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ORIGINAL PAPER

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Structural characterisation of some fatty acids from the brain as biomarkers of BSE risk material

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Abstract Identification of bovine and ovine tissue from the central nervous system (CNS: brain and spinal cord) in meat products is possible by using certain CNS fatty acids as biomarkers in GC–MS analysis. Furthermore, the relationship between the isomers of the tetracosenic acid (C24:1) is important for differentiation of the species and age of the CNS in view of the legal definition of specified risk material (SRM). This has so far been referred to as the "*cis*/*trans* ratio of the isomers of nervonic acid"; however, structural analysis was not performed. Here we present results from GC–MS structural analysis by retention time and DMDS adduct profiling of the even numbered monoenoic fatty acids from C18:1 to C26:1. Retention times and mass spectra of the FAME standards indicated that the so far designated *trans*-nervonic acid has a different isomeric structure in the tetracosenic acid from brain-sample extracts. By performing GC–MS analysis of DMDS adducts we have shown that this isomer was actually *cis*-17-tetracosenic acid in all species so far tested, not *trans*-15-tetracosenic acid (*trans*-nervonic acid). The tetracosenic acid isomer ratio proved to be species-specific in accordance with previous results. Thus, instead of the ratio of *cis*/*trans* isomers of nervonic acid, the ratio of $\omega 9/\omega 7$-tetracosenic acid (15c-C24:1/17c-C24:1) will have to be used as a correct reference in future publications. Although *trans* isomers were not detectable in sheep and cattle brain, porcine brain contained, in addition to *cis*-17-tetracosenic acid, small amounts of the *trans* isomers of the C18:1, C20:1, C24:1, and C26:1 fatty acids, in decreasing quantities. In future, this might be useful as another means of differentiation between porcine CNS (non-SRM) and ovine or bovine CNS (SRM). Extensive follow-up studies must be performed to elucidate the extent to which this GC–MS approach will facilitate the detection of CNS according to the legal SRM definition.

Keywords BSE · SRM · CNS · GC–MS · Fatty acids · Nervonic acid · Structural analysis

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Introduction

In 1996 the emergence of a new variant of Creutzfeldt–Jakob disease was closely connected with the exposure of consumers to bovine risk materials, especially brain and spinal cord (central nervous system, CNS, tissue) via the food chain [1—4]. Since then, methods for detection of CNS tissue in food products have been developed to elucidate and control food-borne human BSE-exposure risk [5—10]. These methods are predominantly based on immunochemical detection of certain CNS-specific proteins, such as neurone specific enolase (NSE) and glial fibrillary acidic protein (GFAP). In particular, two test kits based on NSE western blotting and GFAP ELISA have been shown to be highly applicable in official food control and have been internationally validated in several studies [11—14].

However, these immunochemical procedures suffer from some drawbacks. First, results are only tissue-specific whereas the legal definition of specified risk material (SRM) refers not only to tissue (such as brain and spinal cord) but also to animal species (only bovine, ovine, and caprine) and to the age of the animal (e.g. over 12 months for CNS tissues). Second, protein biomarkers are prone to losses during meat technological

processing, in particular heat treatment. Third, quantification of the CNS content is hard to achieve, especially as the extent of technologically induced partial loss of protein marker is unknown [13]. Furthermore, an additional method for validation of positive results, preferably based on a different analytical principle, is missing.

It was recently shown that certain fatty acids of the CNS could be used as stable biomarkers in GC–MS analysis of food products [15—19]. These fatty acids, which are present in the CNS as complex shingolipids and cerebrosides, were detected and quantified by means of GC–MS after extraction (solvent, solid phase extraction (SPE)), derivatisation to methyl esters (FAME) and, recently, silylation [17].

Overall, the analytical potential of this novel approach was judged to be very promising in terms of specificity, sensitivity ($< 0.01\%$ CNS), stability of markers, and practicability. It was also shown that the ratio of two isomers of C24:1—denoted the *cis*/*trans* ratio of nervonic acid—differed substantially between animal species and ages. Thus, it was hypothesised that the GC–MS approach would not only facilitate the detection of CNS in meat products but would also give information on the species and age of the CNS in accordance with the legal definition of SRM [15—19]. In these publications the two C24:1 isomers were designated according to their retention times and mass spectra as ''*cis*- and *trans*-nervonic acid'' (i.e. *cis*-15- and *trans*-15-tetracosenic acid). In contrast with that, a publication from 1963 showed the presence of only *cis*-tetracosenic acid in porcine brain [20].

Although the greatest importance is ascribed to the isomers of tetracosenic acid for detection of CNS as SRM, their true chemical nature is not clear. Thus, the aim of our study was to characterise the structure of these specific fatty acids of the brain. In particular, we show that C24:1 isomers are present in bovine and ovine brain as *cis*-ω9-tetracosenic acid (15c-C24:1) but not as *trans*-ω9-tetracosenic acid (15t-C24:1), as has been taken for granted until now; instead, this second isomer was identified as *cis*-ω7-tetracosenic acid (17c-24:1).

