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# Determination of volatile products of human colon cell line metabolism by GC/MS analysis

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Colon cancer is one of the most reasons for cancer death worldwide. Thus, it is important to find new prognostic and diagnostic marker, as well as to throw light on the special metabolic pathways of colon cancer cells. This paper highlights for the first time some qualitative differences in the profiles of the volatile metabolites of colon cancer cell lines SW 480 (grade IV, Duke B) and SW 1116 (grade II, Duke A) among themselves and in comparison to the normal colon cell line NCM460, which are mostly represented by ketones and alcohols. These results, which were obtained by applying solid phase micro extraction (SPME) and combined gas chromatography/mass spectrometry (GC/MS), are consistent with Warburg's hypothesis because the found reaction products may indicate that the cancer cells show the Crabtree's effect. Furthermore, compounds like undecan-2-ol and pentadecan-2-one were associated for the first time with the human metabolism. In summary, these findings indicate that the metabolism of colon cancer cells differs extremely from the metabolism of healthy cells and it changes during the progress of the disease. Compounds that are present in the breath, the blood and the tissue of patients represent the differences and they can serve as new biomarker for colon cancer in future.

**KEY WORDS:** metabolomics; human colon cell lines; metabolic pathways; volatile metabolites; SPME; GC/MS.

## 1. Introduction

For a comprehensive description of the colon cancer pathogenesis it is essential to clear up the metabolism of colon cancer cells, especially during the tumor progression. Additionally, the results of the investigations in low-molecular-weight metabolites provide the opportunity for new prognostic and diagnostic marker, which cover a large spectrum of the disease colorectal cancer. In contrast to genomics and proteomics, which data might indicate the potential pathophysiological effect, the results of metabolite analysis incorporate pathway feedback mechanisms (Nicholson and Wilson, 2003). Thus, the results of the metabolome analysis provide essential extension to the achieved results of genomics and proteomics. This will make it possible to get a nearly complete picture of the complex disease colon cancer.

The analysis of the metabolome covers the identification and quantification of all intermediate catabolic products with a molecular mass lower than 1000 dalton (Villas-Bôas *et al.*, 2005). In the post-genomic era, analytical tools such as liquid chromatography coupled to mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) spectroscopy have been developed and improved for the investigation in non-volatile metabolites (Shockcor *et al.*, 1996; Villas-Bôas *et al.*, 2005). For volatile metabolites, gas chromatography coupled to mass spectrometry (GC/MS) were applied but nearly exclusively for determination of metabolic pathways in/of plants (Roessner *et al.*, 2000). These analytical techniques are appropriated to solve problems like low concentration of the single metabolites, a high fluctuation rate and high diversity of compounds with different functional groups, which appear in the evaluation of metabolites.

For the investigation in a rather complex disease like colorectal cancer, it is indispensable to utilize model organisms like human cell lines or animal models. In the case of colon cancer several cell lines are available, but not every cell lines is well classified concerning the grade of the origin tumor and concerning the stage of the disease. Thus, the colon cancer cell lines SW 1116 and SW 480, which were established in the year 1976 by Leibovitz *et al.*, have been selected for these investigations (Leibovitz *et al.*, 1976). They represent excellent model systems for colon cancer of the grade II and stage Duke A in the case of SW 1116 and for colon cancer of the grade IV and stage Duke B in the case of SW 480. To comment on the pathogenesis of colon cancer, the comparison with normal colon epithelial cells is indispensable. Therefore, the normal colon epithelial cell line NCM460 was chosen as model system, additionally. Moyer *et al.* (1996) have obtained this cell line from normal human colon mucosa.

This paper presents for the first time results of the metabolic profiling of volatiles of the two colon cancer

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cell lines SW 1116 and SW 480 in comparison with the non-cancer colon epithelial cell line NCM460 based on a sampling at an SPME-fiber followed by qualitative GC/MS analysis.

## 2. Materials and methods

### 2.1. Cell culture

The colon cancer cell lines SW 1116 and SW 480 were cultivated in Dulbecco's Modified Eagle's Medium/nutrient Mixture F-12 Ham (DMEM/F-12 medium) (PAA), supplemented with 10% fetal bovine serum (PAA) at 37 °C in a humidified atmosphere of 5.5% $CO_2$ (v/v).

The cell line derived form normal mucosa (NCM460) was cultivated in M3$^{TM}$ Base Medium (INCELL Corporation), supplemented with 10% fetal bovine serum (PAA) under the same conditions as the cancer cell lines (Moyer *et al.*, 1996; Schmied *et al.*, 2000). The cultivation in this medium is essential for keeping the appropriate phenotype of this cell line. Only in the frame of the measurements, the NCM460 cells were transferred to the DMEM/F-12 medium, supplemented with 10% fetal bovine serum, to guarantee the comparability of the results with those of the cancer cell lines.

