Zeitschrift: Mitteilungen aus Lebensmitteluntersuchungen und Hygiene = Travaux de chimie alimentaire et d'hygiène

Herausgeber: Bundesamt für Gesundheit

Band: 92 (2001)

Heft: 2

Artikel: Identification of species specific central nervous tissue by gas chromatography-mass spectrometry (GC-MS) - a possible method for supervision of meat products and cosmetics

Autor: Niederer, Markus / Bollhalder, Rita

DOI: https://doi.org/10.5169/seals-981903

Identification of Species Specific Central Nervous Tissue by Gas Chromatography-Mass Spectrometry (GC-MS) – a Possible Method for Supervision of Meat Products and Cosmetics

Markus Niederer and Rita Bollhalder, State Laboratory Basel-City, Basel

Received 13 February 2001, accepted 23 February 2001

Introduction

In Switzerland legal restrictions prohibit the use of bovine nervous tissue (specified risk material) as a component in food (inclusive meat products) and cosmetics (1, 2) because of bovine spongiform encephalopathy (BSE). Hence there is a great interest in a routine analytical method to ensure that the regulations are observed by producers and importers. Recently, an immunohistochemical procedure for the detection of tissues of the central nervous system (CNS) has been developed (3–5) which can be used for market survey purposes. Here we present an alternative approach using gas chromatography and mass spectrometry (GC-MS) for the detection of CNS tissues by using brain specific fatty acids from sphingolipids (SLs) as markers. SLs are typical membrane constituents of mammalian nervous cells composed of ceramide, phosphorylcholine or galactose and fatty acids (fig. 1). Most of their fatty acids are unusual with 22 to 24 carbon atoms. In particular, lignoceric acid (C24), docosahexaenoic acid (C22:6), nervonic acid (C24:1) and cerebronic acid (C24oh) are found predominantely in CNS (6, 7). The cleavage of the fatty acids by acidic methanolysis (8, 9) results in characteristic profiles of their methyl esters (FAMEs). Our preliminary experiments with brain tissues indicate that these profiles can be used to differentiate between some animal species, e.g. cow, pig and sheep. Further we report about first experiences with analysing meat products and cosmetic samples.

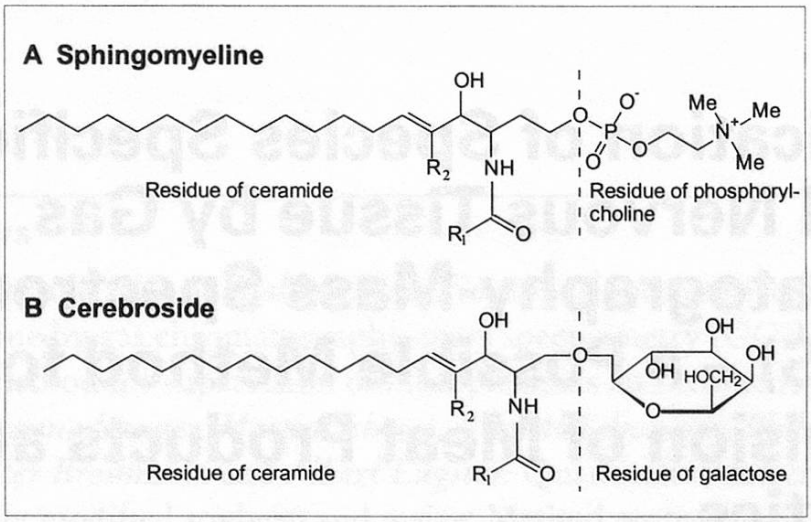

Figure 1 **Structures of sphingolipids** (A = phospholipid, B = glykolipid) **and their residues.** R1 = fatty acids (C22:6, C24, C24:1, C24oh mainly) were cleaved by acidic methanolysis, R2 = Hydrogen for sphingolipids or OH-group for phyto-sphingolipids. Abbreviations see experimental section