Material and methods

Whole brain tissue homogenates

Brains from pig, sheep, and cattle were taken directly after slaughter in a local abattoir. The age of the animals at slaughter was 6, 60, and 61 months, respectively. The brains of sheep and cattle had been officially tested and shown to be negative for BSE. During testing the heads were stored at 4 °C. The material was handled and destroyed after analysis in accordance with current SRM legislation [3] and occupational/laboratory safety rules. 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.

Chemicals

Acetone, diethyl ether, dimethyl disulfide (DMDS), *n*-hexane, iodine, methanol, methanolic HCl 1 mol L^{-1}, 2-methylpropanol, sodium chloride, sodium hydrogen carbonate, sodium sulfate (anhydrous), and sodium thiosulfate were supplied by Merck (Darmstadt, Germany). Sea sand and 3-mL SPE tubes containing 500 mg silica gel (Supelclean) were supplied by Supelco (Deisenhofen, Germany). Standards of the fatty acid methyl esters (FAME) of *cis*-15-tetracosenic acid (C24:1ω9c) and *trans*-15-tetracosenic acid (C24:1ω9t) were supplied by Larodan Fine Chemicals (Malmö, Sweden).

Preparation of FAME

Extraction of lipids from the sample matrix and their derivatisation to FAME have been described in detail recently [18, 21]. In brief, 25 mg homogenised brain tissue was ground with sodium sulfate (anhydrous) and sea sand in a mortar and then twice extracted by shaking with *n*-hexane/2-methylpropanol (9:1, v/v) at 25 °C over 30 min. This was followed by SPE in a vacuum manifold on silica gel. The neutral lipid fraction was discarded; the fraction containing the complex lipids was converted with 1 mol L^{-1} methanolic HCl at 60 °C over 4 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 washed with 1 mL 5% aqueous sodium hydrogen carbonate solution, washed again with 1 mL water, separated and dried with anhydrous sodium sulfate. Aliquots were analysed directly by GC–MS or further treated with DMDS.

Derivatisation using DMDS

Derivatisation of the FAME with DMDS was performed according the procedure reported in detail by Scribe et al. [22]. In brief, 10 μL of each of the FAME standards (1 mg mL^{-1}) was mixed in a 1.5 mL vial with 100 μL *n*-hexane, 20 μL iodine solution (60 mg mL^{-1} ether) and 100 μL DMDS. This mixture was incubated at 50 °C for 48 h. Then 200 μL *n*-hexane and 100 μL of a 5% aqueous solution of sodium thiosulfate were added and vortex mixed until decolouration. The upper phase was separated and dried with anhydrous sodium sulfate. An aliquot was than analysed immediately by GC–MS. The FAME from raw extracts of porcine, bovine, and ovine brain samples (25 mg each) were treated accordingly, however, using five times the amounts of chemicals given above.

Determination by GC–MS

GC analyses were performed on an Agilent 6890 N gas chromatograph (Agilent Technologies, Palo Alto, CA,

USA) employing, if not otherwise specified, a 60 m×0.32 mm×0.25 μm HP-5MS fused-silica capillary column (J&W Scientific from Agilent Technologies). The samples (1 μL) were injected in the splitless (1 min)/split mode using helium as carrier gas at a constant flow of 1.5 mL min^{-1} and an inlet temperature of 300 °C.

The column temperature was maintained isothermal at 90 °C for 1 min then programmed at 20 ° min^{-1} to 140 °C, at 12 ° min^{-1} to 260 °C, at 2 ° min^{-1} to 295 °C, and at 5 ° min^{-1} to 320 °C which was held for 9 min isothermal. MS analysis was performed using an Agilent MSD 5973 N quadrupole mass spectrometer operated in electron-impact (EI) ionisation mode. Electron energy was 70 eV. Data acquisition was performed in scan mode in the mass range from 35 to 550 amu, if not otherwise reported. The temperature of the transfer line was 280 °C and the temperature of the MS was 250 °C. MS data were acquired using Agilent ChemStation G 1701 CA and Computer Vectra P 2074 A (Hewlett Packard, Palo Alto, CA, USA).

In the following text reference is made to the positional isomers of $\omega 9/\omega 7$ fatty acids. As this relationship cannot be easily generalised in IUPAC nomenclature and in view of previous references we will apply the classic $\omega 9/\omega 7$ nomenclature throughout this paper.