### 2.2. Design of the experiments

For the measurements, the cells were cultivated in glass flasks. The attachment of the cells at the glass surface was improved by pre-treating the glass flask with poly-D-lysine (0.01% w/v) (Sigma Aldrich). The measurements were performed at sub confluent cultures in consideration of the different generation times of the cell lines. Prior the insertions of the cells in the glass flasks and after the measurements the cells were counted with a Neubauer counting chamber. For each type of sample, duplicate cell cultures were used and the experiments were repeated once. Together with the measurement of the cell cultures, blank values of the medium were taken. Therefore, the cell culture medium supplemented with 10% fetal bovine serum was incubated in the same way as the cells.

To take care of errors caused by the different components of the experimental set up, the measurements were carried out in a randomized way.

### 2.3. Sampling and GC/MS measurements

For sampling a SPME fiber of the type carbowax/divinylbenzene with a film, thickness of 65 $\mu$m (Sigma Aldrich/Supleco) was placed in the atmosphere of the cell culture flask for 40 min. Afterwards the fiber was directly transferred to the GC injector of the G1800A GC/MS system (Hewlett Packard), which was used for the analysis. The analytes were desorbed for 20 s at 200 °C with a closed split. The separation was performed on a 60 m fused silica capillary column with 0.32 mm inner diameter. As stationary phase, SB 11 with 0.2 $\mu$m film thickness was used (WGA). The carrier gas was helium at a flow rate of 1.0 mL min$^{-1}$. The start temperature of the oven program was 35 °C for 2 min, followed by a heating rate of 5 °C min$^{-1}$ up to the final temperature of 200 °C, held for 10 min.

### 2.4. Identification, assignment

The identification of the measured mass spectra was performed by AMDIS version 2.1 (Automated Mass Spectral Deconvolution and Identification System DETRA/NIST, 2000). In the case of (relative) small and superimposing signals, the automated identification was controlled manually for an additional proof of the results.

In addition, reference measurements with the pure compounds were performed and the substances were confirmed by the retention time and the mass spectra. For a first correlation between the compounds found and metabolic pathways the database BRENDA© was used (Schomburg *et al.*, 2000).

## 3. Results and discussion

For the analysis of the volatile metabolites, a cell culture/sampling arrangement that enables a reproducible sampling and measurement was established. Previous work has confirmed that devices and additives made of plastics such as the cell culture flask of polystyrene disturbs the identification of the mass spectra in GC/MS-analysis of the volatile compounds strongly as demonstrated by the chromatograms in figure 1. Thus, it was decided to set up a cell culture method in glass flasks. To encourage the adherence of the cells on the glass surface of the flask, the surface was coated with poly-D-lysine. Microscopical observations and the counting of the cells showed that the cells grew as well as in normal polystyrene cell culture flask without visible changes in the morphology or the generation time.

For a representative sampling of the volatile analytes it is important not to disturb the surrounding atmosphere of the cells, as it would happen, if an air stream were removed continuously. For the same reason a discontinuous sampling of a certain gas volume must also be avoided. Therefore, the sampling at the fiber of a SPME-unit is the most advantageous method. The disadvantage of the fiber is the constricted selectivity depending on the chemistry of the fiber itself and the physical and chemical properties of the analytes. For collecting the cell metabolites a (semi-)polar carbowax/divinylbenzene phase with 65 $\mu$m thickness was selected as the best compromise, because knowing from initial experience alcohols, esters and ketones were due. The collecting time can be adapted to the analytes; here a time of 40 min were fixed to get analytes in very low concentrations, too.

