analysis of the volatile components in three European PDO ewes' milk cheeses. *Int.Dairy J*., **11**, 911-926.

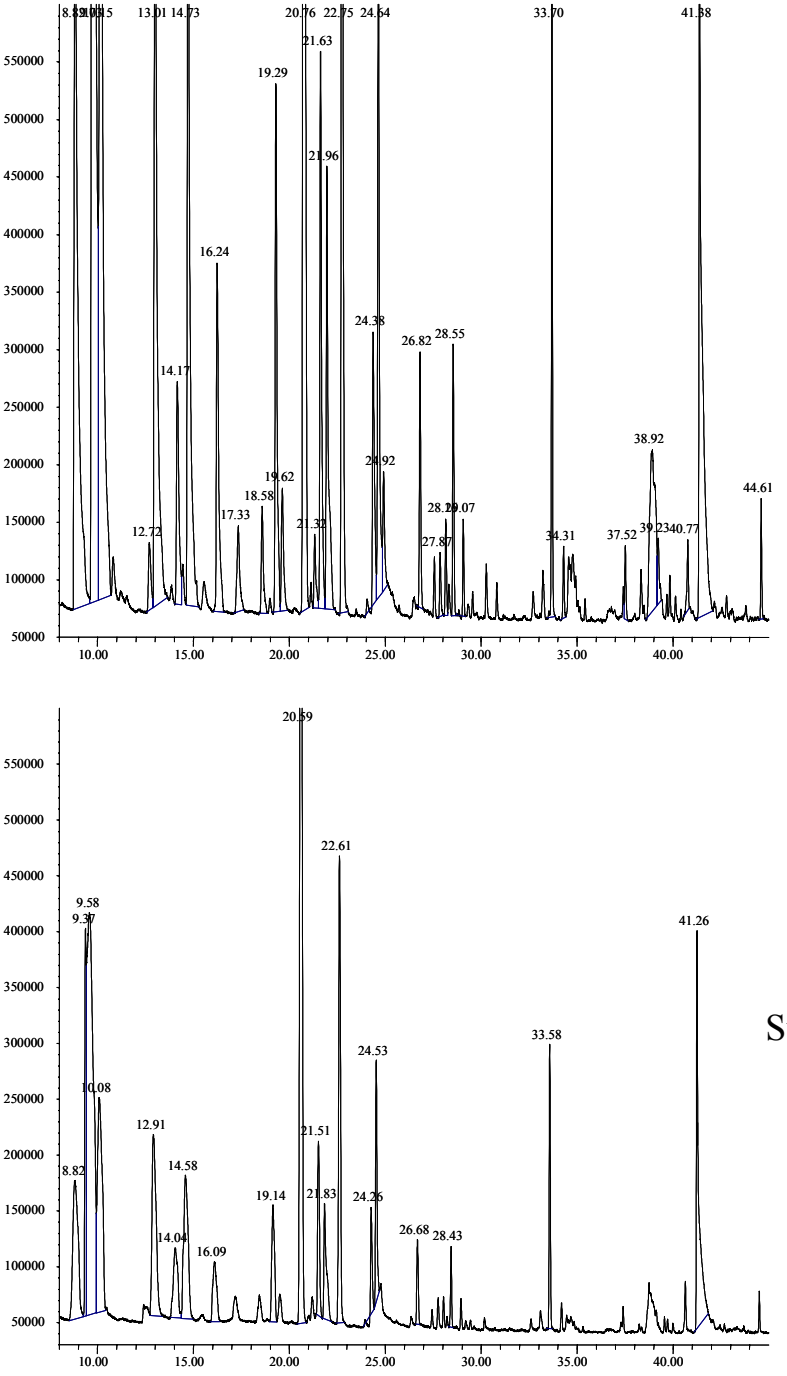

Figure 3 Comparison of two Emmental chromatograms using different liners

The effect of temperature was investigated by placing the whole system (loading cell and trap) in an air oven at 45°C (case **C**). Higher temperatures facilitate the evaporation of volatile compounds. The trap however retains the highly volatile compounds less efficiently. This was clearly demonstrated by homologous series of compounds. The recovery of short-chain ketones and alcohols was lower at 45°C than at 25°C whereas the opposite effect was observed for the higher boiling homologues. The acid peaks were also significantly larger than in **A** (approx. factor 4), leading to the chromatographic overlapping of some compounds. This approach may however be useful if the interest is centred on medium and high boiling compounds.