Results and discussion

Retention times and mass spectra of C24:1 isomers

In the first approach we compared retention times of the FAMEs of pure *cis*-ω9-tetracosenic acid (C24:1ω9c or 15c-C24:1) and *trans*-ω9-tetracosenic acid (C24:1ω9t or 15t-C24:1) standard substances with the FAMEs extracted from brain samples. Figure 1 shows the respective section from the gas chromatograms for the two certified standards (dotted line) and porcine brain (continuous line) as recorded using an HP1 capillary column (30 m × 0.25 mm × 0.25 μm). We noted a slight but clearly reproducible difference in retention times in one of the FAME standards and the respective FAME from brain samples. Whereas the first peak of the standard C24:1ω9c and of the sample were identical, the second peak of the standard, i.e. C24:1ω9t, consistently had a shorter retention time than the respective peak from the sample.

The difference between the retention times for the C24:1ω9t standard and the porcine brain sample extract (Fig. 1) was substantially enhanced when using a weakly polar column (HP5, 60 m × 0.32 mm × 0.25 μm) as shown in Fig. 2. In addition to the peak from porcine brain, the respective peaks from bovine and ovine brain were also recorded under the same conditions. For all species the retention times of the first FAME peak were found to be identical with that of the C24:1ω9c standard. The retention times of the second peak of all brain samples were identical but were significantly longer than the retention time of the peak as obtained for the C24:1ω9t FAME standard. Figure 2 also demonstrates that the height of the second peak in relation to that of the first peak C24:1ω9c differs largely between species. On this basis it was shown that, in principle, species and age differentiation for brain in meat products could be achieved in accordance with the legal definition of SRM [15, 16]. As a conclusion from our results, however, we could no longer identify the second FAME as C24:1ω9t with certainty. There is a clear and consistent difference between this CNS-specific FAME and the FAME of pure C24:1ω9t.

In addition, comparison of the results we obtained for the certified standards of the FAMEs of C24:1ω9c and C24:1ω9t with data from the literature [23] also indicated that, besides C24:1ω9c, this important second fatty acid in the brain of pigs cannot be identical with C24:1ω9t (data not shown).

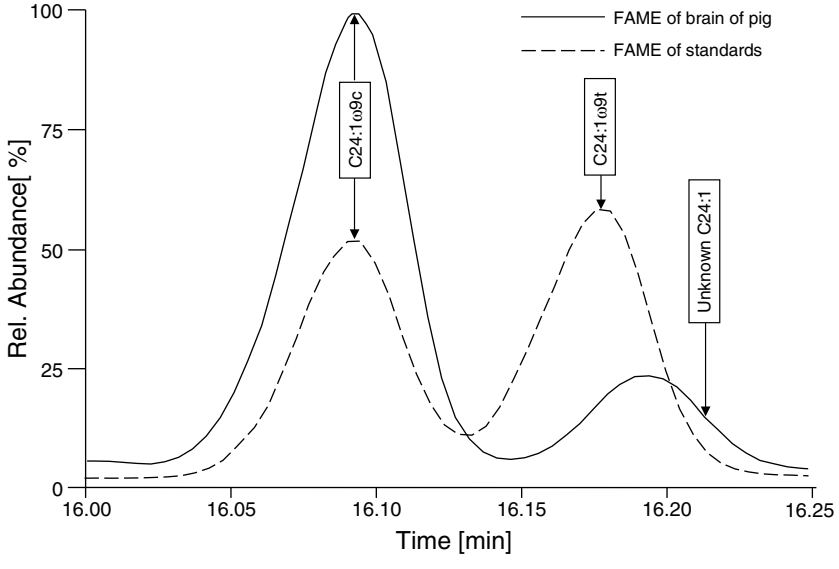

Fig. 1 Gas chromatograms of FAME C24:1 from porcine brain (*continuous line*) and certified standards C24:1ω9c and C24:1ω9t (*dashed line*). Fused-silica capillary column HP1 30 m × 0.25 mm × 0.25 μm

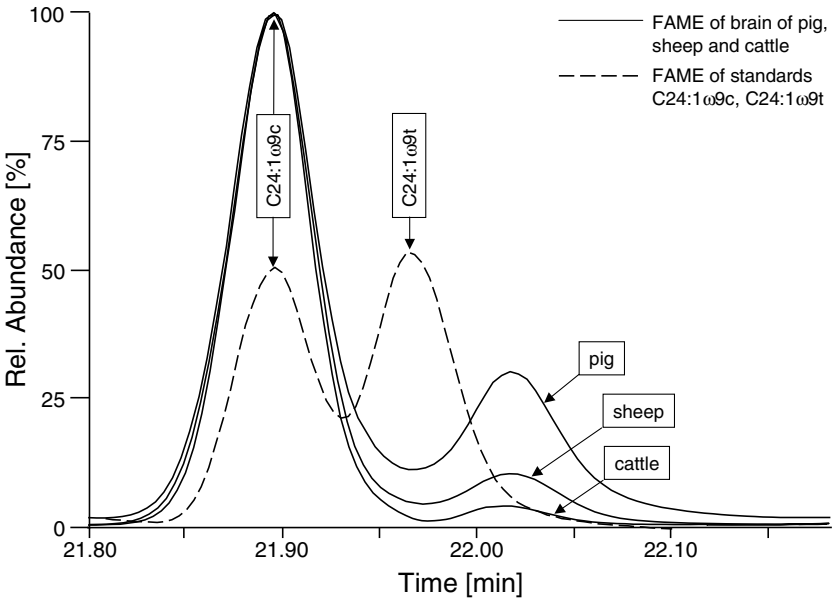

Fig. 2 Gas chromatograms of FAME C24:1 from porcine, ovine, and bovine brain (*continuous line*) and certified standards C24:1ω9c and C24:1ω9t (*dashed line*). Fused-silica capillary column HP5 60 m × 0.32 mm × 0.25 μm

