Technical Note

Polarity-tuning Derivatization-LC-MS Approach for Probing Global Carboxyl-containing Metabolites in Colorectal Cancer

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Polarity-tuning Derivatization-LC-MS Approach for Probing Global Carboxyl-containing Metabolites in Colorectal Cancer

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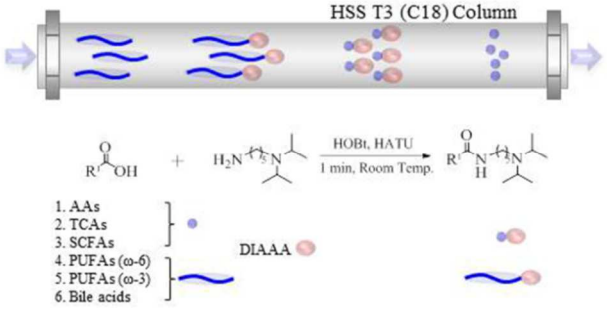
ABSTRACT: Carboxyl-containing metabolites (CCMs) widely exist in living system and are the essential components for the life. Global characteristics of CCMs in biological samples are critical for the understanding of physiological processes and the discovery for the onset of relevant diseases. However, their determination represents a challenge due to enormous polarity difference, structural diversity, high structural similarity, and poor ionization efficiency in mass spectrometry. Herein, 5-(diisopropylamino)amylamine (DIAAA) derivatization coupled with liquid chromatography-mass spectrometry (LC-MS) was developed for mapping the CCMs. With this methodology, the sensitivity was significantly enhanced. More importantly, the hydrophobicity of polar CCMs, amino acids, TCA cycle intermediates and short-chain fatty acids, and the hydrophilicity of low-polar CCMs, long-chain fatty acids and bile acids, were significantly increased, resulting in a remarkable separation efficiency for which 68 CCMs can be simultaneously determined. Furthermore, the polarity-tuning effect was confirmed to be induced by the different impacts of aliphatic chains and nitrogen atom in DIAAA, the latter existing as a cation in the acidic mobile phase, using different derivatization reagents. Finally, this derivatization method was utilized to hunt for the potential biomarkers in colorectal cancer (CRC) patients and 52 CCMs, related with several key metabolic pathways including amino acids metabolism, TCA cycle, fatty acid metabolism, pyruvate metabolism and gut flora metabolism, were identified. This innovative polarity-tuning derivatization-LC-MS approach was proved to be a valuable tool for probing global metabolome with high separation efficiency and sensitivity in various biological samples.



Carboxyl-containing metabolites (CCMs) widely exist in living system and are the essential components for the life. CCMs mainly contain amino acids (AAs), TCA cycle intermediates (TCAs), short-chain fatty acids (SCFAs), long-chain fatty acids (LCFAs), bile acids and so on. AAs have been central to the study of cancer metabolism and are required for the synthesis of protein, nucleotide and cell growth.[1,2] TCA cycle is an important route for oxidative metabolism and also associated with highly proliferating cells, like tumour cells.[3] SCFAs, such as butyric acid (BA) and propionic acid (PA), have beneficial effects on anti-inflammation and are the key determinants of diseases in intestinal microbiota.[4] Long-chain fatty acids (LCFAs), for example, hydroxyeicosatetraenoic acids (HETEs, ω-6), prostaglandins (PGs, ω-6), thromboxanes (TXs, ω-6), and hydroperoxyeicosapentaenoic acids (HEPEs, ω-3), play vital roles in cancer,[5,6] inflammation,[7] and other biological activities.[8] Bile acids influence the composition of the gastrointestinal microbiota and host metabolic pathways through the reciprocal microbe-host crosstalk.[9] Moreover, some SCFAs and TCAs are the precursors of biosynthesis of LCFAs.[10] Therefore, the global determination for CCMs profile is critical for the understanding of physiological processes and the discovery for the onset of relevant diseases.

Colorectal cancer (CRC) is the second most common cancer in the world.[11] The diet, inflammation, and loss of tumor suppressor gene are the key risk factors contributing to the high incidence of CRC. Recent studies further indicated that dysbiosis of commensal gut microbiota and its metabolites

also play an important role in the development of CRC.[12] However, it is unclear which metabolites and how they influence the process of CRC development. Thus, there is an urgent need to clarify the metabolic variations in CRC to improve the diagnosis and treatment of the disease.