Method

Chemicals

Chloroform, toluene, hexane, acetone, methanol, sodium chloride, sodium sulfate (analytical grade or higher), acetic acid 100 % and hydrochloric acid 32 % were obtained from Merck (Germany), α-bromoacetophenone and triethylamine from Fluka (Switzerland). Water was distilled and free from organic compounds. The following FAMEs and SLs were from Larodan Fine Chemicals (Sweden): methyl tetracosanoate 99 % (lignoceric acid, C24), methyl docosahexaenoate 99 % (DHA, C22:6), methyl 15-cis-tetracosenoate 99 % (nervonic acid, C24:1c), methyl 2-hydroxytetracosanoate 98 % (cerebronic acid, C24oh), ceramide galactosides 98 % (bovine cerebosides), D-erythro-sphingomyelin 98 % (bovine), TLC Mix 51 98 % (cerebrosides, sulfatides, sphingomyeline). Tridecanoic acid (C13) and/or methyl tridecanoate (C13-FAME) from Fluka (Switzerland) were used as internal standards for GC-MS and 15-cis-tetracosenoic acid from Aldrich (Germany) as external standard for RP-HPLC.

Samples

Cooked sausages spiked with various amounts of calf CNS tissue (0.00 to 1.00 % w/w) were provided by the Swiss Federal Veterinary Office. Brain (cow, calf, sheep, pig) reference samples were obtained from the regional slaughter-house. These were treated as potential infectious materials and stored at –20° C. Sausages and brains

were cut while still frozen into little pieces immediately before the extraction procedure. Cosmetic samples were bought from commercial shops in Basel and stored at room temperature. Plant samples from phyto-SL containing species were obtained as reference samples from the University of Basel, dried at 60° C, homogenized and stored at room temperature.

Sample extraction and clean up

An overview scheme of the different clean up procedures is shown in figure 2. Samples (0.5–1.0 g fresh weight or plant dry weight) were extracted with 2 ml hexane-acetone (20:1, v/v) in a capped glass tube (10 ml) by intense manual shaking and ultra sonic bath (5 min). After adding 2 ml of a saturated NaCl solution the mixture was again shaken intensely and centrifuged (2 min, 4000 U/min). The organic supernatant (raw extract) was used for purification with SPE (fig. 2a) or for direct derivatization of the fatty acids to methyl esters (FAME, fig. 2b).

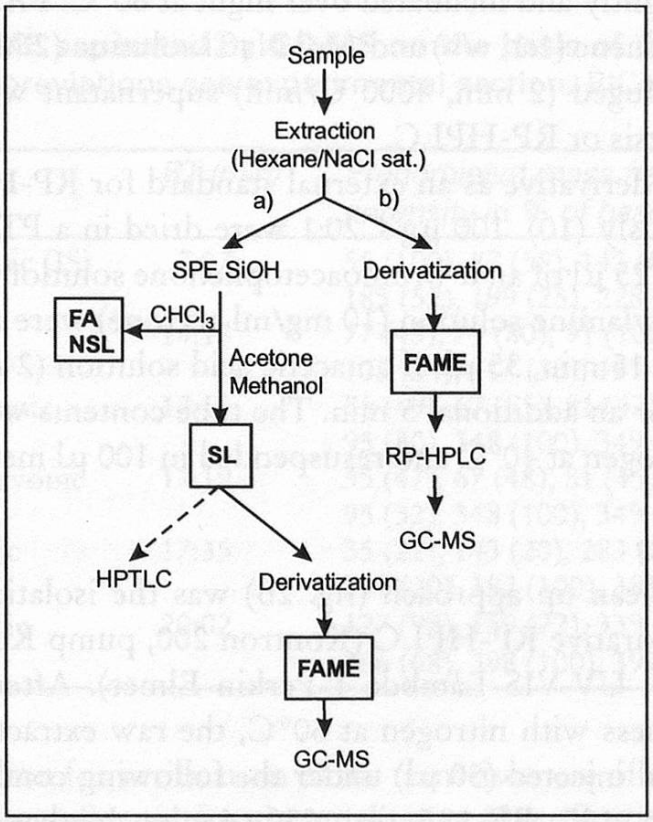

Figure 2 **Clean up overview.** a) solid phase extraction (SPE) preferably used for meat products, b) reverse-phase high performance chromatograpy (RP-HPLC) for cosmetics. FA = free fatty acids, NSL = neutral sphingolipids, further abbreviations see experimental section

Purification of raw extract with SPE

The raw extract was separated into three organic fractions (fig. 2a) differing in polarity by using SPE (SiOH, 500 mg, 3 ml, M&N, overlayered with 0.5 cm dried Na_2SO_4 and conditioned with chloroform). The first apolar fraction of mainly neutral lipids and some free fatty acids was eluted with 5 ml chloroform and discarded. The second more polar fraction of glycolipids was eluted with 10 ml acetone and the most polar fraction of phospholipids with 10 ml methanol as eluant (8, 9). In general, these two fractions were collected together in a distillation flask, spiked with internal standard C13 (5 µg), concentrated by rotary evaporator to minimal volume and resuspended in 1 ml hexane/toluene (1/1 v/v) for derivative preparation.

