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# Development of a supercritical fluid extraction method for determination of lipid classes and total fat in meats and its comparison with conventional methods

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**Abstract**

A method has been developed for the determination of total fat and lipid classes in meat using supercritical fluid extraction (SFE). The results agreed well with results from the conventional Bligh and Dyer and Schmid, Bondzynski and Ratzlaff extraction methods. SFE has advantages compared to the latter methods of a low consumption of hazardous organic solvents and shorter extraction time. After investigation of several different conditions, the most rapid extraction was achieved by adding 1 ml of cyclohexane to a 0.5-g sample mixed with 1 g Hydromatrix in a 7-ml thimble. The optimized SFE parameters were: 370 bar, 50°C, 8% ethanol modifier, 4 ml/min dynamic flow for 30 min and collection with a tube leading to a vial.


*Keywords:* Meat; Extraction methods; Lipids; Fat

## 1. Introduction

The lipid group consists of many types of lipids with different chemical composition. They are insoluble in water but soluble in solvents such as chloroform, diethyl ether and heptane.

Various extraction techniques have been used in methods for the analysis of lipids in meat. Soxhlet [1] extraction with diethyl ether is the most common but if the food sample contains lipids bound to membranes, this method cannot be used directly since the lipids need to be released using acid hydrolysis. Thus it is important to know exactly how the lipids are present in the sample before a suitable extraction method is selected [2]. The most widely used method in Sweden for determination of the total fat content in meat and meat products is based on the work of Schmid, Bondzynski and Ratzlaff (SBR) [3–5]. This method includes an acid hydrolysis step, before extraction with a mixture of diethyl ether and petroleum ether [6]. Other methods, which use acid hydrolysis to give the total fat content, are the Weibull [7], Stolth [8] and European Union [9] methods. With the acid treatment there is a breakdown of the lipids in the sample to more polar substances. Extraction with polar solvents is needed [10–12]. In the widely used Bligh and Dyer (B&D) method the sample is first extracted in a one-phase solvent system and thereafter in a two-phase system after addition of chloroform and water, where the lipids remain in the organic phase [11]. There are problems associated with these conventional extraction techniques. They are labour intensive, time-consuming, difficult to automate, use toxic solvents and often require a post-extraction clean-up.

SFE is gaining increasing interest as an alternative

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sample work-up technique. Liquid-like solvating capabilities combined with almost gas-like transporting properties enable fast and efficient extraction of the target analytes. The merits of the technique for lipid extraction have been thoroughly reviewed [13–23]. Carbon dioxide has almost exclusively been used as the extraction fluid, due to its inertness and non-toxic properties. The solvent power is easily tuned by varying the density and temperature. If required, the polarity of the fluid can be changed by adding polar modifiers.

The aim of this study was to investigate the possibility of replacing the Bligh and Dyer extraction with SFE for the determination of lipid classes in meat and meat products and to outline the potential to also use SFE for total fat determination.

## 2. Experimental

### 2.1. Instrumentations

A Hewlett–Packard 7680T (Wilmington, DE, USA) supercritical fluid extractor was used equipped with 7-ml thimbles as extraction cells and a Hewlett–Packard 1050 pump for modifier delivery. The system was controlled by Windows-based software (Hewlett–Packard). The analytes were trapped on octadecylsilica (ODS) (Hewlett–Packard) trap or directly in tubes as shown in Fig. 1.

The TLC-plates were scanned on an imaging densitometer Model GS-670 (Bio-Rad, Hercules, CA, USA) and the spots were quantified with molecular analyst software, version 1.2 (Bio-Rad).

### 2.2. Materials

Thin-layer plates precoated with silica gel 60 were obtained from Merck (Darmstadt, Germany), Hydromatrix and glass fibre filters were obtained from Metric (Stockholm, Sweden); (Munktell MGC), glass filters (G3), aluminium foil and extraction tube were from Kebo Lab. (Spånga, Sweden). Carbon dioxide from Air Liquide (Malmö, Sweden) was used as Cryo gas (grade: Aligal) and extraction gas (grade: N48). Ethanol (95%) were from Kemetyl (Haninge, Sweden), cyclohexane, methanol, chloroform, diethyl ether, hydrochloric acid, petroleum ether, acetone and resublimated iodine were of analytical grade and came from Kebo Lab and glyceride standard from Larodan Fine Chemicals, (Malmö, Sweden).