DMDS derivatives of FAMEs of C24:1 isomers

To identify the true nature of this second C24:1 isomer it was necessary to analyse its chemical structure. Potential candidates were isomers of C24:1 as branched fatty acids, cyclopropane fatty acids, or, more probably, isomers with differences in the position of the double bond. The common strategy for determination of the position of the double bond is the combined application of chemical, chromatographic, and spectrometric analyses [24]. Good results can be obtained by combination of GC and ozonolysis where characteristic degradation products are obtained [20]. Highly reliable results can be obtained when determining the position of the double bond by analysing the dimethyl disulfide derivatives (DMDS) of FAME [22].

Using ozonolysis and gas chromatography Kishimoto and Radin [20] found, without exception, even-numbered monoenoic fatty acids as isomers with the double bond in positions ω9 and ω7. The main fraction was represented by the two fatty acids C24:1ω9 and C24:1ω7. The fatty acids C22:1 and C26:1, both present at a level of about 8%, were also detected exclusively as positional isomers, with the double bond either in position ω9 or ω7.

In all previous studies on the detection and characterisation of CNS in meat products using GC–MS and nervonic acid isomers as biomarkers, the possible presence of positional isomers has not been taken into account. Instead, the presence of *cis*/*trans*-isomers was exclusively postulated [13, 15—19, 21].

Based on our own findings and the results of Kishimoto and Radin [20], we tried to elucidate the chemical nature of bovine, ovine, and porcine CNS C24:1, and other isomers of fatty acids by analysing their structure by applying a procedure of DMDS derivatisation according to Scribe et al. [22] which we slightly modified.

During the reaction of the FAME of monoenoic fatty acids with DMDS, which is catalysed by iodine, a methyl sulfide group is added to each C-atom of the double bond, saturating the respective π-electron pair. Such DMDS adducts are heat-resistant and thus accessible for GC analysis; they elute at retention times distinctly longer than those of the original FAMEs. During MS the C–C bond between the methyl sulfide groups is specifically broken, generating three typical mass fragments which characterise the position of the original double bond. In addition, the molecular peak will also be present in all mass spectra [22].

The formation and decomposition of DMDS adducts of tetracosenic acid C24:1ω9c and tetracosenic acid C24:1ω7c are illustrated in Figs. 3 and 4, respectively.

Typical key ions, which have to be expected from the mass spectrum of the C24:1ω9c DMDS adduct, are the ions 474 (molecular ion), 173 (alkyl ion), 301, and 269 (ester ion). For C24:1ω7c (Fig. 4) we can expect the masses 474, 145, 329, and 297.

The schemes as shown in Figs. 3 and 4 can also be applied to the degradation of other DMDS derivatives of homologous FAME. The mass fragments expected in the mass spectra of DMDS adducts of FAMEs of monoenoic ω7 and ω9 fatty acids are listed in Table 1.

In the following text the decomposition of the DMDS adducts of C24:1 FAME from porcine brain is used as an example to illustrate the interpretation of the GC and GC–MS results depicted in Figs. 5a–d.

GC and GC–MS results as obtained for the decomposition of DMDS adducts of the FAME of the standards C24:1ω9c and C24:1ω9t are shown in Fig. 5e–g.

In the single-ion chromatogram for mass fragment 173 from porcine brain we obtained a main fraction and a secondary minor fraction eluting at 35.10 and 35.23 min, respectively (Fig. 5a). According to the MS results for the standards of C24:1ω9c and C24:1ω9t