Derivative preparation

FAMEs were prepared by hydrochloric acid catalyzed esterification (8, 9). The raw extract (1.5 ml) for subsequent RP-HPLC or the eluate of SPE was evaporated under a stream of nitrogen (60° C) in capped glass tubes (10 ml). After adding 1 ml derivatization mixture containing methanol-chloroform-HCl (10:1:1, v/v/v), the tubes were sealed tightly and incubated over night at 60° C. FAMEs were extracted with 2 ml hexane-toluene (1:1, v/v) and 1 ml NaCl solution (2 %) by intense manual shaking. The centrifuged (2 min, 4000 U/min) supernatant was ready for subsequent GC-MS analysis or RP-HPLC.

C20:1-phenacyl derivative as an external standard for RP-HPLC was prepared as described previously (10). 100 µg C20:1 were dried in a PTFE-lined screw cap centrifugation tube. 25 µl of an α-bromoacetophenone solution (10 mg/ml acetone) and 25 µl of a triethylamine solution (10 mg/ml acetone) were added and heated in boiling water. After 15 min, 35 µl of an acetic acid solution (2 mg/ml acetone) was added and heated for an additional 5 min. The tube contents were then evaporated to dryness with nitrogen at 40° C and resuspended in 100 µl methanol.

RP-HPLC of FAMEs

An alternative clean up approach (fig. 2b) was the isolation of brain specific FAME by semipreparative RP-HPLC (Kontron 200, pump Kontron T-414, injector Rheodyne 7125, UV-VIS Lambda 1 Perkin Elmer). After derivatisation and evaporation to dryness with nitrogen at 60° C, the raw extract was redissolved in 100 µl methanol and injected (50 µl) under the following conditions: 500 µl loop, column Lichrosorb 100 RP-18 5 µm, 250×4 mm; methanol isocratic 100 % (0–36 min); flow rate 1.0 ml/min, UV 254 nm; fractions: 4.5–5.5 ml for C22:6, 9.5–11.0 ml for C24oh, 12.5–14.5 ml for C24:1 and 17.5–19.5 ml for C24. The UV-active C20:1-phenacyl derivative was used as an external standard for retention time (fraction) correction before analysing samples. The fractions were combined in a distillation flask, spiked with internal standard C13-FAME (500 ng), concentrated by rotary evaporator to minimal volume and resuspended in toluene (50 µl) for subsequent GC-MS analysis. Fractionating of RP-HPLC was very reproducible which

could be confirmed by observing only small shifts in retention time of the external standard C20:1-phenacyl derivative (rel. standard deviation S.D. = 0.5 %) over a series of about 20 injections.

Determination of FAMEs by GC-MS

Identification and determination of brain specific FAME clusters were performed by gas chromatography-mass spectrometry (Carlo Erba HRGC 5160, Fisons and GCQ, Finnigan MAT) under the following conditions: DB-5MS (J&W) fused silica column (30 m×0.25 mm I.D., 0.25 µm film) with a retention gap (0.5 m×0.32 mm I.D., deactivated) used with on column injection of 1.0 µl; temperature programme 90° C(1min)-(30° C/min)-140° C-(12° C/min)-260° C-(2° C/min)-295° C (2 min); carrier gas (helium) 60 kPa; EI mode (trap offset = 10, AGC target = 50, high mass adjust = 80 %, wave form off), mass range 50–400 amu, full scan mode (sec/scan = 1, µscan = 7); transferline = 280° C; ion source = 200° C.

The following FAMEs were used for identification (table 1).