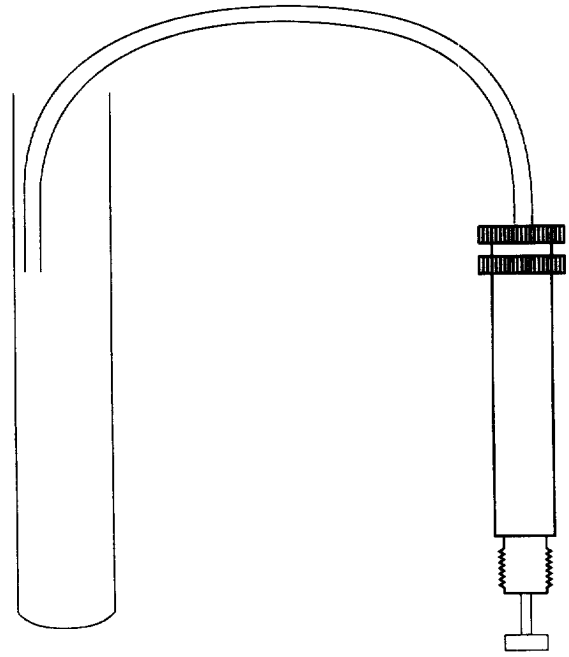

Fig. 1. Drawing of the direct connection line (HP No. 07680-80570: tube collar assembly and HP No. 07680-80700: empty fritted trap).

The following sample types were used in this study: pork loin, smoked pork loin, fermented entrecôte (sterile minced entrecôte inoculated with *Staphylococcus xylosus* stored for 12 days at 25°C), three different sausages and a canned meat reference material SMRI 94-1 (Swedish Meat Research Institute, Kävlinge Sweden).

### 2.3. SFE extraction

Homogenised meat (0.5 g) was weighed and mixed for 1 min with 1 g Hydromatrix using a glass pestle and mortar. The mixture was transferred to an extraction thimble. Extractions were carried out on the Hewlett–Packard equipment. Carbon dioxide was used as the cryo gas, required for cooling different zones in the SFE apparatus and as the extracting medium, in most cases together with 95% ethanol as modifier (0–10%). Hewlett–Packard standard 7-ml

thimbles were used throughout this work with glass fibre filters MGC at each end of the thimble and 1 ml of cyclohexane was added just before the start of the extraction. At optimal conditions the pressure and temperature were set to 370 bar and 50°C respectively, corresponding to a density of 0.91 g/ml without modifier. In the first experiments the extractions were performed with a trap containing octadecylsilica (ODS) Later on the analytes were collected directly in vials (height 20 cm, O.D. 3.8 cm, I.D. 3.5 cm).

The flow-rate of carbon dioxide was set to 4.0 ml/min and the modifier pump containing 95% ethanol was operated at a flow-rate of 0.32 ml/min. Trap and nozzle temperatures were set at 50°C and 55°C, respectively. After extraction the connection line was rinsed with 1.8 ml cyclohexane, pumped at 2.0 ml/min. Trap and nozzle temperatures were set at 50°C and 55°C respectively, during the rinse procedure. The solvent in the vials was evaporated with compressed air and the fat was dried at room temperature overnight in a fume hood. Weighing of the vials before and after solvent evaporation gave the total fat content of the sample. For determination of lipid classes the fat was dissolved in chloroform to a concentration of 10 mg/ml. The lipid classes were determined by thin layer chromatography (TLC) according to the procedure described below.