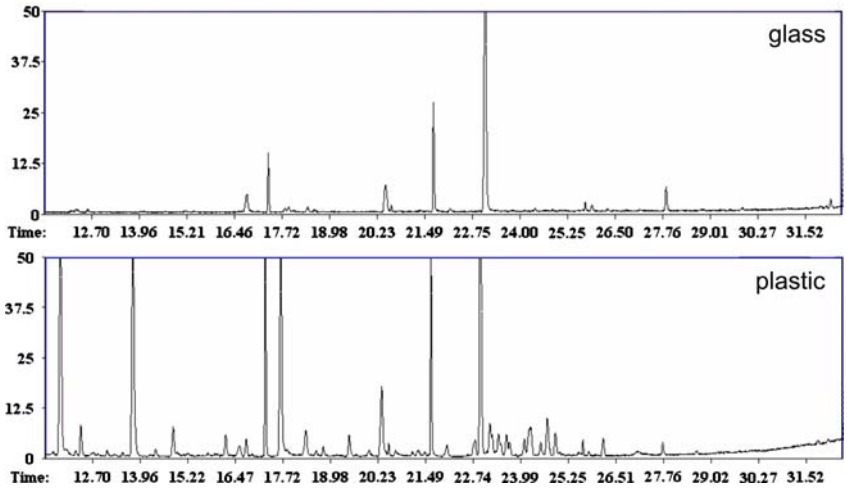


Figure 1. Material influence of the cultivation device on the GC/MS-analysis. (Above) TIC-chromatogram of SW 480 cells, grown in a glass flask; (below) Chromatogram of the same cells, grown in a plastic flask.

The SPME-unit was then transferred to the injector of the GC and the analytes were desorbed from the fiber as described in the experimental chapter.

To get a first impression about the metabolism only such signals that are clearly different within the three cultures and the medium were investigated. The reproducibility of the data from four samples of each cell line was high.

### 3.1. *Comparison between volatile compounds of the medium alone and with the cell lines*

The total ion chromatograms showed similarities in the metabolism of all three cell lines. There are compounds, either which are produced by all cell lines, or which are metabolized by all cell lines (figure 2). Benzaldehyde, decan-1-ol and methyl dodecanoate were found in the chromatograms of the medium, but not in the chromatograms of the medium with cells, indicating, that all three cell lines metabolize these compounds.

In the case of the benzaldehyde, there are several metabolic pathways like the fatty acid metabolism, the glycolysis/gluconeogenesis or the tryptophan metabolism with related enzyme like the NADP-alcohol dehydrogenase (Enzyme Commission (EC) number 1.1.1.2) and the NAD-aldehyde dehydrogenase (EC number 1.2.1.3), this compound is involved in (Wermuth *et al.*, 1977; Klyosov, 1996; Schomburg *et al.*, 2000). For decan-1-ol the data base and literature search specify the enzymes NADH-alcohol dehydrogenase (EC number 1.1.1.1) the long-chain-alcohol dehydrogenase (EC number 1.1.1.192) and the alcohol sulfotransferase (EC number 2.8.2.2) for the conversion of this alcohol in mammalians as part of several pathways like the sulfur metabolism, the androgen estrogen metabolism, the

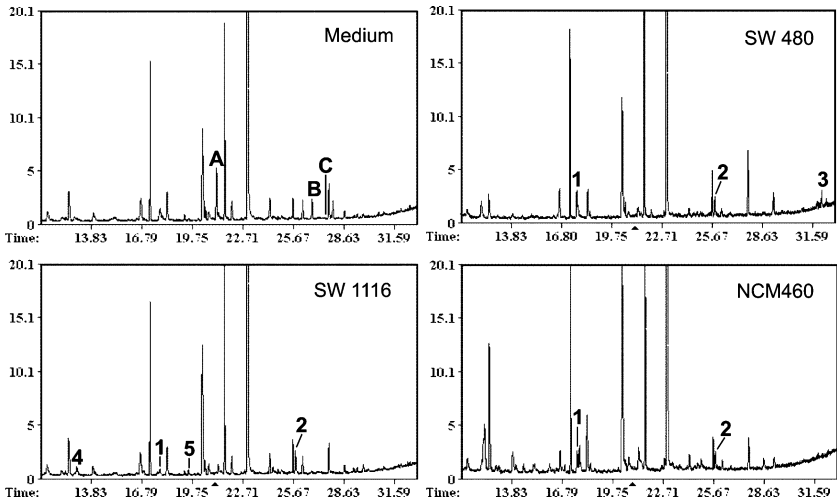


Figure 2. Comparison of TIC-chromatograms of the medium alone and with the three different cell lines SW 480, SW 1116, and NCM460 grown in it. A = benzaldehyde, B = decan-1-ol and C = methyl dodecanoate were metabolized by the cells. 1 = nonan-2-one, 2 = undecan-2-ol, 3 = pentadecan-2-one, 4 = 3-methylbutan-1-ol and 5 = heptan-1-ol were formed during cell growing.

fatty acid metabolism and the glycolysis/gluconeogenesis (Bosron *et al.*, 1979; Lee, 1979; Chen *et al.*, 1996; Schomburg *et al.*, 2000). In the case of methyl dodecanoate no information concerning metabolic pathways and enzymes are given.