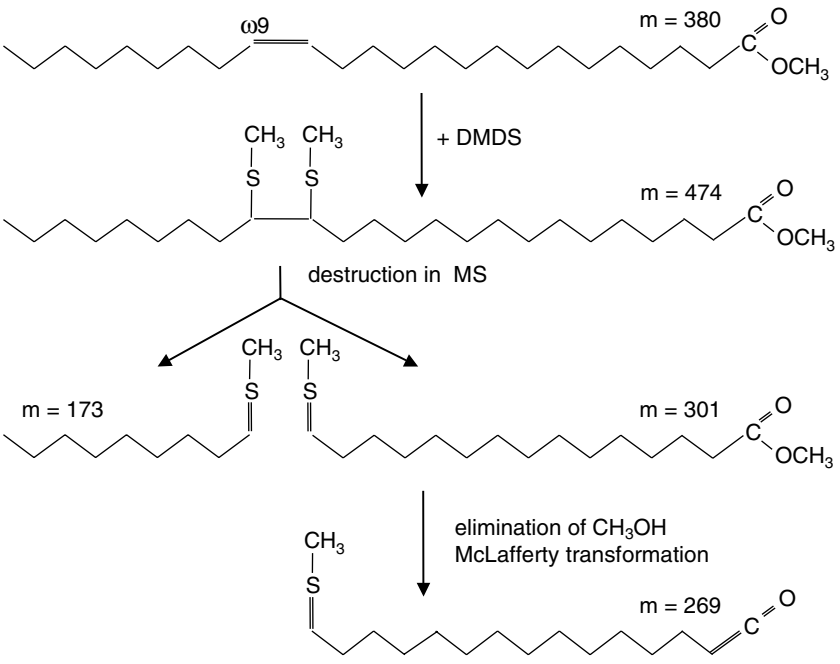

Fig. 3 Formation and decomposition of DMDS derivatives of FAME C24:1ω9c

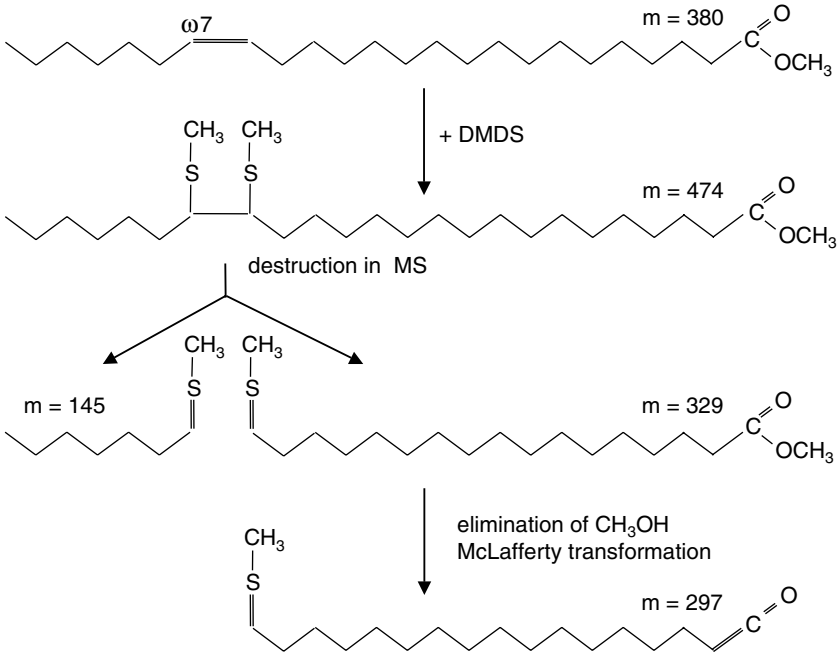

Fig. 4 Formation and decomposition of DMDS derivatives of FAME C24:1ω7c

(Fig. 5e) the first peak in Fig. 5a can be identified as C24:1ω9c and the second peak as C24:1ω9t. The decomposition in the MS of both of these *cis*/*trans*-isomeric standards produced the same mass fragments, namely, 173, 269, 301,and 474 (Figs. 5f, g). This was also true for the C24:1ω9 FAMEs from porcine brain as illustrated in Fig. 5b.

In the single-ion chromatogram for the mass fragment 145, which is characteristic of ω7 FAMEs, we obtained a well pronounced peak at 35.26 min (Fig. 5c). In this case we found mass fragments 145, 297, 329, and 474 characteristic of C24:1ω7 FAMEs (Fig. 5d). These mass fragments were to be expected according to the theoretical approach based on the data given in Table 1. Overall, our results strongly indicate the presence of *cis*-ω9-tetracosenic acid (C24:1ω9c) and *cis*-ω7-tetracosenic acid (C24:1ω7c) in porcine brain.

DMDS derivatives of FAMEs of some other fatty acids

In view of the presence of other isomers of monoenoic even-numbered fatty acids in the brain besides the C24:1 isomers, we also considered the DMDS adducts of the

Table 1 FAME of monoenoic fatty acids $\omega 9$ and $\omega 7$, DMDS derivatives, and expected mass fragments in mass spectrometry

FAME	MW		$\omega 7$ (MW of mass fragments)		$\omega 9$ (MW of mass fragments)	
	FAME	DMDS derivative	Methyl end group	Ester end group	Methyl end group	Ester end group
18:1	296	390	145	245 (213)	173	217 (185)
19:1	310	404	145	259 (227)	173	231 (199)
20:1	324	418	145	273 (241)	173	245 (213)
21:1	338	432	145	287 (255)	173	259 (227)
22:1	352	446	145	301 (269)	173	273 (241)
23:1	366	460	145	315 (283)	173	287 (255)
24:1	380	474	145	329 (297)	173	301 (269)
25:1	394	488	145	343 (311)	173	315 (283)
26:1	408	502	145	357 (325)	173	329 (297)

