ASSESSING ETHYL CHLOROFORMATE FOR DERIVATIZATION OF SELECTED TASTE COMPOUNDS FOR ANALYSIS BY SPME AND GC-MS

Monika Gibis[1*], Peter J. Watkins[2], Robyn D. Warner[2], and Jochen Weiss[1]
[1] Institute of Food Science and Biotechnology, Department of Food Physics and Meat Science, University of Hohenheim, Garbenstr. 25, D-70593 Stuttgart, Germany
[2] CSIRO, Division of Animal, Food and Health Sciences, Sneydes Road, Werribee, Vic., 3030, Australia

Abstract – A fast analytical method has been developed and assessed for the determination of taste compounds in cooked meat using ethyl chloroformate (ECF) derivatization and solid-phase microextraction (SPME) with gas chromatography-mass spectrometry (GC-MS). After extraction with water from meat, the free amino and organic acids were derivatized to *N*-ethoxycarbonyl methyl or methyl esters while the fatty acids were esterified as methyl esters, and then extracted using a divinylbenzene(DVB)/Carboxen®/polydimethylsilicone(PDMS) SPME fiber, and determined using GC–MS using single ion monitoring. Response surface methodology with a Box-Behnken experimental design was used to assess the effect of extraction temperature and time on the abundance of the derivatized compounds with the SPME fiber. Both temperature and time with the interaction term were statistically significant for the extraction of the compounds. For the derivatized amino acids, the optimal conditions were 80 °C and 45 min while, for the methyl esters, these were 50 °C and 45 min. The limits of detection (LOD) and quantification (LOQ) of all derivatized acids was determined in the range of 0.22-25.2 mg/L, and 0.49-56.9 mg/L, respectively. Finally, the method was applied to the analysis of selected taste compounds in pork, beef and lamb (raw and cooked). Further work is required to fully validate the method prior to routine application.

Key Words – GC-MS, SPME, Ethyl chloroformate derivatization, Organic acids, Amino acids, Fatty acids

I. INTRODUCTION

Flavour is an important component of the eating quality of meat, and consists of taste, the sensation perceived by the taste buds, and odour, the sensation perceived by the olfactory organ. In its uncooked state, meat has a very little flavour and it only develops as a result of cooking. Historically, considerable focus has been given to the measurement of volatile compounds, which contribute to the odour associated with the cooked meat product. In comparison, considerably less is known of the contribution that non-volatile taste compounds make to the overall meat flavour [1]. Usually, liquid chromatography (LC) can be used to measure the non-volatile compounds, which can be time-consuming. In contrast, gas chromatographic (GC) techniques are simpler and a fast alternative to LC methods. With a suitable derivatization step to increase the volatility of the taste compounds, GC could be used for the measurement of many different compounds such as organic, fatty or amino acids [2]. Derivatization of taste compounds with ethyl chloroformate is one possible approach since this reagent allows derivatization in aqueous systems [3]. Solid-phase microextraction (SPME) is a sampling technique popular for aroma analysis since it is solvent free, offers rapid sampling with low cost, ease of operation and sensitivity; all of which can be performed in a single step [4, 5]. Given the advantages of this technique, our aim was to evaluate the use of ethyl chloroformate for derivatization of selected taste compounds with SPME and measurement by GC-mass spectrometry. We also optimised the sample extraction temperature and time to find the optimal response for the different analytes.

II. MATERIALS AND METHODS

Sample material

Ground beef, pork and lamb were purchased from a local retailer.

Chemicals

The standards for the amino acids (Glu, Phe, Leu,), organic and fatty acids (lactic, octanoic, nonanoic, decanoic, dodecanoic and tetradecanoic acids) and the two internal

standards L-norleucine and benzoic acid, as well as methanol, pyridine, and ethyl chloroformate were purchased from Sigma-Aldrich (Sydney, Australia). Hydrochloric acid, sodium hydrogen carbonate, sodium hydroxide, and acetonitrile were obtained from Merck (Darmstadt, Germany).

Sample preparation

Minced meat (10 g, raw and cooked (85 °C for 45 min in closed system)) was stirred in 9.4 mL of ultrapure water plus 300 µL of L-norleucine (5.226 mg/mL) and 300 µL benzoic acid (27.33 mg/mL) for 5 min using a blender (Sorvall Omni-Mixer, Newton, Connecticut, USA). After chilling (15 min) in a freezer, the mixture was centrifuged (12,100 g, 10 min, Beckman J2-MC, Palo Alto, CA, U.S.A.). An aliquot of supernatant (6 mL) was mixed with acetonitrile (3 mL) and methanol (1 mL), and chilled once more in a freezer (15 min). After membrane filtration (0.45 µm, PTFE, Phenomenex, Lane Cove, NSW Australia), 1.0 mL of the filtered extract was added to a 10 mL vial followed by the sequential addition of methanol (100 µL), pyridine (50 µL), ethyl chloroformate (50 µL), and a mixture of 1 M sodium hydroxide and 1 M sodium hydrogen carbonate (100 µL). The vials were then rapidly closed and the contents were mixed using a Vortex blender (30 s). After the addition of saturated sodium chloride solution (200 µL) and mixed (30 s), the samples were ready for GC-analysis.