### 2.4. Bligh and Dyer extraction

Approximately 5 g of homogenised meat sample was weighed and mixed with 40 ml methanol, 20 ml chloroform and 2 ml water using an Omnimixer at the highest speed for 4 min while cooling with ice. Another 20 ml of chloroform was added and mixed for 30 s as above. A third homogenisation was performed after the addition of 20 ml of water. The homogenate was filtered through a glass filter G3 with light vacuum and the filtrate collected in a flask. The filter cake was washed with 20 ml chloroform and filtered as above using the same collecting flask. The filtrate in the flask was transferred to a beaker with approximately 15 ml of chloroform. The beaker was left standing for 2 h at 4°C for phase separation.The chloroform phase was siphoned off to a round bottomed flask. The chloroform was evaporated at 40°C and the residual fat was dried overnight in a fume hood at room temperature. The fat was dissolved in chloroform to give a concentration of 10 mg/ml before determination of the lipid classes by TLC.

### 2.5. Fat determination by SBR

Approximately 3–5 g homogenised meat sample was weighed on aluminium foil and transferred to the extraction tube. A volume of 10 ml 8 *M* hydrochloric acid was added and the tube was placed for 1 h in a boiling water bath. When the sample has cooled down to approximately 30°C, 10 ml of 95% ethanol was added and the sample was mixed. Then 25 ml diethyl ether was added and mixed and thereafter 25 ml petroleum ether was added and mixed. The tube was allowed to stand overnight for phase separation. The ether phase was siphoned off into a flat bottomed flask. The sample was extracted with further 30 ml of an diethyl ether–petroleum ether (50:50, v/v) mixture. After phase separation the organic phase was siphoned off into the same flask as before. This extraction was repeated a third time and the organic phases collected in the same flask as before. The solvent in the flask was evaporated and the flask was placed in a drying oven for ~2 h at 102–105°C. Finally the fat in the flask was weighed.

### 2.6. TLC analysis

The lipid classes were separated on TLC plates, with $R_f$ values for triglycerides (TG) of 0.9, diglycerides (DG) of 0.6, free fatty acids (FFA) of 0.25, monoglycerides (MG) of 0.1 and phospholipids (PL) of 0. Acetone in chloroform (4%, v/v) was used as the eluent and the spots were visualised by iodine vapour. The TLC-plates were scanned on an imaging densitometer Model GS-670 and the spots were quantified with molecular analyst software, version 1.2. A glyceride mixture, TLC MIX 94, with monoolein, diolein, triolein and oleic acid was used as standard.

## 3. Results and discussion

The development of the supercritical extraction

methodology was performed using the triglycerides as a model lipid class and the optimized procedure was then applied to diglycerides, monoglycerides and free fatty acids. For phospholipids further development was performed incorporating ethanol as modifier for the extraction procedure.

### 3.1. Optimization of the extraction procedure

The solubility of fat in supercritical carbon dioxide increases with the density and the temperature [24]. Thus, extraction of fat in a fat matrix should preferably be performed at the highest possible temperature and density [25]. The first optimization concerning the temperature effect on the extraction was performed by measuring the content of triglycerides in pork loin and in SMRI 94-1. Due to pressure limitation of the instrument a temperature of 50°C was selected to achieve the high density needed for good recoveries.

The influence of time and density at 50°C on the extracted amount of TG, for a meat sample with low fat content, is shown in Fig. 2. The best recovery was obtained at a density of 0.91 g/ml of carbon dioxide. This density was used in further work. At this density a plateau was reached within ~2 h. Of course, such a plateau does not necessarily prove that 100% recovery was achieved. However, at this stage of the method development this information was judged to be sufficient. Later on, when the parameters had been optimized for the SF extraction procedure the recoveries obtained were compared with results from standard methods.

The recovery was shown to be independent of the sample size in the range of 0.3–1.0 g. The small variation in recovery in this range was considered to depend on sample inhomogeneity. A sample size of 0.5 g was used in further work.