MW molecular weight, FAME fatty acid methyl ester, DMDS dimethyl disulfide

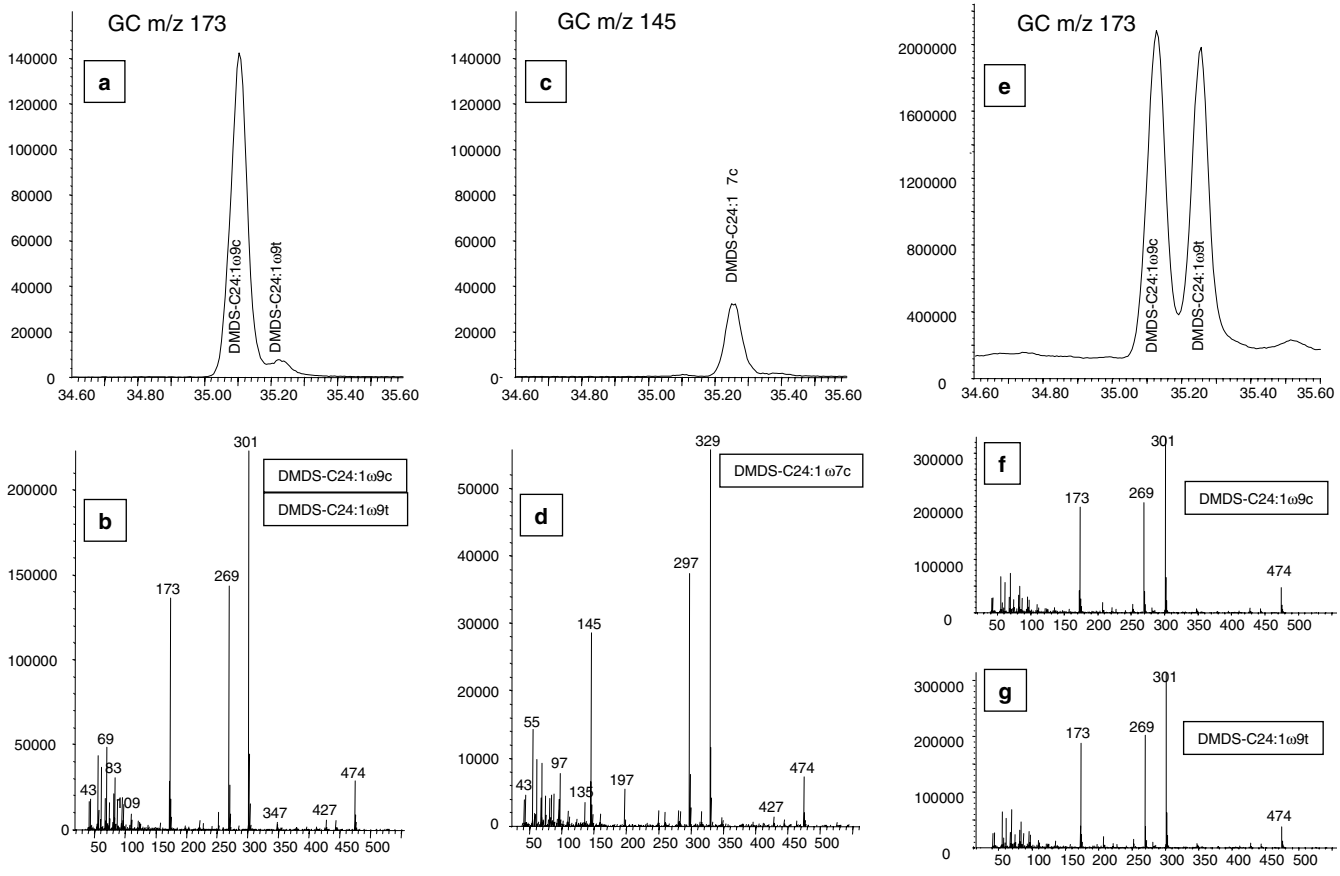

Fig. 5 Gas chromatograms and mass spectra of DMDS derivatives of porcine FAME C24:1 (**a–d**) and standards C24:1ω9c and C24:1ω9t (**e–g**). **a, c** Gas chromatograms of C24:1ω9 (MZ 173) and of C24:1ω7 (MZ 145). **b, d**: Mass spectra of C24:1ω9 and of C24:1ω7. **e** Gas chromatogram of C24:1ω9c and of C24:1ω9t (MZ 173). **f, g** Mass spectra of C24:1ω9c and of C24:1ω9t