Theoretically, the best solution would be to heat the sample and cool the trap. For this purpose, the loading cell was placed in a water bath at 45°C and the trap left at room temperature on the lid. But another problem occurred due to the very large amount of water condensed on the walls of the trap, making dry purge almost useless and introducing too much water into the GC system. Though the extraction technique of the Purge&Trap from Tekmar is similar to that in the current experiment, great differences were found in the resulting chromatograms (Chapter 9). Using the Tekmar instrument, no water problems were observed and the signals for the acids were much lower than in cases **A** or **C** (results not shown). The single difference possibly explaining the disparity between these two extraction systems is the longer distance between the warm loading cell including the sample and the trap itself in the Tekmar instrument. In the latter, water condensation could occur in the transfer line and not in the trap. Acids and probably other compounds with a high affinity for water are trapped in the condensed water. A further experiment was designed to check this hypothesis. The loading cell was placed in a water bath as in **C**, but the trap was connected to the lid with a 80 cm long Teflon tube held at room temperature (case **D**). The whole system was purged and dried with nitrogen after each loading cycle. Indeed no water condensation was observed in the cartridge and the signals for the acids were much lower. Propanoic and butanoic acid were even below the detection limit. The recovery for highly volatile compounds such as ethanol, acetone, 2-methylpropanal was better than in cases **A** and **C**. A large loss was however observed for less volatile compounds such as 2-heptanone, 2-nonanone or octanal. The loss of molecules with low polarity such as the benzene derivates was less marked. It should be possible to find a good compromise between the attenuation of the acids and the loss of high and medium boiling compounds by changing the length or the temperature of the transfer line. Further investigations would be necessary to optimise such a loading system if it has to be run using a DB1 column.

The last experiment was repeated using grated instead of suspended cheese (**E**). In this case, the amount of acid compounds was effectively reduced in comparison to **B**, but still lay in the same range as in **C**, possibly overlapping important compounds. The advantage of this methods is clearly the enhanced signal intensity for highly volatile compounds.

Table 1 Effect of various loading conditions of the trap on the recovery of some selected volatile compounds from Emmental cheese(Peak height in arbitrary units)

	Boiling point	A	B	C	D	E
Acids						
Acetic acid	118	110	3423	393	23.4	591
Propanoic acid	141.4	364	5661	1779	< 10	1560
Butanoic acid	165.5	102	977	442	< 10	552
Alcohols						
Ethanol	78	264	786	80.2	667	1970
iso-Propanol	82	289	1083	132	1056	2430
1-Propanol	97	160	513	116	653	1402
Pentanol	138	138	185	256	389	-[2]
2-Butanol	99.5	30.9	-[1]	-[1]	30	-[1]
Ketones						
Acetone	56	640	2245	196	2051	3171
2,3-Butanedione	88	46.6	146	523	194	223
2-Butanone	80	218	609	226	702	797
3-Hydroxy-2-butanone	148	605	4028	1946	490	3441
2-Heptanone	151	1166	814	1546	586	972
2-Nonanone	195	139	88.6	292	< 10	< 10
Aldehydes						
2-Methylpropanal	64	25	58.4	< 10	58.9	68.3
Butanal	76	22	38.2	< 10	35.8	51.8
3-Methylbutanal	90	51	-[3]	45.3	92.3	96
Heptanal	154	114	< 10	129	62.2	69.1
Octanal	171	139	< 10	83	39	26.6
Aromatics						
Ethylbenzene	136	16.9	18.2	31.4	23.8	35.8
p-Xylene	138	26.2	33.5	30.7	42.2	59.1
1,2,3-Trimethylbenzene	176	16	18.2	25.6	21.7	25.8

A Suspension; whole system at 25°C, cartridge on the lid of the loading cell
B Grated cheese; whole system at 25°C, cartridge on the lid of the loading cell
C Suspension; whole system at 45°C, cartridge on the lid of the loading cell
D Suspension; in a water bath at 45°C, cartridge connected to the lid of the loading cell with a 80 cm long Teflon tube at 25°C
E Grated cheese; under part of loading cell in a water bath at 45°C, cartridge connected to the lid of the loading cell with a 80 cm long Teflon tube at 25°C

[1] overlapped by butanoic acid
[2] overlapped by 3-methyl-2-butanol
[3] overlapped by acetic acid

Conclusion

Various parameters were investigated for loading the TDS-Tenax traps. To minimise the amount of water entering the GC-system, the *standard mode* should be preferred to the *sample remove mode* in the TDS-Software. Furthermore the cartridge should be dried using nitrogen outside the TDS-system instead of the *solvent vent mode* inside the oven. However the abnormally high amounts of water found in the chromatograms indicate a possible leak in the system. Both conclusions stated above should be re-examined after the system has been meticulously checked. Two liners were also compared. The Tenax-filled liner delivered narrower peaks than the empty one. Finally the performances of the extraction were investigated varying i) the temperature, ii) the form of the matrix (grated or suspended cheese) and iii) the length of the connection between the loading cell and the trap. The following conclusions are only valid for separation on a non-polar column such as the DB1 column used. Grated cheese was generally better for highly volatile compounds but the high amount of acids extracted in comparison to suspended cheese made the grated form less interesting. In conclusion, the best results were obtained with suspended cheese heated at 45°C, where the loading cell was connected to the trap, held at room temperature with a 80 cm long plastic tube. The signal for acids was very low whereas the recovery rate for low and medium boiling compounds was very good. Only some long-chain compounds such as 2-nonanone or octanal were poorly extracted.