Based on these experiments conditions for the extraction was selected. A set of experiments was then performed at a carbon dioxide flow-rate of 2.0 ml/min with the trap temperature set to 45°C and the

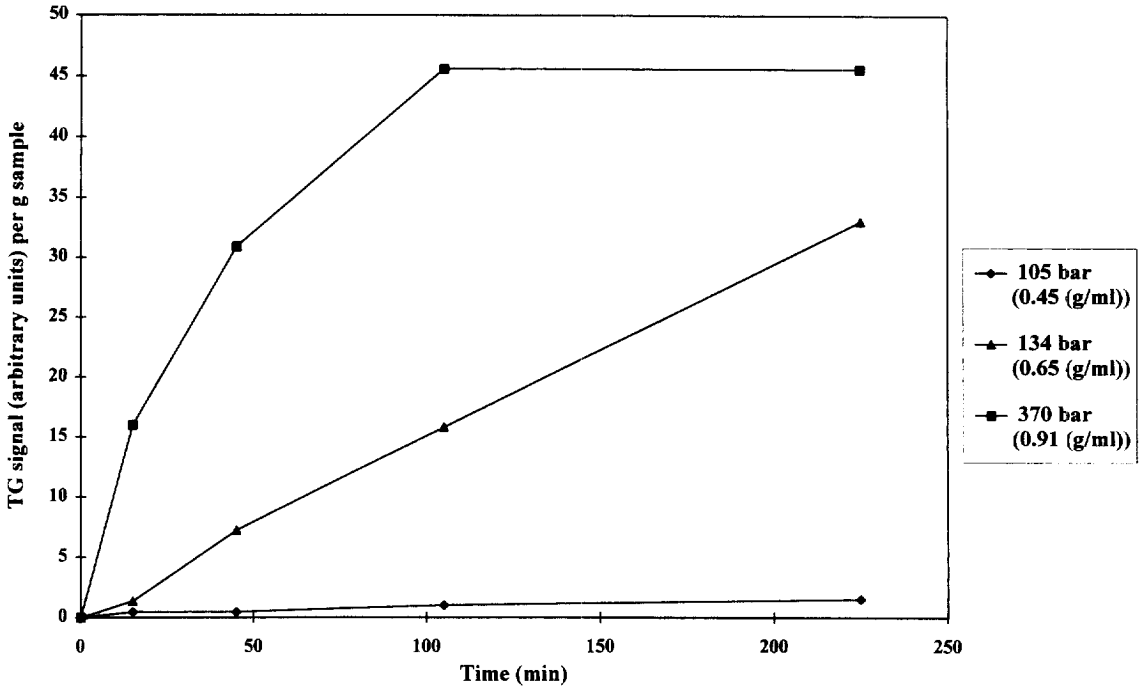


Fig. 2. Extraction profiles for different densities. Pork loin 0.5 g. Dynamic extraction with pure $CO_2$ (flow-rate: 2.0 ml/min), temperature: 50°C, trap: ODS (trap temperature: 50°C, nozzle temperature: 55°C), rinse solvent: cyclohexane at a flow-rate of 1.0 ml/min, eluted volume: 1.4 ml.

nozzle temperature set to 50°C. After completing the extraction the trap, containing the lipids, was rinsed with 1.4 ml cyclohexane pumped at 1.0 ml/min. During the rinse procedure the trap temperature was set to 50°C. The extract was collected in standard vials and diluted with chloroform for the determination of triglycerides by TLC. For samples with a high fat content, like SMRI 94-1, an extraction time in the order of 5 h was needed to reach the plateau indicating 100% recovery.

This SFE procedure was compared with B&D extraction for seven different samples. The results showed that B&D gave higher amount of lipids and it was suspected that this depended on problems with trapping using ODS trap.

The problem with losses may be solved in different ways. Recent results using a trap (E. Björklund et al. personal communication, Department of Analytical Chemistry, University of Lund, Sweden) have shown that fractionated extraction and elution, i.e. repeated short extractions, rinsing of the trap and collection of the extracted fat in vials, can be used for samples with high fat content. Samples with fat amounts up to 500 mg have been processed with this technique, but this also means relatively long extraction time even for pure fat samples (1.5 h). This methodology has not yet been investigated for samples containing as much as 500 mg fat with a modifier such as ethanol passing the trap. However, it is well known that modifiers may cause trapping problems, since the trap must be set to a temperature, at which condensation of the modifier solvent does not take place. This problem has been solved with methanol as modifier up to ~8% [26] but has, as far as we know, not been studied in detail with ethanol as modifier for fat samples.