SPME and gas chromatography-mass spectrometry (GC-MS)

Headspace SPME GC-MS analysis was performed using an Agilent system (Palo Alto, CA, USA), comprising a Model 6890 GC and a Model 5973 mass selective detector, with a CombiPAL SPME autosampler (CTC, Switzerland). For the optimisation study, aliquots of standard solutions (1.5 mL) were placed in 10 mL glass headspace vials and sealed with PTFE/silicone septa and steel caps. The vials and their contents were pre-heated at one of three temperatures (50, 65 or 80 °C) for 2 min prior to the insertion of a DVB/Carboxen®/PDMS fibre into the headspace and held for one of three times (15, 30 or 45 min). The autosampler withdrew the fibre and inserted it into the GC injector and held for 7 min. The volatile compounds were separated on HP-5 fused silica capillary column (30 m, 0.32 mm i.d., 0.25 µm film thickness, J&W Agilent, Mulgrave, VIC, Australia). The GC oven temperature was initially held at 45 °C for 2 min, increased to 280 °C at a rate of 15 °C min^{-1} and held for 7.33 min. The injector was in the splitless mode for 2 min, and held at 170 °C for 1 min and heated to 250 °C at 200 °C min^{-1} where it was held for the remainder of the analysis. Helium was used as the carrier gas (1.5 mL min^{-1}). The MS was operated in electron ionisation mode (70 eV) and data acquired in single ion monitoring mode (SIM) with the detector maintained at the autotune voltage. The temperature of the source and the detector were 150 °C and 230 °C respectively while the MS transfer line was held at 280 °C. The analyte response was quantified by measuring the abundance of a characteristic target ion using Chemstation software. The target ions (amu) were as follows: norleucine as *N*-ethoxycarbonyl methyl ester (internal standard for amino acids), *m/z* = 158, glycine (*N*-ethoxycarbonyl methyl ester) *m/z* = 102, leucine (*N*-ethoxycarbonyl methyl ester) *m/z* = 158, glutamic acid (*N*-ethoxycarbonyl dimethyl ester), *m/z* = 188, phenylalanine (*N*-ethoxycarbonyl methyl ester), *m/z* = 162, methyl benzoate (internal standard for fatty and organic acids), *m/z* = 105, lactic acid (*O*-ethoxycarbonyl methyl ester), *m/z* = 45 while for methyl trimethyl-3,3,5-hexanoate, methyl octanoate, methyl nonanoate, methyl decanoate, methyl dodecanoate and methyl tetradecanoate, *m/z* = 74.

Response surface methodology (RSM)

RSM was used to evaluate the best combination of extraction temperature and time for sampling the selected compounds. The central point (65 °C, 30 min) was replicated three times to estimate the experimental reproducibility. The ratio of the measured abundance for each analyte to the respective internal standard was used to estimate the coefficients (a_i) of a polynomial model as given by $R = a_0 + a_1 t_e + a_2 t_i + a_3 t_e.t_i$ where R is the relative ratio of measured abundance to the internal standard, t_e and t_i are the encoded variables for extraction temperature and extraction time, respectively using multilinear regressions in R [6]. These were used to generate contour plots to identify the optimal extraction conditions for temperature and time.

III. RESULTS AND DISCUSSION

A class of compounds normally requires the use of a specific analytical method, and so a range of different methods are needed to characterize the profile of taste compounds within a food system. Ethyl chloroformate is useful for derivatizing amino, organic and fatty acids in a complex mixture without the need of multiple steps for sample preparation [3], yet for SPME, the extraction conditions need to be optimized for each class of substances.

Response surface methodology was used to find the optimal set of SPME extraction temperature and time (Fig 1 and 2). For the *N*-ethoxycarbonyl methyl ester of leucine, the optimal temperature and time was 80 °C and 45 min. This was also the case for the other amino acids. For methyl decanoate (C10), the optimal temperature and time was 50 °C and 45 min, which was also applicable for the other fatty acids. The range of linearity was evaluated for the selected compounds along with the associated limits of detection (LODs) and quantitation (LOQs) (Table 1). For SPME, the fatty acids have much lower LODs and LOQs when compared to the amino acids. A comparable LOQ value of 0.3 mg/L was found for valproic acid using ethyl chloroformate derivatization and SPME in human plasma [7].