Nonan-2-one and undecan-2-ol were not found in the chromatograms of the medium but in the chromatograms of the cells. These results point out that the cells produce these two compounds. Analog to the methyl dodecanoate these compounds are for the first time correlated with the human metabolism; related enzymes must be figured out in separate experiments. Probably there is a similar enzyme like the secondary-alcohol oxygenase (EC number 1.1.13.8) available in the human body. In bacteria, this enzyme is involved in the anabolism of nonan-2-one (Sakai *et al.*, 1985).

### 3.2. *Differences in the metabolic profiles of the cell lines*

Differences in the metabolic profile of all three cell lines were also detected (figure 2).

The cell line SW 480 exclusively produces pentadecan-2-one. This indicates that in contrast to the cell lines NCM460 and SW 1116 in the cell line SW 480 different pathways are active. At that time, an appropriation to metabolic pathways is impossible, because the role of the substance pentadecan-2-one in the human metabolism has not been described yet. The cell line SW 1116 exclusively produces the alcohols 3-methylbutan-1-ol and heptan-1-ol. Even if heptan-1-ol has not been described in the human metabolism yet, in other mammalians it is the product of a reaction, which is catalyzed by the enzyme carbonyl reductase (EC number 1.1.1.184) as part of the arachidonic acid metabolism (Oritani *et al.*, 1992; Forrest and Gonzalez, 2000; Schomburg *et al.*, 2000).

Analog to heptan-1-ol the 3-methylbutan-1-ol is a new compound in human metabolism and has only been described as part of the glycerolipid metabolism and the glycolysis/gluconeogenesis of sacchromyces cerevisiae with the NADP-alcohol dehydrogenase (EC number 1.1.1.2) as responsible enzyme (Larroy *et al.*, 2002). Analog to the pentadecan-2-one these results indicate that there are differences in the active pathways between the cell lines NCM460/SW 480 and SW 1116. Octan-1-ol was detected in the chromatograms of the medium and in the chromatograms of the cancer cell lines SW 1116 and SW 480, but not of the non-cancer cell line NCM460. This suggests that the cell line NCM460 metabolizes octan-1-ol. There is one enzyme known, which change over octan-1-ol in human – the NADH-alcohol dehydrogenase (EC number 1.1.1.1) (Chou *et al.*, 2002). The NADH-alcohol dehydrogenase is part of several pathways like the fatty acid metabolism, the glycerolipid metabolism and the glycolysis/gluconeogenesis (Schomburg *et al.*, 2000).

Table 1
Summary of the volatile compounds metabolized and produced by the cell lines

| Compound | Medium | NCM460 | SW 1116 | SW 480 |
|---|---|---|---|---|
| Benzaldehyde | × | | | |
| Decan-1-ol | × | | | |
| Methyl dodecanoate | × | | | |
| Nonan-2-one | | × | × | × |
| Undecan-2-ol | | × | × | × |
| Pentadecan-2-one | | | | × |
| 3-Methylbutan-1-ol | | | × | |
| Heptan-1-ol | | | × | |
| Octan-1-ol | × | | × | × |

(×) Compound found in the chromatogram.

## 4. Concluding remarks

Even if this study was focused on volatile compounds, the results furnish findings about collective and different metabolic pathways in the different types of colon cells (table 1). Based on these experimental results in combination with a literature and data base research it could be postulated, that the colon cancer cell lines SW 1116 (grade II, Duke A) and SW 480 (grade IV, Duke B) and the non-cancer cell line NCM460 differ in several pathways like the glycerolipid metabolism, the glycolysis/gluconeogenesis and the arachidonic acid metabolism. The differences seem to be concentrated in the two main groups of metabolism – the carbohydrate metabolism and the lipid metabolism, what corresponds to the findings of Schulz *et al.*, who provided the experimental proof for Warburg's hypothesis based on investigations in the mitochondrial frataxin protein (Schulz *et al.*, 2006). Furthermore, in the frame of these experiments methyl dodecanoate, decan-1-ol, heptan-1-ol, 3-methylbutan-1-ol, undecan-2-ol, nonan-2-one and pentadecan-2-one appeared as metabolites, which have never been described before associated with the human metabolism. In future projects, these compounds should be correlated to metabolic pathways in human beings.

In conclusion, this paper show for the first time qualitative differences in the volatile metabolome of colon cancer cell lines representing different disease stages in comparison with the volatile metabolites of a non-malignant colon epithelial cell line. These compounds are one piece of the puzzle "colon cancer metabolome" and they represent a progress towards a deeper understanding of the colon cancer biochemistry, but also to new colon cancer biomarker, because the compounds are represented for example in the body fluids and the tissue of the patients.