Since the purpose of this work was also to determine phospholipids, which need a polar modifier, we decided to bypass the trap and collect the extracted lipids directly in an empty glass tube, according to a suggestion of Eller (F. Eller, personal communication, US Department of Agriculture, Peoria, USA). This set-up was expected to give less problems than collection on a solid sorbent, when modifier was used. One disadvantage with this set-up is that the whole procedure is less automated. Normally eight samples can be run sequentially automatically. With this new set-up the collecting tube must be replaced manually after each extraction, which takes ~2 min. However with appropriate instrumentation the method could be automated.

The potential of ethanol as modifier for polar analytes has been discussed by Timelli [27]. Ethanol as modifier for the extraction of phospholipids from meat samples was investigated in the range 0–10%. For good recoveries a modifier concentration of at least 6% was needed. A concentration of 8% was used subsequently.

The main limitation of the SF extraction procedure developed to this point was still the long extraction time needed for full recovery. Different attempts were made to reduce this time. The extraction fluid contained 8% v/v of ethanol as modifier. In one set of experiments the extractions were performed with sea sand (1:3, w/w) mixed with the sample on a filter paper using a glass rod. In other experiments the sample was mixed with steel balls with and without the addition of 1 ml cyclohexane to the thimble immediately before the extraction. Since water could decrease the rate of mass transfer thereby prolonging the extraction time, experiments were performed with Hydromatrix as a water adsorbent mixed at a ratio sample:Hydromatrix of 1:2 (w/w). Hydromatrix has been established as a good water sorbent for SF extractions [28,29]. In a final set of experiments 1 ml of cyclohexane was added to the thimble just before the extraction, in order to further facilitate the solution procedure of the sample. The results are shown in Fig. 3.

The extraction time needed in all the three procedures (without the water adsorbing Hydromatrix) is ~210 min for full recovery, which is a modest reduction compared to previous results. Using Hydromatrix the time is reduced to 2 h and with addition of 1 ml cyclohexane it is further reduced to only 30 min. It seems that the water acts as polar protection for the fat, so that it takes a longer time for the carbon dioxide to penetrate the sample and reach the fat. If Hydromatrix is added to the sample, the water is adsorbed and the carbon dioxide will have better access to the fat in the sample. The large surface area of the absorbent gives a high dispersion of the sample, which further facilitates mass transfer, thereby reducing the extraction time. The solvation procedure is further facilitated by addition of a small amount of cyclohexane to the thimble.