C18:1, C20:1, and C26:1 fatty acids in this study. In particular we concentrated on the $\omega 9/\omega 7$ isomers according to the results of Kishimoto and Radin [20], which indicated a predominance of these isomers. As an example Fig. 6 shows the respective single-ion chromatograms and mass spectra of the DMDS FAME adducts in the range from C18:1 to C26:1 from the brain of pig (data for sheep and cattle are not shown). The results for the fatty acid C18:1 in porcine brain (Fig. 6) demonstrate the presence of all combinations of $\omega 9$, $\omega 7$, *cis* and *trans* isomers: C18:1ω9c (oleic acid), C18:1ω9t (elaidic acid), C18:1ω7c (*cis*-vaccenic acid), and C18:1ω7t (*trans*-vaccenic acid). Consistently, the *cis* isomers were eluted before the *trans* isomers; *trans* isomers were also found for the C20:1 and for the C24:1 fatty acids, although in smaller amounts. No *trans* isomer was detected in the C26:1 fatty acid.

We failed to detect any *trans* isomer in the ovine and bovine brain (data not shown). In addition, we noted that the ratio of C24:1ω9c to C24:1ω7c fatty acids was substantially greater than in the porcine brain, in accordance with our previous results as depicted in Fig. 2.

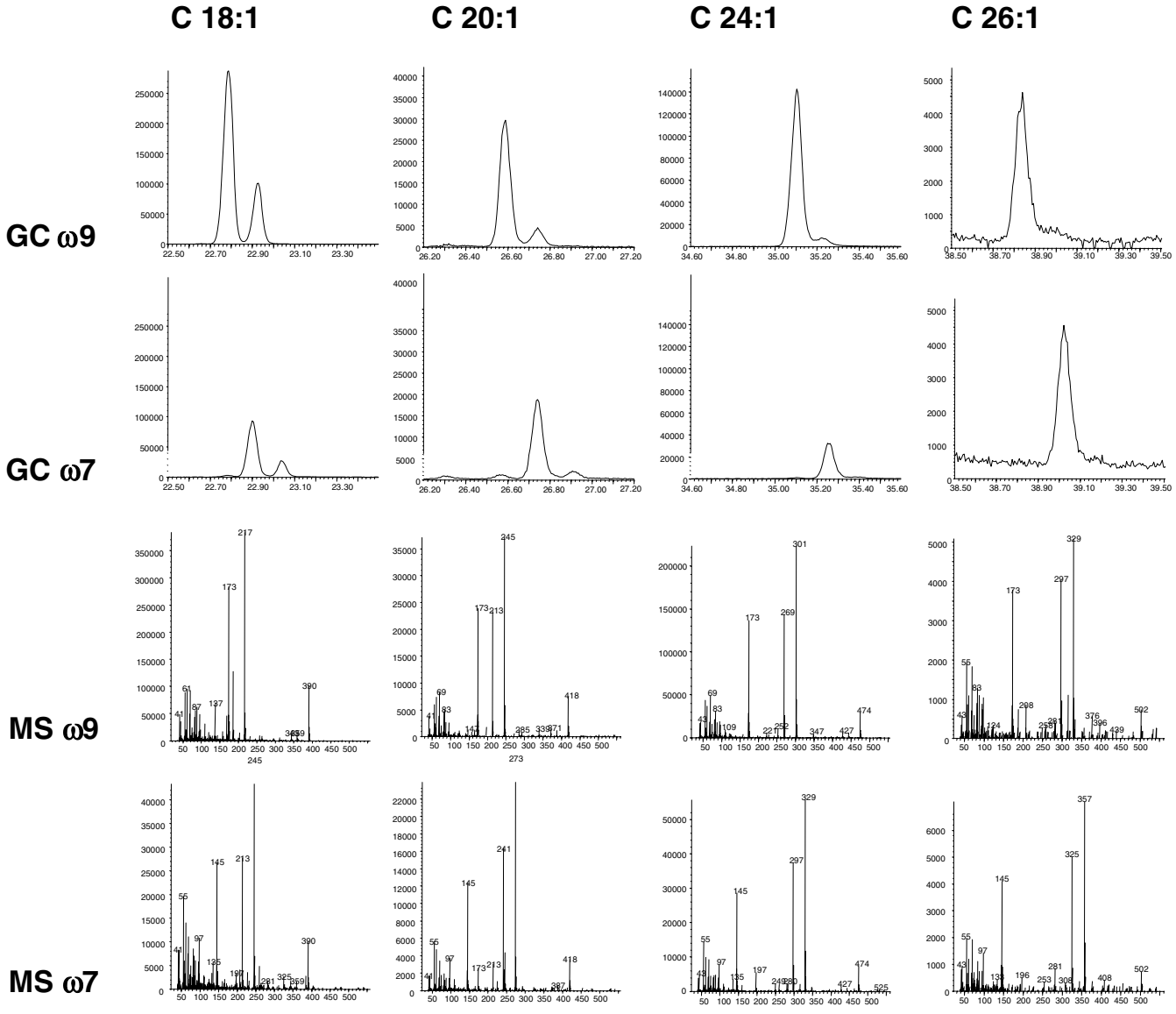

Fig. 6 Porcine brain. Gas chromatograms and mass spectra of DMDS derivatives of ω9 and ω7 isomers of FAME C18:1, C20:1, C24:1, and C26:1