Table 1
Identification of FAME separated by GC-MS on the basis of retention times (RT) and mass (m/z). Abbreviations see experimental section. RIC = reconstructed ion counts

Abbrev.	*FAME*	*RT (min)*	*Predominant mass fragments (intensity in % of base peak)*	*Quant. mass*
C13	Tridecanoic (IS)	7:07	55 (100), 87 (58), 143 (65), 185 (52), 199 (25), 228 (15)	RIC
C22:6	DHA	14:18	77 (45), 79 (80), 91 (100), 105 (29), 117 (33), 131 (18)	91
C24:1c	cis-Nervonic	17:16	55 (49), 67 (55), 81 (47), 95 (30), 348 (100), 349 (45)	348
C24:1t	trans-Nervonic	17:19	55 (47), 67 (48), 81 (45), 95 (32), 348 (100), 349 (45)	348
C24	Lignoceric	17:35	55 (22), 143 (20), 283 (15), 339 (20), 382 (100), 383 (28)	382
C24oh	Cerebronic	20:02	127 (98), 159 (72), 339 (99), 366 (68), 398 (100), 399 (33)	398

A reference solution for verification of the trans-isomere of c24:1 was not at our disposal. GC-MS was validated for the qualitative identification of brain specific fatty acid profiles, therefore quantitative aspects as linearity and precision are of low priority. The limits of detection for standard solutions were between 10 pg (all C22-C24) to 150 pg (C24oh). Processing and interpretation of mass spectra were carried out with Masslib™ V.8.6 (Max Planck Institute, Germany).

Results and Discussion

Comparison of sample purification procedures

Taking into account the extremely complex fatty matrix of meat and cosmetic products two different clean up procedures were tested in order to achieve the most efficient and sensitive method for the determination of brain specific fatty acids. These are solid phase extraction (SPE) and semipreparative RP-HPLC. Additionally, for screening purpose high performance thin-layer chromatography according to (11) was applied but with no success for samples with small amounts of CNS tissues. Therefore, this method will not be further described here.

The principle of the purification methods is different. With the first variant the intact macromolecules of SLs were separated by SPE prior to splitting off the fatty acids by acidic methanolysis (fig. 2A). Alternatively, the raw extract was derivatized and the resulting free FAMEs were separated with RP-HPLC thereafter (fig. 2B). The advantage of SPE compared to RP-HPLC is that SPE is easier to perform and allows the determination of cholesterol as a CNS marker for screening purposes (4). On the other hand, in the raw extracts free fatty acids and those derived from fragments of degraded SLs are lost with SPE (degradation of SLs during manufacturing processes of sausages and cosmetics has to be taken into account). Therefore, with SPE the resulting FAME profile may not exactly correspond to the original profile anymore and is less informative than with RP-HPLC particularly for cosmetic samples.

FAME profiles of brain and plant reference samples

In initial experiments, extracts of three brains per species and plant reference samples (phyto-SLs containing species *Borrago officinalis, Lunaria annua, Lunaria rediviva, Brassica napus, Hesperis matronalis, Primula spec.*) were purified by SPE or RP-HPLC and screened with GC-MS for dominant SL-specific fatty acids (fig. 3). Although phyto-SLs contain also several fatty acids which are generally regarded as brain (animal) specific, we can observe some differences to the brain profile: phyto-SLs lack DHA and the trans-isomere of nervonic acid (fig. 3C). Therefore, SLs of brain and of plant origin can be distinguished. Furthermore, the profiles indicate that in the case of brain samples we can differentiate between animal species due to their characteristic cis/trans-ratios (c/t) of nervonic acid (table 2). C/t-ratios were very well reproducible. Interestingly even the brains of cow and calf show some significant differences and we therefore presume that the bovine TLC Mix 51 is derived from calf brain. Maybe the isomers of DHA could additionally be used for distinguishing purposes providing a better resolution of the peaks can be achieved.

