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SHORT COMMUNICATION

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GC-MS detection of central nervous tissues as TSE risk material in meat products: analytical quality and strategy

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Abstract The detection of central nervous system (CNS) tissue (i.e. brain and spinal cord) by the use of GC-MS and certain fatty acids (FAs) as their methyl esters (FAMEs) was previously shown to be a very promising approach towards identification of CNS tissue as a specified risk material (SRM) in meat products, contrasting available immunochemical methods. This GC-MS method promised to allow quantification of CNS material as low as 0.01%. Here, we show that the CNS-relevant FAMEs C22:6, C24:1ω9, C24:1ω7, C24:0 and C24-OH are present in pure muscle and adipose tissue samples in detectable amounts. Thus, limits of detection are not feasible as quality parameters in this analytical GC-MS approach. Instead, cut-off values have to be applied as calculated from the baseline content of the respective FAME in CNS-free samples and its variation for a given statistical security. Furthermore, the FAs used for quantification of the CNS showed distinct differences depending on species and age. This finding is in accordance with previous studies where it had been concluded that species and age differentiation of CNS might be possible with GC-MS. However, it was not taken into account that it also necessitates a strict analytical strategy for quantification of the CNS content: identification of the presence of CNS (step 1); identification of species and age (step 2); and quantification by use of a species- and age-specific CNS calibration (step 3). Differences between the FA content of the CNS used for calibrating and the CNS in the sample will cause up to fivefold deviation from the true CNS content. Our results show that the FA best suited for identification (step 1) and quantification (step 3) purposes is cerebronic acid C24-OH after silylation. Further in-depth studies are needed in order to elucidate variability of brain FA content and to determine analytical limits. However, the present GC-MS approach is already a highly promising supplement to existing immunochemical methods for the detection of traces of CNS material in meat products.

Keywords GC-MS · TSE · SRM · CNS · Fatty acids · Cerebronic acid

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Introduction

Official food control demands analytical methods for the detection of tissues of the central nervous system (CNS; i.e. brain and spinal cord) in meat products, considering the introduction of the new food labelling regulations [1] and concerns pertaining to a potential human exposure to the BSE agent via the food chain [2–4]. Since 1996, several analytical approaches have led to the commercial introduction of at least two immunochemical methods based on the detection of neuron-specific enolase (NSE) and glial fibrillary protein (GFAP) as indicators for CNS in meat products using western blotting [5, 6] and ELISA [7], respectively. These methods have been tested and validated in several studies with considerable success (e.g. see ref. [8]). They facilitate the detection of CNS tissue irrespective of species and age. The current legal definition of specified risk material (SRM) [4] implies, however, some specifications as pertaining to species and age (e.g. only CNS of bovine and ovine animals

older than 12 months). Immunochemical methods can be successfully applied in labelling law enforcement but not for identification of CNS as SRM in the legal sense. Thus, a method for the species- and age-specific identification is needed. Furthermore, an alternative method based on a non-immunochemical detection principle for reference purposes is needed. Finally, the use of heat-resistant markers for CNS tissue in such an alternative method would be advisable when considering the disadvantageous heat-induced losses of protein markers in immunochemical methods.

All of these three requirements were—tentatively—met by a recently published GC-MS approach [9, 10]. It was shown that certain fatty acids (FAs) of the sphingolipid fraction of the brain can be successfully used as markers for CNS tissue in meat products. Detection and quantification of brain in meat products were achieved by means of GC-MS following solid-phase extraction and derivatisation to methyl esters (FAMEs). Moreover, using the ratio of C24:1 isomers (C24:1ω9/C24:1ω7 [9, 11]), it was suggested that this analytical approach might facilitate the discrimination between CNS tissue of different animal species, in particular between ovine and bovine brain on the one hand and brain of pigs and poultry on the other hand. Moreover, this ratio proved to be age-dependent, thus suggesting a potential for age-specific discrimination between for example calf brain and cow brain. Detection limits for quantification of CNS tissue were reported to be as low as 0.01% (non-species specific) and 0.05% (including species differentiation); however, it was not reported how these limits of detection were statistically validated. Several follow up studies corroborated in principal the analytical potential of this GC-MS approach [10–12]. However, considerable uncertainty remains with regard to the method's potential for quantification of the CNS content. In particular, inconsistent results on FA baseline contents in muscle tissue and detection limits were reported.

In the present study, we compare the suitability of several FAs (C22:6, C24:1ω9, C24:1ω7, C24:0, C24-OH) as markers for CNS and show that cerebronic acid (C24-OH) is the best choice regarding identification and quantification of CNS, albeit with an additional silylation step needed. Our results also confirm that the C24:1ω9/ω7 ratio is most suitable for identification of the species and estimation of the age of the CNS tissue. Finally, we demonstrate that it is impossible to directly quantify the CNS content in meat products without first determining species and age of CNS tissue. Our finding that traces of the FAs were also detectable in muscle and adipose tissue necessitates the introduction of cut-off values and possibly a considerable up-correction of the achievable lowest limit of detection. Although more data are needed to determine the analytical limits, the GC-MS approach for the detection of traces of CNS tissue in meat products is ready for application in official food control as a supplement to existing immunochemical methods.