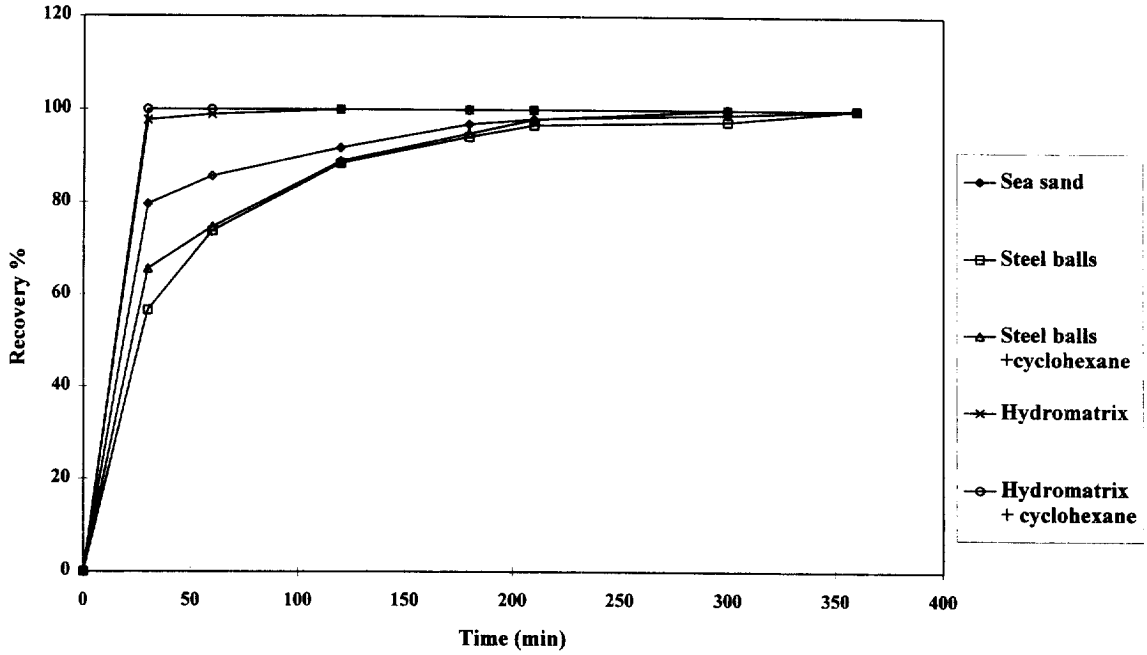


Fig. 3. Influence of extraction time on different sample modifying procedures. 8% ethanol was used as modifier; the analytes were collected directly in vials; the rinse solvent pump was operated at 2 ml/min; other conditions were as in Fig. 2.

The same recoveries were obtained at the same extracting volume at different flow-rates (1.0–4.0 ml/min). This means that the recovery largely depends on the solubility of the lipids in the extracting fluid. Accordingly, the highest flow-rate of 4.0 ml/min is optimal with respect to extraction time. A higher flow-rate was not possible to obtain with this instrument.

### 3.2. TLC analysis

One factor, which has large influence on the measured value of the TG content after the different extraction procedure, is the concentration of fat/ml solvent applied on the TLC plate. Too low concentrations should be avoided, since the spots tend to be difficult to measure. Samples with fat concentrations greater than 10 mg/ml gave very dark spots and thus small changes in concentrations could not be detected. In further measurements the extracted samples were prepared to give a concentration of 10 mg/ml.

This TLC technique is not optimal for analysing PL because the $R_f$ value is approximately zero. Thus any substance with very high affinity for the TLC material might interfere. However, in samples, which did not contain PL, no spot on the TLC plate at a $R_f$ value of zero was observed from any of the investigated samples. This implies that the risk of interferences is small and that this TLC technique is suitable for getting an overview of the lipid classes in the food.

### 3.3. Validation

The developed SFE method was validated using six different samples with each sample being analysed in duplicate. For total fat content, the SFE values were compared with values obtained by SBR and B&D. For the different lipid classes, the SFE values were compared with values obtained after a B&D extraction. The extracted amount of fat using SFE or B&D was obtained by weighing the glass tubes before the TLC evaluation of the lipid classes. The values obtained for total fat content in the samples are illustrated in Fig. 4. The total fat content was between ~1 and 40% in the samples.