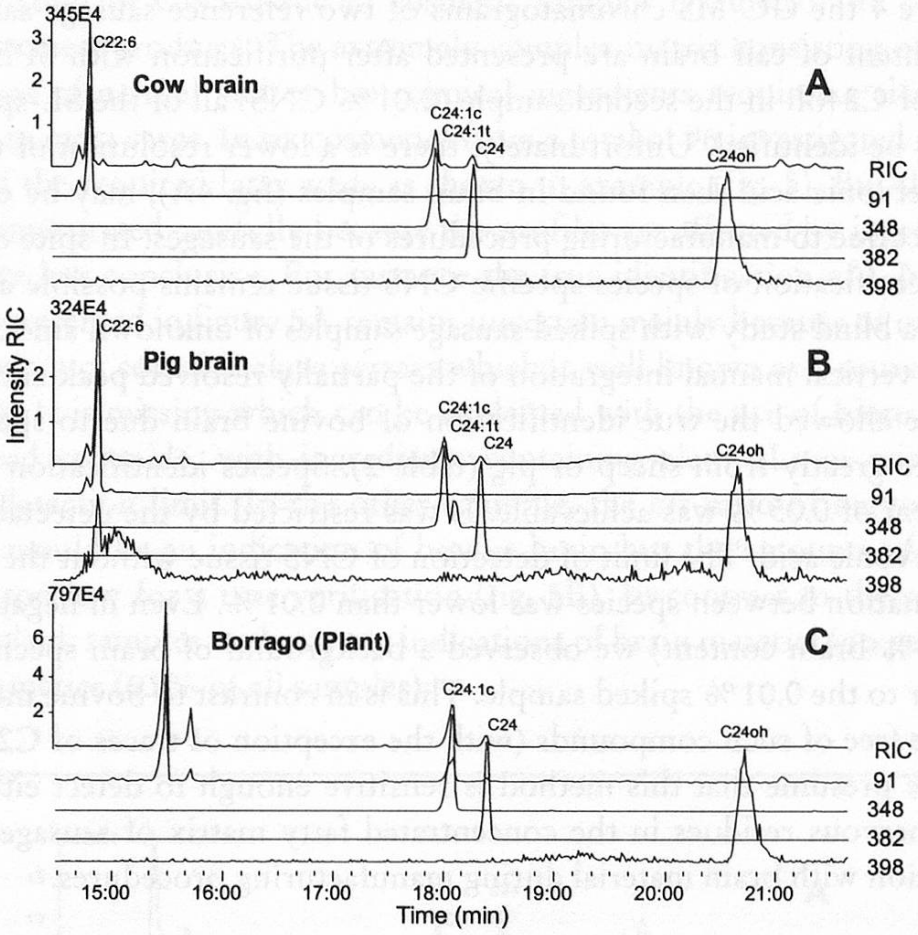

Figure 3 **GC-MS chromatograms of representatives reference samples (RP-HPLC extracts).** A) cow brain, B) pig brain, C) plant extract of borrago *(Borrago officinalis)* containing phyto-sphingolipids. Reconstructed ion (RIC) and single-ion chromatograms at m/z 91 (DHA), 348 (nervonic acid), 382 (lignoceric acid) and 398 (cerebronic acid). Abbreviations see experimental section

Table 2
Ratio of cis/trans-nervonic acid of reference materials. Abbreviations see experimental section

Reference material	*c/t-ratio C24:1*		*number of individual samples*	*t-test p <0.005*
	mean ± S.D.	*range*		
1) Pig brain	2.9 ± 0.1	2.8–3.1	3	1) vs 2)
2) Sheep brain	5.2 ± 0.6	4.5–5.9	3	2) vs 4)
3) Calf brain	6.3 ± 0.7	5.4–7.1	3	3) vs 4)
4) Cow brain	11.0 ± 0.7	10.4–12.0	3	4) vs 1)
5) Spiked sausages (calf)	9.5 ± 1.8	7.5–11.9	6	5) vs 1), 2)
6) TLC MIX 51 (bovine)	7.0 ± 1.7	5.8–8.2	1	not tested

Identification of CNS tissue in reference sausages

In figure 4 the GC-MS chromatograms of two reference sausage samples with varying content of calf brain are presented after purification with SPE. With the exception of C24oh in the second sample (0.01 % CNS) all of the SL-specific fatty acids could be identified. Unfortunately, there is a lower resolution of the c/t-isomeres of nervonic acid than found in brain samples (fig. 4A), may be caused by a matrix effect due to manufacturing procedures of the sausages. In spite of this artefact, the identification of species specific CNS tissue remains possible as we could confirm in a blind study with spiked sausage samples of unknown amount of CNS tissue. The vertical manual integration of the partially resolved peak areas down to the baseline allowed the true identification of bovine brain due to specific ratios which differ greatly from sheep or pig (table 2). Species identification down to a brain content of 0.05 % was achievable. It was restricted by the detectable amount of trans nervonic acid. The limit of detection of CNS tissue without the possibility of discrimination between species was lower than 0.01 %. Even in negative control sausages (0 % brain content) we observed a background of brain specific FAMEs very similar to the 0.01 % spiked sample. This is in contrast to bovine meat samples which were free of such compounds (with the exception of traces of C24, data not shown). We presume that this method is sensitive enough to detect either natural occurring nervous residues in the concentrated fatty matrix of sausages or cross-contamination with brain material during manufacturing procedures.