Materials and methods

Brains from pig, sheep and cattle were taken directly after slaughter in a local abattoir. The age of the animals at slaughter was 1 (turkey), 6 (pig), 9 (calf), 48 (sheep) and 61 (cattle) months, respectively. In sheep and cattle, the brains were officially tested negative for BSE. During testing the heads were stored at 4°C. Homogenisation was performed using a Retsch knife-mill Grindomix 200 (Haan, Germany). The material was stored in close-fitting PE vessels at −25°C until analysis. Materials not containing CNS tissue as used in this study were: porcine muscle (*M. longissimus dorsi*), adipose tissues (pure back fat) and filling (emulsion type sausage). Standard material of emulsion-type sausage filling was prepared containing 1 and 3% of bovine brain (8 months). For calibration purposes, emulsion-type sausage standards were produced using a previously reported recipe [5]; these contained 0, 0.1, 0.5, 1, 2, 4, and 10% bovine brain (24 months).

Acetone, *n*-hexane, methanol, methanolic HCl 1 N, 2-methyl-1-propanol, sodium chloride, sodium sulfate (anhydrous), pyridine, trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS), and triacontane as internal standard were supplied by Merck (Darmstadt, Germany); sea sand and 3-mL solid-phase extraction (SPE) tubes LC-Si containing 500 mg silica gel (Supelclean) were supplied by Supelco (Deisenhofen, Germany); the standards of the fatty acid methyl esters (FAMEs) of *cis*-15-tetracosenoic acid (C24:1ω9c), docosahexaenoic acid (DHA, C22:6), tetracosanoic acid (C24:0) and 2-hydroxy-tetracosanoic acid (C24-OH) were supplied by Larodan Fine Chemicals (Malmö, Sweden).

Lipid extraction from the sample matrix and derivatisation to FAME have been described in detail elsewhere [13, 14]. In brief, approximately 25 mg of homogenised brain tissue or a 300- to 500-mg sample sausage was ground with 3 g sodium sulfate (anhydrous) and 1 g sea sand and extracted by shaking with 5 mL *n*-hexane/2-methyl propanol (90/10, v/v) at 25°C over 30 min. A volume of 5 mL saturated NaCl solution was added and vortexed twice over 10 s. After separating the upper hexane phase the rest was treated with 3 mL *n*-hexane on a shaker over 10 min and, again, the upper phase was separated. Then, both hexane phases were mixed and dried with 200 mg sodium sulfate. This was followed by SPE in a vacuum manifold in an SPE tube 3 mL/500 mg silica gel. The first fraction containing mainly the neutral lipids was eluted using 5 mL *n*-hexane/2-methyl propanol (90/10, v/v) and discarded; the second and third fractions (3 mL acetone; 8 mL methanol) containing the complex lipids were mixed with 10 µg triacontane as internal standard, concentrated, and converted with 1 mL 1 N methanolic HCl at 60°C over 12 h into the methyl esters. The solution was cooled at room temperature and shaken after addition of 3 mL water and 2 mL *n*-hexane. The *n*-hexane fraction was

dried with anhydrous sodium sulfate. Aliquots were analysed directly by GC-MS.

Silylation to the trimethylsilyl ethers of the 2-hydroxy-tetracosanoic acid C24–OH was achieved by adding 0.5 mL of a mixture of pyridine, HMDS and TMCS 10:3:1 (v/v/v). The mixture was allowed to react for 5 min at room temperature and was then directly transferred to GC-MS analysis.

Gas chromatographic analyses were performed on an Agilent 6890 N gas chromatograph (Agilent Technologies Inc. Palo Alto, CA, USA) employing a fused silica capillary column HP-5MS 60 m×0.32 mm×0.25 μm (J & W Scientific from Agilent Technologies). The samples (1 μL) were injected in the splitless (1 min)/split mode using He as carrier gas at a constant flow of 1.5 mL min^{-1} and inlet temperature of 300°C. The column temperature was programmed at 90°C (1 min isotherm), 20°C min^{-1} to 140°C, 12°C min^{-1} to 260°C, and 2°C min^{-1} to 295°C, 4 min isotherm.