Though gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) have been widely used for CCMs analysis,[13,14] the thermal instability of some CCMs hinders the use of GC-MS,[15] and LC-MS has to face the problems of retention and separation on the column owing to the marked polarity differences between hydrophilic CCMs (AAs, TCAs and SCFAs) and hydrophobic CCMs (LCFAs and bile acids), high polarity similarities among different hydrophilic or hydrophobic CCMs, as well as poor ionization efficiency.[16] Various derivatization methods, e.g. diisopropylphosphoryl,[17] *p*-dimethylaminophenacyl bromide[18] for hydrophilic CCMs; N,N-dimethylaminobutylamine,[19] cholamine[20] and 2-dimethylaminoethylamine[21] for hydrophobic CCMs have been developed to solve these difficulties. However, until now, the comprehensive analysis of global CCMs could not be reached yet.

In this research, 5-(diisopropylamino)amylamine (DIAAA) was first applied to establish a highly sensitive derivatization ultra-high performance liquid chromatography-quadruple-time of flight mass spectrometry (UHPLC-Q-TOF/MS) approach, that achieved the simultaneous determination of 68 CCMs belonging to 6 different types, which have a large polarity differences (Scheme 1 and Figure 1). Furthermore, utilizing the newly developed approach, the potential biomarkers in the sera of CRC patients were determined and the influence of microbial metabolites on the process of CRC was disclosed.

EXPERIMENTAL SECTION

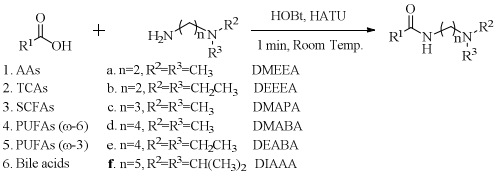
Chemicals and reagents. Amino acids mixtures, TCA cycle intermediates, short-chain fatty acids, bile acids, and 4-chloro-DL-phenylalanine were purchased from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO). All long-chain fatty acid standards mixtures and the isotope labeled internal standards were provided by Cayman Chemical (Ann Arbor, MI). The detailed information of CCMs was shown in supporting information.

5-(Diisopropylamino)amylamine (DIAAA), N,N-dimethylethylenediamine (DMEEA), N,N-diethylethylenediamine (DEEEA), 3-(dimethylamino)-1-propylamine (DMAPA), 4-dimethylaminobutylamine (DMABA), 4-(diethylamino)butylamine (DEABA), *O*-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxybenzotriazole hydrate (HOBt), triethylamine (TEA) and DMSO were bought from Sigma-Aldrich Laboratories, Inc. LC-MS grade acetonitrile and HPLC grade methanol were obtained from Anaqua Chemicals Supply Inc., Ltd. (Houston, TA). Other reagents were purchased from Sigma-Aldrich Laboratories, Inc. Deionized water was prepared using a Millipore water purification system. DIAAA was purified by prepared HPLC before use.

Sample preparation. Fifty μL of serum was first mixed with 4 times volume of cold methanol to remove the proteins by centrifugation at 13,000 rpm for 5 min at 4 °C. The extraction was repeated 3 times and the combined supernatants were dried under a nitrogen stream. The residue was stored at -20 °C prior to derivatization. In addition, to assess the influence of other endogenous components in serum on the determination of CCMs, the recovery and matrix effects were assessed according to the previously described procedure (Supporting Information).[20] As well, the stability was also evaluated.

Derivatization. The reaction solutions were firstly prepared as follows, i.e. HOBt and HATU were separately dissolved in DMSO at the concentration of 20 mM, while the diamine-TEA solution, i.e. DMEEA-, DEEEA-, DMAPA- DMABA-, DEABA- and DIAAA-TEA solution, was prepared by dissolving 100 μmol of corresponding diamine and 200 μmol of TEA in 1 mL of DMSO. Thereafter, the dried residue of real samples was sequentially mixed with 5 μL HOBt, 5 μL DIAAA-TEA solution, and 5 μL HATU followed by 1 min incubation at room temperature. As well, the dried residue of standards were sequentially mixed with 5 μL HOBt, 5 μL DMEEA-, DEEEA-, DMAPA- DMABA-, DEABA- or DIAAA-TEA solution, and 5 μL HATU followed by 1 min incubation at room temperature. Finally, 35 μL acetonitrile was added to make up to the final volume of 50 μL, and 1 μL was directly injected into UHPLC-Q-TOF/MS.

UHPLC-Q-TOF/MS analysis. An Agilent 1290 Infinity LC system (UHPLC, Santa Clara) and binary pump with Waters ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm) was employed for the separation of CCMs. The mass spectrometry was conducted on an Agilent 6550 UHD accurate-mass Q-TOF/MS system with a dual Jet stream electrospray ion source (dual AJS ESI). The instrument was operated in positive (POS) and negative (NEG) ion modes. For the detailed parameters of UHPLC-Q-TOF/MS, please see the Supporting Information.