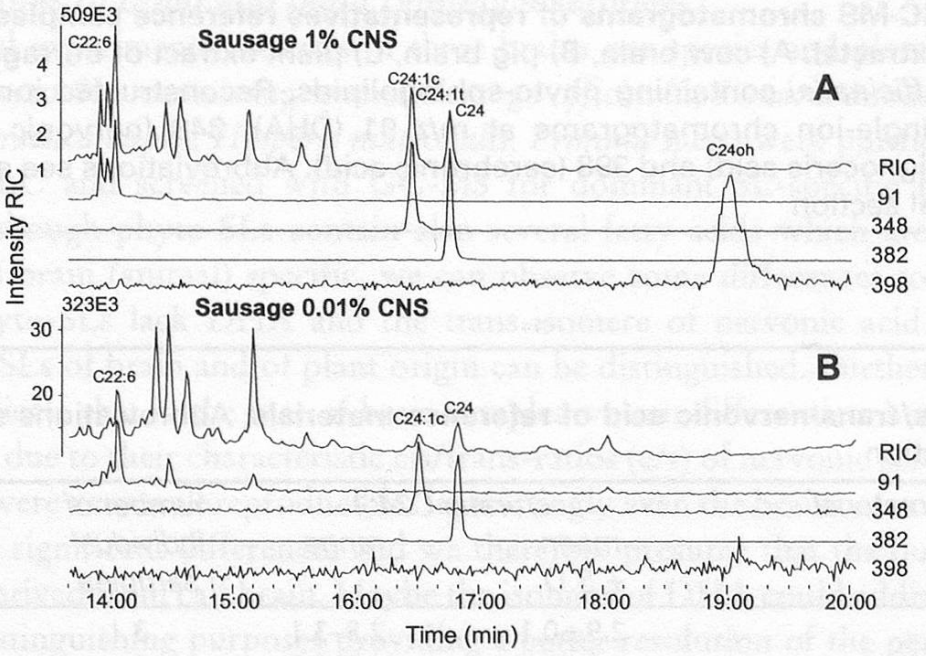

Figure 4 **GC-MS chromatograms of reference sausages** (SPE extracts). A) sausage spiked with 1 % of calf brain, B) sausage spiked with 0.01 % of calf brain. Ion monitoring as described in figure 3

Identification of CNS tissue in cosmetics

Identification of CNS-tissue in cosmetic samples is much more complicated compared to meat products. The extremely complex matrix consisting of numerous combinations of natural and/or biotechnical ingredients requires a clean up with RP-HPLC in most cases. In six cosmetics from a total of 96 investigated samples we found all of the required fatty acids as shown in example (fig. 5). But these results have to be interpreted carefully because the profiles are affected by ingredients and therefore are less conclusive. For instance the true identification of CNS tissue in the sample presented in figure 5A remains uncertain mainly because of two reasons: First, this product contains algae extract which is well known as a source of DHA. Second, C24:1t is missing which can be explained with the use of biotechnogically manufactured ceramide, with ingredients containing plant SLs or with amounts below the detection limit. In the other example, the c/t-ratio of nervonic acid of approx. 15 would be an indication of bovine brain but the amounts of DHA and C24oh are too low for a true verification (fig. 5B). In contrast to the sausages we found real blank samples without any indications of brain material (no peaks or C24 only) in cosmetics (63 % of all samples).