A summary of these results is given in Table 2. For all species C18:1 is the dominating fatty acid comprising approximately 47 to 73% of the sum of all acids. The fatty acids C24:1ω9c (19–27%) and C20:1ω9c (6–13%) were also found in substantial amounts. The *trans* fatty acids in porcine brain are predominantly elaidic acid C18:1ω9t (16%) and *trans*-vaccenic acid C18:1ω7t (11%); *trans* isomers of the other fatty acids were detected in only very small amounts (up to 1%). The ratio C24:1ω9/C24:1ω7 fatty acids, as calculated from the integrated peak areas of the mass fragments 173 and 145, was 35:1 in the bovine brain, 15:1 in the ovine brain, and only 4:1 in the porcine brain. In principle this is consistent with our findings as depicted in Fig. 2. It is also consistent with previous results reported in literature where, however, the isomers were incorrectly identified as *cis*- and *trans*-nervonic acid [13, 15—19].

Overall, it can be concluded that the brains of sheep and cattle contain monoenoic fatty acids 18:1 to 26:1 as ω9 and ω7 positional isomers but not as *cis*/*trans* isomers. The importance of this ω9-/ω7-C24:1 isomeric relationship for discerning between the animal species of the CNS tissue detected in meat products necessitates correct nomenclature when considering the legal definition of specified risk material. In view of our findings future reference should not be made to the ratio *cis*-*trans* isomers of nervonic acid but to the ratio of *cis*-ω9-/*cis*-ω7-tetracosenic acid (C24:1ω9c/C24:1ω7c or 15c-C24:1/17c-C24:1).

This analytical GC–MS approach offers potential advantages over established immunochemical methods. It might indicate the presence of SRM, i.e. CNS from particular species of a specific age. It can, moreover, be expected that the fatty acids are pronouncedly more

Table 2 Monoenoic fatty acids from porcine, ovine, and bovine brain

FA	Cattle brain			Sheep brain			Pig brain		
	$\omega9/\omega7$[a]	$\omega9$ (%)	$\omega7$ (%)	$\omega9/\omega7$[a]	$\omega9$ (%)	$\omega7$ (%)	$\omega9/\omega7$[a]	$\omega9$ (%)	$\omega7$ (%)
17:1c	1.4	0.2	0.7	5.8	0.3	0.3	ND	ND	ND
18:1c	5.2	57.2	72.8	5.8	65.0	69.5	3.2	47.3	47.0
18:1t	ND	ND	ND	ND	ND	ND	4.4	15.6	11.2
19:1c	ND	0.1	ND	4.9	0.1	0.1	ND	ND	ND
20:1c	9.4	12.6	9.0	6.8	8.7	8.0	1.6	5.5	11.1
20:1t	ND	ND	ND	ND	ND	ND	2.5	0.8	1.1
21:1c	ND	0.2	ND	8.0	0.2	0.1	ND	ND	ND
22:1c	5.2	1.9	2.5	3.2	2.4	4.5	0.8	1.9	7.4
22:1t	ND	ND	ND	ND	ND	ND	ND	0.2	ND
23:1c	4.1	0.2	0.3	3.7	0.6	0.9	ND	ND	ND
24:1c	34.9	22.5	4.3	14.7	18.7	7.9	4.3	25.6	18.9
24:1t	ND	ND	ND	ND	ND	ND	ND	1.5	ND
25:1c	10.5	3.1	1.9	11.1	3.0	1.7	3.7	0.7	0.7
26:1c	1.5	1.9	8.5	1.0	1.1	7.0	1.0	0.9	2.7
27:1c	ND	0.2	ND	ND	ND	ND	ND	ND	ND
Sum	ND	100.0	100.0	ND	100.0	100.0	ND	100.0	100.0

ND—not detected
[a]calculated from areas of mass 173/mass 145 in gas chromatogram

heat-stable as CNS biomarkers than the proteins (NSE, GFAP) used in immunochemical methods. Drawbacks of the GC–MS approach are high cost, long duration, and the sophisticated nature of the analyses. Thus, GC–MS might be suitably applied in future food monitoring to confirm CNS-positive samples from immunochemical screening and to narrow down the possibility of the presence of SRM. Prior to its application in routine food monitoring as a confirmatory or even reference method, however, extensive follow-up studies will be necessary to validate the species and age specificity of CNS detection in meat products by means of GC–MS.

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