Scheme 1. Derivatization of carboxyl-containing metabolites with different reagents.

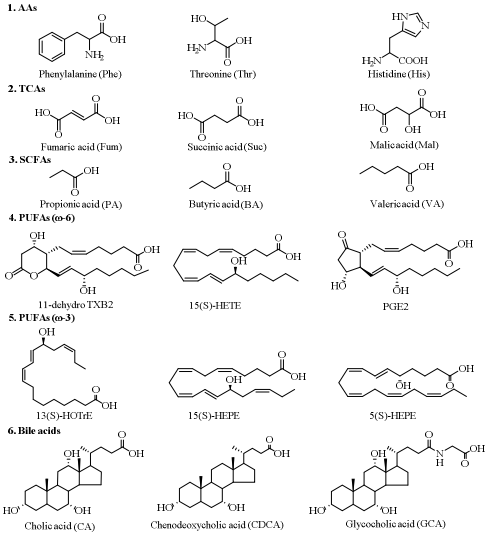


Figure 1. Representative structures of carboxyl-containing metabolites.

Linearity. The calibration curves were established by plotting the peak area ratios of the standards to the corresponding internal standard against the concentrations.

CRC and healthy human serum. All the experiments were performed in accordance with the first affiliated hospital, Sun Yat-sen University Ethics Committee's guidelines and regulations. The serum samples of 20 healthy human and 20 CRC patients were recruited and CRC was diagnosed by the histopathological methods according to the Union Internationale Contre Le Cancer (UICC), while the healthy volunteers were selected by a routine physical examination. The samples were stored at −80 °C before use.

Animals and experimental design. $Apc^{Min/+}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME) for breeding. Mice were housed in a 12-h light-dark cycle facility with controlled room temperature and humidity and were fed with PicoLab®Rodent Diet 20-5053 (LabDiet, St. Louis, MO). Blood was collected from the main celiac vein of 14-weeks old $Apc^{Min/+}$ and wide type C57BL/6J mice as described.[22] Fresh blood sample was centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatant was taken out and stored at −80 °C for later usage. The mice were sacrificed for ploys count in the intestines. The $Apc^{Min/+}$ mice yielded 30 polyps in average, while the wild-type mice produced none detectable polyp.

RESULTS AND DISCUSSION

Improvement of sensitivity and separation efficiency of CCMs by DIAAA-derivatization. As mentioned in Introduction, CCMs play pivotal roles in various physiological functions and may serve as potential biomarkers in many diseases, thus a simple, rapid, sensitive and global method is required to quickly detect the metabolites in biological samples. In this research, a derivatization method using DIAAA, was developed. First, the reaction conditions were optimized using the representative CCM standards (Figure 1), phenylalanine (Phe), threonine (Thr), histidine (His), fumaric acid (Fum), succinic acid (Suc), malic acid (Mal), PA, BA, valeric acid (VA), 11-dehydro TXB2, 15(S)-HETE, PGE2, 13(S)-HOTrE, 15(S)-HEPE, 5(S)-HEPE, cholic acid (CA), chenodeoxycholic acid (CDCA), and glycocholic acid (GCA). It was found that the reaction could complete within 1 min at room temperature (Figure S1). And, no evident by-product was observed.

Then, using the above optimized method, 68 CCMs were derivatized with DIAAA followed by UHPLC-Q-TOF/MS analysis. It was found that the ionization efficiency of DIAAA-derivatized CCMs remarkably enhanced, which induced the improvement of detection sensitivity up to 2000 times (Table S1). More importantly, the retention times for hydrophilic CCMs, AAs, TCAs and SCFAs, were greatly prolonged from 0.5-3 min to 0.5-16.5 min (Figure 2, from region A1 to B1) after derivatization. Conversely, the retention times for hydrophobic CCMs, LCFAs and bile acids, shortened from 27-41 min to 21.5-39 min (Figure 2, from region A2 to B2). As a result, 68 CCMs can be well separated within 40 min, otherwise, only 45 CCMs can be detected with very weak MS signals and heavy peak overlap using non-derivatization method. For example, the isomers of HETEs and epoxyeicosatrienoic acids (EETs) could reach to baseline separation, which is essential for the identification and quantification of these compounds. Over all, the DIAAA-derivatization not only achieved the simultaneous determination of hydrophilic and hydrophobic metabolites, but also improved the separation resolution (Figure 2).

Good linearities of calibration curves were obtained for most of CCMs with the coefficients of determination (R^2) greater than 0.99 (Table S2). The derivatized metabolites were stable in 4 °C for at least 3 days. Other endogenous components in serum were found to have no significant influence on the determination of CCMs from the matrix effect and recovery (Table S3).