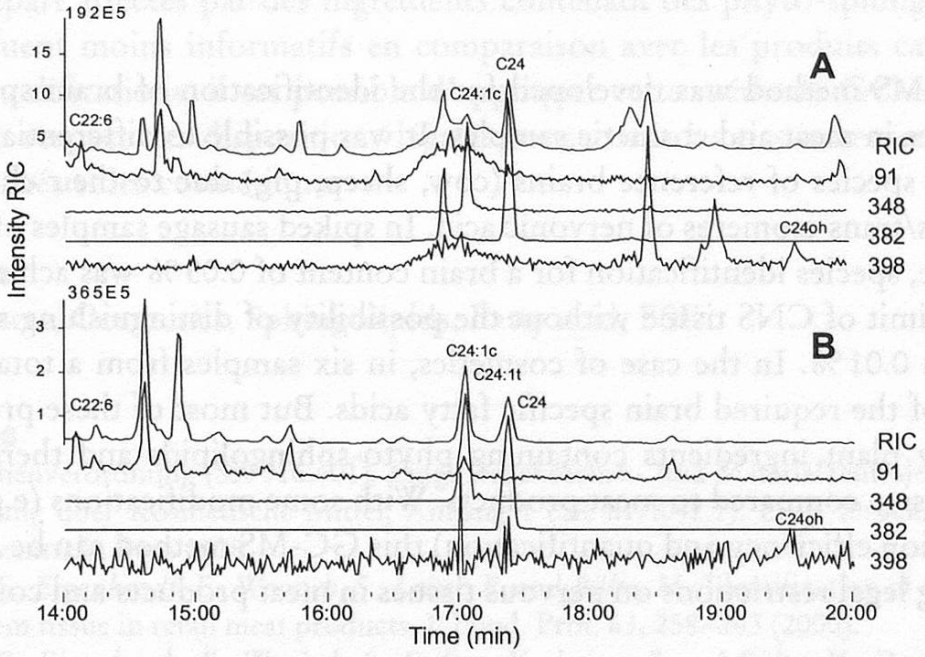

Figure 5 **GC-MS chromatograms of cosmetic samples (RP-HPLC extracts).** A) anti wrinkle cream containing plant and algae extracts and biotechnological ceramide, B) day cream containing plant extracts and vitamine A + E. Ion monitoring as described in figure 3

Conclusion

The results of preliminary experiments for the detection and identification of nervous tissue presented in this study are promising. Nevertheless there is some need to optimize the extraction efficiency and a reproducible quantification in order to define an accurate and usable detection limit of CNS tissue in meat products. In general a purification with SPE leads to acceptable results whereas for cosmetics the more efficient RP-HPLC method should be used. We also have to accept a certain error probability for brain detection in cosmetics due to ingredients of different sources, but we can get reasonable indications and therefore minimize the amount of cosmetic products which have to be checked further in detail by administrative means. With the modifications mentioned above this GC-MS method can be recommended as a simple routine procedure for supervising legal restrictions on nervous tissues in meat products and cosmetics and as a real alternative to immunohistochemical applications.

Acknowledgements

We wish to thank *Christopher Hohl* (State Laboratory Basel-City) and *Endre Laczko* (Solvit, Kriens) for helpful discussions and the Swiss Federal Veterinary Office for giving sausage reference samples at our disposal.

Summary

A GC-MS method was developed for the identification of brain specific fatty acid profiles in meat and cosmetic samples. It was possible to differentiate between the animal species of reference brains (cow, sheep, pig) due to their characteristic ratios of cis/trans-isomeres of nervonic acid. In spiked sausage samples of unknown CNS tissue, species identification for a brain content of 0.05 % was achievable. The detection limit of CNS tissue without the possibility of distinguishing species was lower than 0.01 %. In the case of cosmetics, in six samples from a total of 96 we found all of the required brain specific fatty acids. But most of these profiles were affected by plant ingredients containing phyto-sphingolipids and therefore were less conclusive compared to meat products. With some modifications (e.g. optimizing extraction efficiency and quantification) this GC-MS method can be applied for supervising legal restrictions on nervous tissues in meat products and cosmetics.

Zusammenfassung

Für die Identifikation von hirnspezifischen Fettsäureprofilen in Fleischprodukten und Kosmetika wurde eine gaschromatographisch-massenspektrometrische Methode entwickelt. In Versuchen mit Hirnreferenzmaterial war die Unterscheidung von Tierarten (Rind, Schaf, Schwein) aufgrund von charakteristischen Verhältnissen der cis- und trans-Isomeren der Nervonsäure möglich. In gespikten Referenzwürsten mit unbekanntem Nervengewebe gelang die Tierartenidentifizierung bis zu einem Gehirnanteil von 0,05 %. Die Nachweisgrenze von Nervengewebe

ohne Unterscheidung der Tierart lag unter 0,01 %. Bei den Kosmetika konnten wir in sechs von insgesamt 96 Proben alle wichtigen charakteristischen Fettsäuren nachweisen. Die Profile waren jedoch oft durch pflanzliche Inhaltsstoffe mit Phytosphingolipiden beeinflusst und somit weniger aussagekräftig als bei den Wurstwaren. Mit einigen Verbesserungen, v.a. bezüglich Extraktion und Quantifizierung, kann diese Methode für die Überwachung von gesetzlichen Bestimmungen bezüglich Nervengewebe in Fleischprodukten und Kosmetika eingesetzt werden.