Additionally, as a side effect, this work demonstrates the suitability of metabolomics for the investigation in metabolic pathways of mammalian cells as well as for the experimental proofs of hypothesis related to the tumor metabolism.

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## References

Bosron, W.F., Li, T.K., Dafeldecker, W.P. and Vallee, B.L. (1979). Human liver pi-alcohol dehydrogenase: kinetic and molecular properties. *Biochemistry* **6**, 1101–1105.

Chen, G., Banoglu, E. and Duffel, M.W. (1996). Influence of substrate structure on the catalytic efficiency of hydroxysteroid sulfotransferase STa in the sulfation of alcohols. *Chem. Res. Toxicol.* **9**, 67–74.

Chou, C.F., Lai, C.L., Chang, Y., Cuester, G. and Yin, S.J. (2002). Kinetic mechanism of human class IV alcohol dehydrogenase functioning as retinol dehydrogenase. *J. Biol. Chem.* **277**, 25209–25216.

Forrest, G.L. and Gonzalez, B. (2000). Carbonyl reductase. *Chem. Biol. Interact.* **129**(1–2), 21–40.

Klyosov, A.A. (1996). Kinetics and specifity of human liver aldehyde dehydrogenases toward aliphatic, aromatic, and fused polycyclic aldehydes. *Biochemistry* **35**, 4457–4467.

Larroy, C., Fernandesz, M.R., Gonzalez, E., Pares, X. and Biosca, J.A. (2002). Characterization of the Saccharomyces cervisiae YMR318C (ADH6) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction. *Biochem. J.* **361**, 163–172.

Lee, T. (1979). Characterization of fatty alcohol: $NAD^+$ oxidoreductase from rat liver. *J. Biol. Chem.* **254**, 2892–2896.

Leibovitz, A., Stinson, J.C., McCombs, W.B., McCoy, C.E., Mazur, K.C. and Mabry, N.D. (1976). Classification of human colorectal adenocarcinoma cell line. *Cancer Res.* **36**, 4562–4569.

Moyer, M.P., Manzano, L.A., Merriman, R.L., Stauffer, J.S. and Tanzer, L.R. (1996). NCM460, a normal human colon mucosal epithelial cell line. *In Vitro Cell. Dev. Biol. Anim.* **32**, 315–317.

Nicholson, J.K. and Wilson, I.D. (2003). Understanding global systems biology: metabonomics and the continuum of metabolism. *Nat. Rev. Drug Discov.* **8**, 668–676.

Oritani, H., Deyashiki, Y., Nakayama, T., Hara, A., Sawada, H., Matsuura, K., Bunai, Y. and Ohya, I. (1992). Purification and characterization of pig lung carbonyl reductase. *Arch. Biochem. Biophys.* **292**, 539–547.

Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N. and Willmitzer, L. (2000). Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant J.* **23**, 31–142.

Sakai, K., Hamada, N. and Watanabe, Y. (1985). Purification and properties of secondary alcohol oxidase with an acidic isoelectric point. *Agric. Biol. Chem.* **49**, 817–825.

Schmied, B.M., Ulrich, A., Matsuzaki, H., Batra, S.K. and Pour, P.M. (2000). Maintenance of human islets in long-term culture. *Differentiation* **66**, 173–180.

Schomburg, I., Hofmann, O., Bänsch, C., Chang, A. and Schomburg, D. (2000). Enzyme data and metabolic information: BRENDA, a resource for research biology, biochemistry and medicine. *Gene Funct. Dis.* **3, 4**, 109–118.

Schulz, T.J., Thierbach, R., Voigt, A., Drewes, G., Mietzner, B., Steinberg, P., Pfeiffer, A.F.H. and Ristow, M. (2006). Induction of oxidative metabolism by mitochondrial frataxin inhibits cancer growth: Otto Warburg revisited. *J. Biol. Chem.* **281**(2), 977–981.

Shockcor, J.P., Unger, S.E., Wilson, I.D., Foxall, P.J.D., Nicholson, J.K. and Lindon, J.C. (1996). Combined HPLC, NMR spectroscopy, and ion-trap mass spectrometry with application to the detection and characterization of xenobiotic and endogenous metabolites in human urine. *Anal. Chem.* **68**, 4431–4435.

Villas-Bôas, S.G., Mas, S., Åkesson, S.M., Smedsgaard, J. and Nielsen, J. (2005). Mass spectrometry in metabolome analysis. *Mass Spectrom. Rev.* **24**, 613–646.

Wermuth, B., Munch, J.D. and von Wartburg, J.P. (1977). Purification and properties of NADPH-dependent aldehyde reductase from human liver. *J. Biol. Chem.* **252**(11), 3821–3828.