Table 1 Analytical calibration parameters

Analyte[1]	m[2]	c[3]	R^2[4]	LOD[5]	LOQ[6]
Gly*	0.002	-0.012	0.937	25.2	56.9
Leu	0.005	0.133	0.933	22.6	51.2
Glu	0.003	0	0.999	3.8	7.9
Phe	0.003	0	0.998	4.6	9.3
Lac	0.002	0.137	0.848	10.1	24.9
Tri	0.182	0.044	0.956	0.31	0.68
C8	0.241	0.028	0.961	0.22	0.49
C9	0.236	0.063	0.955	0.23	0.52
C10	0.233	0.111	0.961	0.25	0.56
C12	0.205	0.113	0.947	0.36	0.8
C14	0.186	0.076	0.881	0.39	0.94

[1]amino acids as *N*-ethoxycarbonyl methyl ester, fatty and organic acids as methyl esters, [2] m = calibration slope, [3] c = calibration intercept, [4] R^2 = adjusted correlation coefficient [5]LOD = limit of detection (mg/L), [6]LOQ = limit of quantification (mg/L)

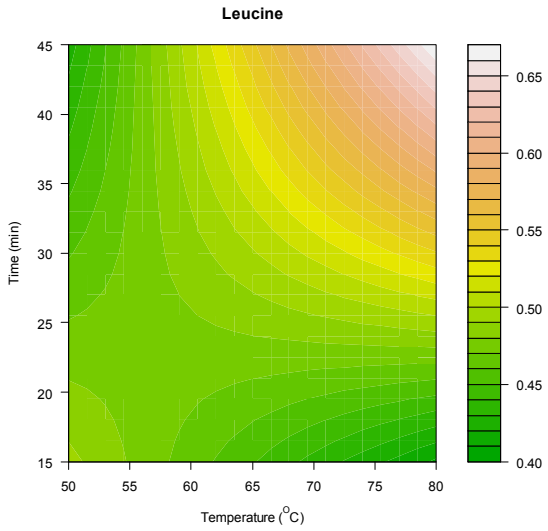

Figure 1. Response surface contour plot of relative abundance ratio of *N*-ethoxycarbonyl L-leucine methyl ester to *N*-ethoxycarbonyl L-norleucine methyl ester (internal standard) as a function of temperature and time

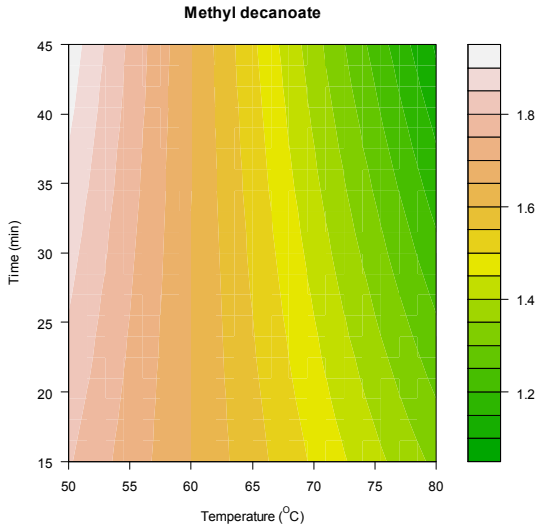

Figure 2. Response surface contour plot of relative abundance ratio of methyl decanoate to methyl benzoate (internal standard) as a function of temperature and time

For this study, we used 80 °C and 45 min for the analysis of the raw and cooked due to the sensitivity found with these conditions. Recoveries of glycine and leucine were found to be in the range of 89–90% for the cooked and raw meat samples, respectively.

The method was applied for the measurement of selected taste compounds in raw and cooked meat (beef, lamb and pork, Table 2). Lactic acid was the most abundant compound found for both raw and cooked meat. The typical slight sour taste present in meat is due to the lactic acid content in meat. Low concentrations of octanoic acid were also found in the aqueous extract taken from the meat which is close to the LOQs for this compound. This preliminary study indicates that there are some significant differences between raw and cooked states of the meat samples (Table 2). Further work is required to examine the effect of these compounds on meat taste as well as the application of this method for other compounds of interest.

IV. CONCLUSION

Ethyl chloroformate derivatization followed by SPME/GC-MS analysis offers an effective analysis for free short chain fatty, organic and some amino acids in complex matrices such as raw or cooked meat. This proposed method is rapid, and can simply be done in aqueous media at room temperature without needing lengthy sample preparation for GC-MS analysis.

Table 1 Concentrations (mean ± standard deviation, *n* = 4) of selected taste compounds in pork (P), beef (B) and lamb (L) (raw (R) and cooked (C))

Meat	Concentration[1]				
	Gly[2]	Leu	Phe	Lac	C8
RP	196[a]	146	20	900[b]	47
	± 54	± 20	± 6	± 66	± 16
CP	121	170	17	1284	43
	± 33	± 80	± 13	± 249	± 40
RB	168	195	16	1109	67
	± 35	± 28	± 4	± 293	± 4
CB	127	165	13	1364	40
	± 27	± 14	± 5	± 122	± 29
RL	266[b]	418	33	1149[b]	96
	± 29	± 51	± 13	± 290	± 20[c]
CL	186	363	20	1732	30
	± 49	± 28	± 6	± 289	± 7

[1]mg/kg except for C8, µg/kg [2]Letters denote statistical significance between raw and cooked within species [a]$P < 0.1$, [b]$P < 0.05$ [c]$P < 0.001$

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ABBREVIATIONS

Gly - glycine, Glu – L-glutamic acid, Leu – L- leucine, Phe – L-phenylalanine, C8 – octanoic acid, C9 - nonanonic acid, C10 – decanoic acid, C12 – dodecanoic acid, C14-tetradecanoic acid, Tri – 3,5,5 trimethylhexanoic acid

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