Résumé

La méthode GC-MS décrite permet d'identifier les profils des acides gras spécifiques du cerveau dans les produits carnés et les cosmétiques. A l'aide du rapport des isomères cis/trans de l'acide nervonique, il a été possible dans le cadre des essais effectués sur du matériau de référence de différencier certaines espèces animales (bœuf, porc, mouton). La différenciation a également été possible jusqu'à une concentration de cerveau de 0,05 % à l'aide de produits carnés enrichis, pour lesquels la concentration initiale en tissus nerveux était inconnue. La limite de détection de la méthode, sans différencier l'espèce animale, est inférieure à 0,01 %. Concernant les cosmétiques, tous les acides gras caractéristiques ont été mis en évidence dans six échantillons (96 examinés au total). Les profils étaient cependant pour la plupart affectés par des ingrédients contenant des phyto-sphingolipides et par conséquent moins informatifs en comparaison avec les produits carnés. Avec quelques modifications, il est possible d'appliquer cette méthode GC-MS pour le contrôle du respect des dispositions légales concernant les tissus nerveux dans les produits carnés et les cosmétiques.

Key words

Meat products, Cosmetics, Sphingolipids, Fatty acid, BSE

References

1 Tierseuchenverordnung (SR 916.401). Eidg. Drucksachen- und Materialzentrale, Bern 1995.

2 Verordnung über Kosmetische Mittel, Anhang 1 (SR 817.042.1). Eidg. Drucksachen- und Materialzentrale, Bern 1998.

3 *Lücker, E., Eigenbrodt, E., Wenisch, S., Leiser, R.* and *Bülte, M.:* Identification of central nervous system tissue in retail meat products. J. Food. Prot. **63**, 258–263 (2000).

4 *Lücker, E., Eigenbrodt, E., Wenisch, S., Failing, K., Leiser, R.* and *Bülte, M.:* Development of an integrated procedure for the detection of central nervous tissue in meat products using cholesterol and Neuron-Specific Enolase as markers. J. Food. Protect. **62**, 268–276 (1999).

5 *Wenisch, S., Lücker, E., Eigenbrodt, E., Leiser, R.* and *Bülte, M.:* Detection of ventral nervous tissue in meat products. – An immunohistochemical approach – Nutrition Research **19**, 1165–1172 (1999).

6 *Lehninger, A.L.:* Biochemie. Verlag Chemie GmbH, Weinheim 1975.

7 *Christensen, M.M., Lund, S.P., Simonsen, L., Hass, U., Somonsen, S.E.* and *Hoy, C.E.:* Dietary structured triacylglycerols containing docosahexaenoic acid given from birth effect visual and auditory performance and tissue fatty acid profiles of rats. J. Nutrition **128**, 1011–1017 (1998).

8 *Frostegard, A., Tunlid, A.* and *Baath, E.:* Microbial biomass measured as total lipid phosphate in soils of different organic content. J. Microbiol. Methods **14**, 151–163 (1991).
9 *Zelles, L.* and *Bai, Q.Y.:* Fractionation of fatty acids derived from soil lipids by solid phase extraction and their quantitative analysis by GC-MS. Soil Biol. Biochem. **25**, 495–507 (1993).
10 *Wood, R.* and *Lee, T.:* High-performance chromatography of fatty acids. J. Chromat. **254**, 237–246 (1983).
11 *Müthing, J.* and *Kemminger, S.E.:* Nondestructive detection of neutral glycosphingolipids with lipophilic anionic fluorochromes and their employment for preparative high-performance thin-layer chromatography. Anal. Biochem. **238**, 195–202 (1996).

Corresponding author: Dr. Markus Niederer, Kantonales Laboratorium Basel-Stadt, Postfach, CH-4012 Basel, E-mail: markus.niederer@bs.ch