The MS analyses were performed using an Agilent MSD 5973 N quadrupole mass spectrometer (Agilent Technologies Inc. Palo Alto, CA, USA), which was operated in electron impact ionisation mode (EI) at 70-eV electron energy. Data acquisition was performed in SIM mode for FAME of C22:6 ($m/z = 91$), C24:1 ($m/z = 348$), C24:0 ($m/z = 382$), C24-OH ($m/z = 398$), C24:O–TMS (trimethylsilyl ether of C24-OH, $m/z = 411$), and for internal standard triacontane ($m/z = 85$). The temperature of the transfer line was 280°C; temperature of MS 250°C. MS data were acquired using ChemStation G 1701 CA (Agilent Technologies Inc. Palo Alto, CA, USA) and a Vectra P 2074 A computer (Hewlett Packard, Palo Alto, CA, USA).

Results and discussion

The resulting content of the FAs C22:6, C24:1ω9, C24:1ω7, C24:0, C24-OH of GC-MS analyses following derivatisation to FAMEs from brain and muscle tissue samples of different species is listed in Table 1. The FA contents we analysed agreed well with previous results [9–11]. However, we observed considerable variations of respective FA contents within different brains of the same species (and age group): in terms of relative standard deviation they ranged from 8.0% (C24:1ω9, adult bovine brain) to 21.2% (C24:1ω7, turkey brain). Thus, the data on the variability of the FA content will have to be extended considerably in order to evaluate the potential of the present analytical approach for species and age differentiation. However, the data clearly demonstrate that all of these FAs can be detected not only in brain but also in muscle and adipose tissue, albeit in very small amounts. The FAME content of non-CNS tissue ranged from 0.4 to 11.7 mg kg^{-1} (wet weight). The lowest baseline content was found for C24:1ω7 and C24-OH whereas those of C22:6, C24:1ω9, and C24:0 were distinctly higher. When comparing the FA content of CNS-free raw material (muscle and adipose tissue) with pure brain, cerebronic acid showed the by far highest ratio with a factor of more than 3,000. C24:1ω7, like all the other FA we studied, showed ratios ranging from 147 to 355, which are one order of magnitude lower than we obtained for cerebronic acid. First, we can conclude that cut-off values have to be preferred to detection limits when using these FAs as markers for CNS identification in meat products (Table 1). In addition to that, species- and age-dependent differences (see below) would considerably affect the detection limit, whereas the cut-off values, as determined in our study, showed no significant ($P < 0.01$) difference between species or age of the raw material. Even though the instrumental limits were as low as 0.01% CNS or even lower [9, 15], the practical limits ranged from 0.1 to 0.5% CNS (C24-OH, adult cattle brain) due to the FA baseline content in the non-CNS raw material. Secondly, we can conclude that cerebronic acid is by far the best choice for identification of CNS tissue in meat products when considering absolute amounts and distances to the baseline content of raw material. Although C24:1ω7 showed a comparably low baseline content in non-CNS samples and a slightly lower cut-off value then cerebronic acid, its CNS/baseline ratio is unfavourable.

Cerebronic acid showed, as was to be expected for all hydroxylated fatty acids in GC-MS analyses, a very pronounced tailing (Fig. 1, peaks A, B). In addition, the integrated peak area of the C24-OH FAME is considerably hindered by an interfering peak of the FAME C26:0, as shown in the 50-times y-axis amplification in Fig. 1 (inset, peak D). Notwithstanding the above-noted advantages of cerebronic acid as a marker for CNS tissue in meat products, observed variances in C24-OH analyses can be higher than in the other FAMEs, as was the case in the baseline analysis for cut-off value determination (Table 1). We further noted a distinct decrease in peak height accompanied by an increase of the tailing over time (Fig. 1, line A and B). It should be noted that this effect was hard to detect as it became apparent only

Table 1 Results of GC-MS quantification of selected FAMEs (mg kg^{-1}) in brains of different species (age in bovines; $n = 29$), in muscle/adipose tissue (bovine and porcine; $n = 22$), and ratio between brain (cattle) and raw material (muscle, adipose tissue)

Species, matrix	C22:6	C24:1ω9	C24:1ω7	C24	C24OH
Cattle, brain	3,730	3,270	136	1,470	1,820
Calf, brain	3,840	2,290	168	1,040	960
Sheep, brain	3,780	2,740	240	960	1,330
Pig, brain	3,310	2,530	660	950	1,440
Turkey, brain	2,890	1,750	382	620	470
Muscle, adipose tissue[a]					
MV	10.5	11.7	0.4	10.0	0.6
SD	8.3	8.7	0.3	7.7	0.7
Cut-off	29.83	32.01	1.08	27.83	2.34
Ratio cattle brain/ muscle, adipose tissue	355	279	340	147	3,033

[a] *mv* arithmetic mean value; *SD* standard deviation; *Cut-off* MV + 2.326×SD; statistical security 99%