The analysis of water-rich food samples is not trivial in the Purge&Trap technique. It will not be easy to load the traps in a reproducible way while avoiding the problem of the acids using the current chromatographic column. A more polar column should solve the problem of broad acid peaks, allowing the use of grated cheese which gives less problems with water. Otherwise further investigations on the temperature and the length of the transfer line between the loading cell and the trap should be carried out to optimise the system for both high and low boiling compounds.

Annex C
List of publications, posters and oral presentations

Journal or book articles

Pillonel, L., Bütikofer, U., Tabacchi, R. and Bosset, J.O.: Authenticity of provenance of Swiss cheeses: conclusion of the project, recommendation to food control laboratories and perspective for the future. *Mitt. Lebensm. Hyg*. (submitted)

Pillonel, L., Badertscher, R., Casey, M., Meyer, J., Rossmann, A., Tabacchi, R. and Bosset, J.O.: Geographic origin of European Emmental cheese. 1. Characterisation and descriptive statistic. *Int. Dairy. J.* (in press)

Pillonel, L., Bütikofer, U., Tabacchi, R. and Bosset, J.O.: Geographic origin of European Emmental cheese. 2. Use of discriminant analysis and artificial neural network for classification purposes. *Int. Dairy. J.* (submitted)

Pillonel, L., Bütikofer, U., Rossmann, A., Tabacchi, R. and Bosset, J.O.: Analytical methods for the authentication and traceability of Raclette Suisse® and Fontina PDO cheese. *Mitt. Lebensm. Hyg*. (in press)

Pillonel, L., Tabacchi, R. and Bosset, J.O.: Comparison of efficiency and stability of two preconcentration techniques (SPME and INDEx) coupled to an MS-based electronic nose. *Mitt. Lebensm. Hyg*. **95,** 85-98 (2004).

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Pattern recognitions for the authenticity of European and Swiss Emmental cheese. Pillonel L., Badertscher R., Casey M., Meyer J., Rossmann A., Tabacchi R. and Bosset J.O. 2003. 7th International Symposium on Food Authenticity and Safety, 14-17 October 2003, Nantes, France.

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Oral presentations

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Annex D
List of abbreviations

AAS	Atomic Absorption Spectroscopy
ALP	Agroscope Liebefeld-Posieux
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
ATR	Attenuated Total Reflection
BPN	Back-Propagation Network
CAR	Carboxen
CF	Continuous Flow
CLA	Conjugated Linoleic Acid
DA	Discriminant Analysis
DFA	Discriminant Function Analysis
DH	Dynamic Headspace
DNA	Deoxyribonucleic Acid
DTD	Direct Thermal Desorption
DVB	Divinylbenzene
ECOC	Enterococci
EN	Electronic Nose
FA	Fatty Acid
FAA	Free Amino Acid
FAM	Forschungsanstalt für Milchwirtschaft (new ALP)
FFA	Free Fatty Acid
FHL	Facultative Heterofermentative Lactobacilli
FID	Flame Ionisation Detector
GC	Gas Chromatography
HPLC	High Performence Liquid Chromatography
HSSE	Headspace Sorptive Extraction
ICP-MS	Inductively Coupled Plasma - Mass Spectrometry
INDEx	Inside Needle Dynamic Extraction
IRMS	Isotope Ratio Mass Spectrometer
LAP	L-leucine-aminopeptidase
LDA	Linear Discriminant Analysis
MAE	Microwave Assisted Extraction
MCM	Moisture Control Modul
MCS	Moisture Control System
MIR	Mid-Infrared
MOS	Metal Oxide Sensor
MS	Mass Spectrometry
NIR / DR	Near Infrared / Diffuse Reflection
NPN	Non Protein Nitrogen
OHL	Obligate Heterofermentative Lactobacilli
OLMT	On-Line Microtrap
OPA	ortho-Phtalaldehyde
OTT	Open Tubular Trap
PA	Polyacrylate
PAB	Propionic Acid Bacteria
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PDO	Protected Denomination of Origin
PGI	Protected Geographical Indication
PLS	Partial Least Square
PTV	Programmable Temperature Vaporization
QMB	Quartz Microbalance
RSD	Relative Standard Deviation
SBSE	Stir Bar Sorptive Extraction
SDS-Page	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SMOW	Standard Mean Ocean Water
SPDE	Solid Phase Synamic Extraction
SPME	Solid Phase Microextraction
STB	Salt Tolerant Bacteria
TCA-SN	Trichlor Acetic Acid - Soluble Nitrogen
TDS	Thermodesoption System
TIC	Total Ion Current
TN	Total Nitrogen
Tr	Transmission
VOC	Volatile Organic Compound
WSN	Water Soluble Nitrogen