Mechanism of polarity tuning induced by DIAAA-derivatization. However, why did DIAAA-derivatization reverse the retentions of hydrophobic and hydrophilic CCMs on C18 column?

As shown in Scheme 1, DIAAA-derivatization introduced a new amino group into the CCMs. The introduced amino group could be protonated in the acidic mobile phase, which made the LCFAs and bile acids derivatives acted as the cationic surfactants[23,24] and reduced their interaction with C18 solid phase. Compared to the large lipophilic groups in LCFAs and bile acids, the introduction of small hydrophobic groups in DIAAA should have relatively low contribution. Finally, the retention times shortened (Figure S2).

Differently, an aliphatic chain with five methylene and two side chains of N-isopropyls in DIAAA have big influence on the hydrophobicity of polar CCMs (AAs, TCAs and SCFAs), which might be the major reason for the increase of retention time. To confirm it, similar diamine derivatization reagents with various main chain lengths and side chains were investigated, and they were DMEEA (a, $C_4H_{12}N_2$), DEEEA (b, $C_6H_{16}N_2$), DMAPA (c, $C_5H_{14}N_2$), DMABA (d, $C_6H_{16}N_2$), and DEABA (e, $C_8H_{20}N_2$) (Scheme 1). The results showed that the derivatization with DMEEA, DMAPA, or DMABA, which had two N-CH_3 and a chain with 2-4 carbons, decreased the retention times for all hydrophilic CCMs as expected. When derivatizing with DEEEA and DEABA, which containing two N-CH_2CH_3 groups and a 2- or 4-carbon chain respectively, the retention times were longer than that of DMEEA, DMAPA or DMABA derivatives, and the retention for most of DEABA-derivatized AAs and TCAs were even longer than that of non-derivatized CCMs, while they were still shorter than that of corresponding DIAAA derivatives (Table 1 and Figure S3). Consequently, the lipophilic groups in diamine reagents indeed influenced the retentions of hydrophilic CCMs on C18 column. Moreover, the side chains had more effect than the main chain, and more carbons the chain had, longer the retentions for most of hydrophilic CCMs were. Finally, DIAAA with two N-$CH(CH_3)_2$ and five methylene chain were found to be the best choice (Table 1). On the other hand, the change of lipophilic groups in the derivatization reagents had the similar influence on the retentions of LCFAs and bile acids derivatives, while all derivatizations induced the shorter retentions compared to non-derivatization metabolites. The above experiments confirmed that the contributions of the lipophilic and hydrophilic groups in derivatization reagents resulted in the polarity-tuning of DIAAA-derivatized CCMs (Figure S2).

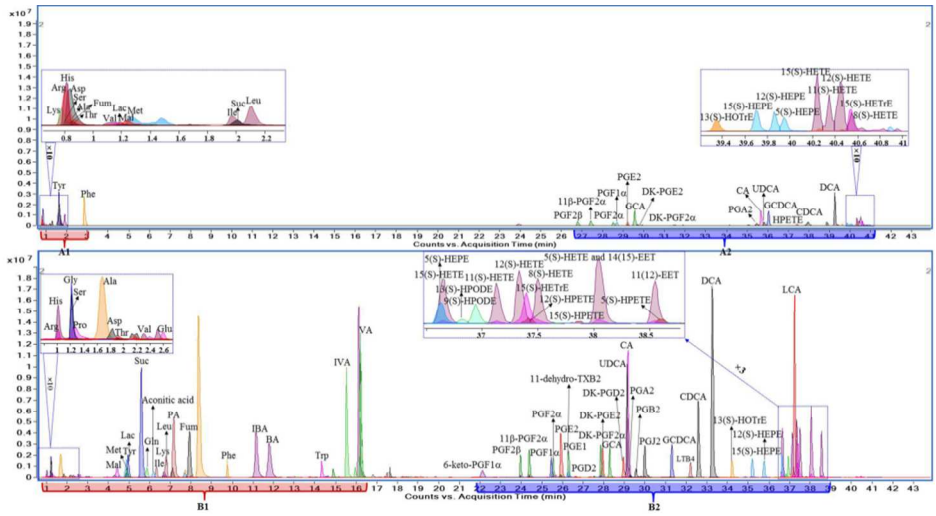


Figure 2. LC-MS chromatograms of carboxyl-containing metabolite standards (A) and DIAAA-derivatives (B). After derivatization, the carboxyl-containing metabolites in regions A1 and A2 appeared in regions B1 and B2, respectively.

Table 1. Retentions of carboxyl-containing metabolite standards and corresponding derivatives on HSS T3 (C18) column.