No significant difference, at 95% significance

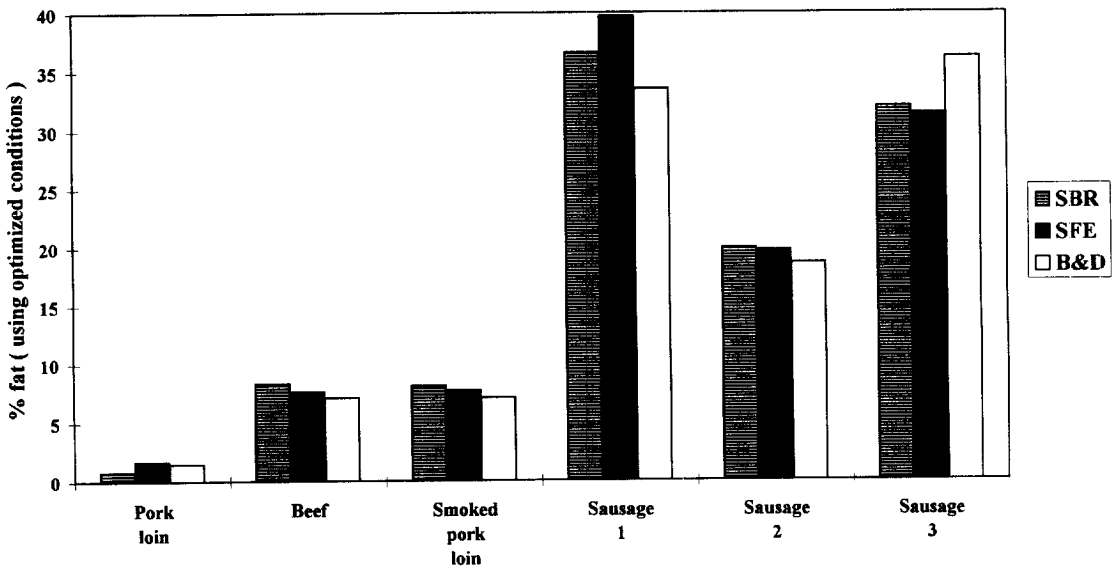


Fig. 4. Comparison of total fat determination between SFE, SBR and B&D. The samples were mixed with Hydromatrix (1:2, w/w) and 1 ml cyclohexane was added. Flow-rate was 4.0 ml/min. Conditions were as in Fig. 3.

level, could be seen with a paired sample $t$-test [30] between the different fat determination methods.

The results obtained with SFE or B&D for different lipid classes are shown in Table 1.

An interesting difference was found in the determination of monoglycerides, where the risk of losses seems to be higher using the B&D method, since no monoglycerides are detected using B&D at these low concentrations. In Table 1 the same signal (arbitrary unit) per g sample was used. To estimate the uncertainty for both methods the relative deviation from the average value for each point in the table was calculated. Since the average deviation between SFE and B&D did not differ significantly the largest uncertainty in the determination comes from the TLC measurements: thus TLC values from both SFE and B&D were treated in the same way. For values up to 2.0 (15 points) the average deviation was calculated to be 14.0%. In the range between 2.1 and 9 (20 points) it was 11.8% and above 9 (12 points, only TG) it was 4.3%. For values below 2 the precision in the determination is low, which can explain the differences obtained in both directions, when considering meat samples with low lipid concentration in Table 1. There is, however, a relatively large difference (ratio>2) between SFE and B&D in the content of diglycerides and free fatty acids from fermented beef, which most probably is

Table 1
Comparison between SFE and B&D in the recovery of different lipid classes (arbitrary units per g sample)

| Sample | TG | | DG | | MG | | FFA | | PL | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SFE | B&D | SFE | B&D | SFE | B&D | SFE | B&D | SFE | B&D |
| Pork loin | 72 | 65 | 1.2 | 0.3 | – | – | 0.7 | 0.4 | 3.5 | 6.5 |
| Fermented entrecôte | 139 | 123 | 4.8 | 1.6 | – | – | 6.8 | 2.8 | 8.5 | 7.4 |
| Smoked pork loin | 62 | 67 | – | – | – | – | 1.1 | 0.5 | 6.9 | 8.5 |
| Sausage 1 | 68 | 69 | 3.1 | 2.6 | – | – | 2.5 | 2.5 | 0.5 | 2.4 |
| Sausage 2 | 54 | 52 | 1.3 | 1.1 | – | – | 1.7 | 1.3 | 1.6 | 4.1 |
| Sausage 3 | 101 | 98 | 3.1 | 2.6 | 2.0 | – | 2.5 | 2.5 | 1.7 | 2.5 |

Six different samples were analysed in duplicate for each extraction method. Other conditions were as in Fig. 4.

due to losses during the B&D extraction procedure. The two methods are roughly equal with respect to accuracy. No significant difference, at 95% significance level, could be seen with a paired sample *t*-test [30] between the SFE extraction and the B&D extraction for respective lipid classes. Factors such as use of organic solvent, sample throughput, the time needed for manual operation and the operator skill should be considered, when choosing between the different analytical methods.

## 4. Conclusions

This study has shown that SFE can be used in the analytical laboratory for extraction of total fat as well as giving the relation between different lipid classes in meat and meat products with an accuracy equal to conventional solvent extraction methods. Under optimal conditions, with ethanol modified carbon dioxide as the supercritical fluid, with Hydromatrix as water adsorbent and with a small amount of cyclohexane added to the sample, the extraction time is reduced to 30 min. This time is considerably shorter than the B&D extraction. Other advantages are the reduction of manual manipulations leading to less labour costs and the reduced consumption of organic solvents in the sample preparation step.

The method might replace other extraction methods used for different food formulas, where the objective is to determine total fat and the lipid classes.

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