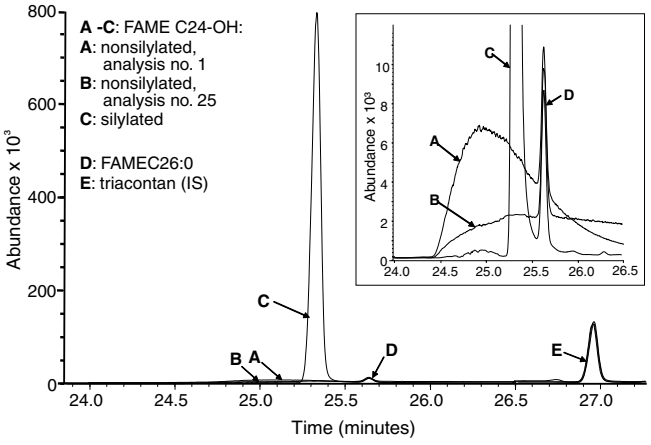

Fig. 1 GC-MS SIM chromatogram of the first *(A)* and twenty-fifth *(B)* analysis of non-silylated ($m/z = 398$) and silylated ($m/z = 411$) *(C)* FAME of cerebronic acid (C24-OH). *Inset* approximately 50-times enlargement of *y*-axis

after high numbers of repetition of a standard sample, whereas it remained unobserved in optimisation studies and routine analyses. All of these drawbacks could be solved by silylation (Fig. 1, peak C; Table 2). When considering the sometimes extreme differences in FAME content between brains of different species or even different ages (Table 1), it becomes apparent that it is impossible to quantify the CNS content without having some prior information about the species and age of the CNS present in the respective sample. An example of the errors introduced when not adhering to this principle is shown in Table 3. In worst cases the factor for the deviation from the true CNS content is nearly 5; this means that when a CNS calibration with C24:1ω7 from for example cattle brain is used to calculate the CNS content of a sample actually containing only 2% porcine brain, the result would be 10% CNS. Thus, we can conclude that quantification of the CNS content in meat products by GC-MS must adhere to a strict sequence of analytical steps: Firstly, the question whether CNS tissue is present in a sample or not has to be answered. It is obvious (Table 1) that C24-OH is best suited for that purpose as the distance to the baseline content is the largest among all tested FAMEs. Secondly, in case of a positive result, species and age of the CNS must be determined. For this purpose the ratio C24:1ω9/ω7 [11]—originally designated as the *cis*/*trans* ratio of nervonic acid [9, 10]—was shown to be the best choice, as the differences between species and age of the CNS was distinctly larger than in any other case. It might be possible to quantify the CNS content; however, the prerequisite is that we use a calibration with standards containing CNS of the adequate species and age. Thus, we have to establish the identity of the CNS, in terms of species and age, prior to its quantification. For the purpose of quantification of the CNS content cerebronic acid—as its trimethylsilyl ether—is best suited for the same reasons as given above.

Table 2 Comparison of results of GC-MS analyses of non-silylated ($n = 12$) and sylilated ($n = 6$) cerebronic acid (as the corresponding FAME) in standardized meat products containing 0, 1 and 3% cattle brain

CNS (%)		Non silylated		Silylated
		Sample 1–6	Sample 25–30	($n = 6$)
0	Peak height			
	MV	68	20	8,300
	Peak area			
	MV	21,583	12,216	306,856
	RSD (%)	12.3	8.8	7.0
1	Peak height			
	MV	1,900	550	284,000
	Peak area			
	MV	1,245,745	554,895	10,127,375
	RSD (%)	6.4	6.5	3.2
3	Peak height			
	MV	6,800	2,400	812,000
	Peak area			
	MV	4,571,289	2,509,829	29,390,451
	RSD (%)	3.4	2.4	3.5

MV arithmetic mean value, *RSD* relative standard deviation

Table 3 Factors of deviation from the true CNS (brain) content of samples as a function of calibration with CNS (brain) from different species (and age in bovines) using C24:1ω7

Sample	Calibration				
	Cattle	Calf	Sheep	Pig	Turkey
Cattle	1.00	0.81	0.57	0.21	0.36
Calf	1.24	1.00	0.70	0.25	0.44
Sheep	1.76	1.43	1.00	0.36	0.63
Pig	4.85	3.93	2.75	1.00	1.73
Turkey	2.81	2.27	1.59	0.58	1.00

Conclusion

Overall, GC-MS is a sound method for the detection of CNS tissue in meat products. It shows the potential to become a reference method supplementing immunochemical methods in official food control. The fact that profiling FAs could facilitate species and age differentiation of traces of CNS in meat products is highly fascinating. It stands in line with innovative analytical approaches in microbiology using lipid biomarker profiling [16]. Our results demonstrate the necessity to respect some inherently limiting characteristics of this attractive analytical approach. Further extensive studies, using suitable experimental models and statistical approaches, are needed to elucidate the limits of species- and age-specific detection and quantification of traces of CNS tissue in meat products.

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