Classification	Name	No.	derivatization reagent	Retention time (min)
AAs	Phe	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	Thr	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	His	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
TCAs	Suc	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	Fum	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	Mal	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
SCFAs	BA	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	PA	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	VA	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	

Classification	Name	No.	derivatization reagent	Retention time (min)
PUFAs ω-6	11-dehydro-TXB2	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	15(S)-HETE	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	PGE2	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
PUFAs ω-3	13(S)-HOTrE	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	15(S)-HEPE	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	5(S)-HEPE	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
Bile acids	CA	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	CDCA	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	
	GCA	1	Non-derivatization	
		2	DMEEA	
		3	DEEEA	
		4	DMAPA	
		5	DMABA	
		6	DEABA	
		7	DIAAA	

Application in sera of CRC patients. Finally, the DIAAA derivatization was applied to probe the changes of CCMs between CRC patients and healthy volunteers. By comparison with the corresponding standards, 52 CCMs were determined, as well, 30 CCMs in CRC patients including 11 AAs, 3 TCAs, 5 SCFAs, 9 PUFAs, and 2 bile acids were significantly decreased. Furthermore, the detected CCMs were found to be involved in several key metabolic pathways including amino acids metabolism, TCA cycle, fatty acid metabolism, pyruvate metabolism and gut flora metabolism (Table S4).

Among the CCMs, the SCFAs group has profound effects on the health of gut microenvironment. It regulates gut epithelial proliferation and immune responses. The decrease of SCFAs in feces and the SCFA-producing bacteria were found to be associated with the etiology of CRC.[25,26] Our results showed that five SCFAs, PA, BA, isobutyric acid (IBA), isovaleric acid (IVA) and VA, were all decreased in the sera of CRC patients compared to the normal controls (Figure 3), which well echo the previous findings.[25,26] The area under the receiver operating characteristic (ROC) curves of PA (0.81), BA (0.80), IBA (0.79), IVA (0.80) and VA (0.80) indicated they should be potential markers. To further confirm the relationship between SCFAs and CRC, SCFAs in $Apc^{Min/+}$ mice, a well-established animal model of intestinal cancer, were examined and compared with that in wild type mice. In this model, *Clostridiaceae*, *Lachnospiraceae* and *Ruminococcaceae* bacteria, which generate SCFAs, were found to be decreased.[27] Similarly to CRC patients, IBA, IVA and VA significantly decreased in $Apc^{Min/+}$ mice compared with wild type C57BL/6J mice (Figure S4). The contents of PA and BA were lower than that in control group, while the difference was not statistically significant. Collectively, these data presented here clearly indicated that variations of these SCFAs had a close relationship with CRC. This significant differentiation between the normal and CRC in the levels of SCFAs may provide another non-invasive diagnosis for CRC. In addition, this is the first report on the SCFAs in the sera of CRC patients.

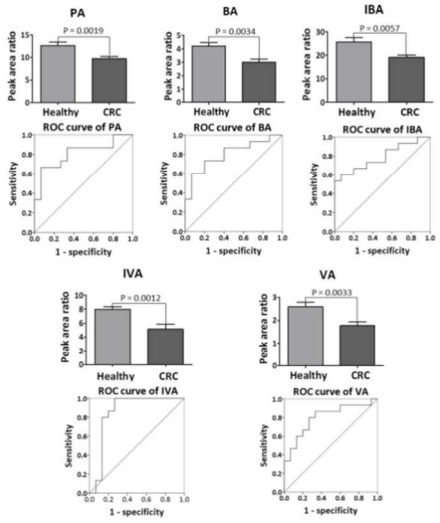


Figure 3. Comparisons and ROC curves of SCFAs in CRC human serum. All SCFAs decreased in the sera of CRC patients, compared to the normal controls.

Additionally, DIAAA-derivatization method was also successfully applied to the determination for the metabolites in cell line, serum, feces, cerebrospinal fluid and tea. Compared with non-derivatization method, more metabolites could be sensitively detected after DIAAA-derivatization (Figure S5).

CONCLUSION

In summary, DIAAA-derivatization coupled with an UHPLC-Q-TOF/MS analysis was developed for mapping global CCMs. The aliphatic chains and nitrogen atom in DIAAA induced the polarity tuning of polar and low-polar CCMs, which facilitated the high separation efficiency. At the same time, the sensitivities were also enhanced up to 2000 times. This method was successfully applied for the investigation on the potential biomarkers in CRC patients and animal model. This innovative CCMs probe was proved to be a valuable tool for in-depth study on global metabolome, especially for CCMs with high polarity differences.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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