Relationship between cancer tissue derived and exhaled volatile organic compound from colorectal cancer patients. Preliminary results

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ABSTRACT

New insight into the omic sciences suggests that volatile organic compounds (VOCs) contained in exhaled breath can reflect the healthy or disease state of patients, representing an attractive, promising and non-invasive method of medical investigation. This approach has recently been proposed as a new potential screening tool in colorectal cancer (CRC) patients. However, a possible correlation between the exhaled VOCs and those produced by the cancerous tissue has never been investigated. In this preliminary study, we compare the VOCs exhaled by seven patients affected by CRC with those produce by own cancer tissue and normal colonic mucosa.

The VOCs contained in the exhaled breath were sampled with the ReCIVA breath sampler©, while those produced by ex-vivo human tissues weresampled by headspace solid-phase microextraction (HS-SPME) at different incubation times after surgery. In both cases, the collected VOCs were analyzed by Gas Chromatography with Mass Spectrometry (GC–MS).

Benzaldehyde, benzene ethyl, benzene methyl, butanoic acid, dodecanoic acid, indole, nonanal, octanoic acid, pentanoic acid, phenol and tetradecane were the VOCs most frequently detected both in the exhaled breath and secreted by tissues.

The results showed that cancer tissue and normal colonic mucosa from the same patient produced a similar VOCs pattern but with different fingerprints. In particular, the concentrations of benzaldehyde, benzene ethyl and indole were significantly different in cancer tissue respect the normal colonic mucosa.

In conclusion, these preliminary data suggest the involvement of the three compounds in CRC by encouraging further investigation.

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1. Introduction

Colorectal cancer is one of the main causes of cancer related death of the last twenty years, both in USA and in Europe [1–4], and the third most diffuse malignant tumour worldwide [5]. However, the 5-years survival rate could be increased up to 90 %, if CRC is diagnosed in its early phase [4]. Therefore, the development of an effective and reliable screening tool is of paramount importance in any national health system program.

The most commonly used screening tests in Europe, the faecal immunochemical tests for human haemoglobin (FIT) or the faecal occult blood test (FOBT), suffer of a very low patient compliance and insufficient reliability (positive predict value of 10 %) [6]. The detection of several specific cancer biomarkers in the blood, e.g., the carcinoembryonic antigen and the carbohydrate antigen 19-9, the long DNA fragments, the miRNA, the liquid biopsy has been experimented with low success, due to the poor sensitivity and specificity for CRC [5,7,8].

Colonoscopy remains the most accurate and reliable method for CRC diagnosis, but its use as a screening tool is prevented by the invasiveness of the procedure, the high costs, patient low compliance and by the risk of iatrogenic colonic perforation [5,9].

Therefore, non-invasive cancer tests based on the quantification of VOCs released by urine, blood, stool or contained in the breath have received increasing attention and represents an interesting expanding area of research [10,11].

The volatile fraction of metabolome, or volatilome [12], contains the VOCs generated within the human organism and reflects the metabolic processes in the body, which may change after exposure to toxic compounds or in the presence of diseases, particularly in cancer [13–15].

It is believed that wherever the cancer is in the body, the VOCs released by the cancer tissue into the bloodstream are subsequently eliminated from the human body through the lung alveoli as components of exhaled air [16,17]. Breath analysis represents an attractive, promising and non-invasive mean of access to the volatilome, despite its complexity, mainly due to the possible environmental pollution [18,19].

Several investigations have confirmed that different patterns of VOCs are exhaled by lung [20,21], breast [22], gastric [23] and colorectal cancer patients [24],as a consequence of the abnormal cancer tissues metabolic processes, and preliminary studies pointed out that the analysis of exhaled VOCs can discriminate CRC patients from normal individuals [24,25].However, none of the proposed potential biomarkers sets has yet reached clinical relevance for each type of cancer, since up to now, no biomarkers have been demonstrated to be exclusive of different cancers and no specific threshold of VOCs concentration has been demonstrated to be able to identify cancer patients from healthy subjects. Furthermore, different patterns of VOCs with good reliability in identification of CRC patients have been identified [11,24]. Previous in vitro studies on different cell lines from human cancers showed that the released VOCs are "fingerprints" of each type of cancer [26].

This study is aimed to find any correlation between the VOCs contained in the exhaled breath of patients affected by CRC and the VOCs released by their surgical resected cancer tissue.

2. Material and methods

2.1. Chemicals and standards

Helium and nitrogen (purity 99.999 %,) were purchased from *Sapio s.r.l.* (TA, Italy), air (purity 99.988 %) form *Sol s.p.a.* (MB, Italy), while all chemicals (purity ≥ 97 %) from *Sigma–Aldrich* (MI, Italy).

Stock solutions of each volatile compound ($20 \, mg \, mL^{-1}$) were prepared in methanol, stored at 8 °C and daily dilute, in fresh culture medium (Dulbecco's Modified Eagle Medium, *Euroclone,* MI, Italy), to prepare working solutions.

2.2. Exhaled breath sampling and analyses

After obtaining written informed consent, the breath of seven CRC patients (four men and three women, mean age of 65 ± 10 years, cancer located in right colon, stage III) was sampled. Furthermore, the exhaled breath of twenty healthy subjects (eight men and twelve women, mean age 60 ± 7 years) with a negative colonoscopy performed within the last 2 years, was also sampled and compared with CRC patients. All the subjects were fasted from at least 4 h and the breath was sampled using a ReCIVA breath sampler© always in the same room, aerated for 30 min before each sampling. The ReCIVA is a portable device which allows selection of different volumes of exhaled breath fraction (alveolar, bronchial), using an infra-red CO_2 sensor when respiratory rate is between 15−25 rpm. To prevent contamination from environmental polluting agents, the patient breathed medical air in a suitable mask during the breath sampling. For each patient, 500 mL of alveolar air was gathered on stainless steel biomonitoring sorbent tubes, containing a mix of Tenax© and Carbograph© phases, able to retain C4-C30 compounds.

The VOCs contained in the exhaled breath, collected in the ReCIVA sorbent tubes©, were desorbed with a thermal desorber (TurboMatrix 350, PerkinElmer, MA, USA), directly connected to the gas chromatograph with a heated transfer line. The desorber was provided with a carousel which allows to process up to 55 sorbent tubes in sequence. Each sorbent tube was heated for 20 min at 250 °C and the desorbed VOCs were directly transferred in the gas chromatograph injector at 200 °C, operating in splitless mode, utilizing helium as carrier gas, at the linear velocity of 0.6 cm s^{-1}.

The separation and quantification of desorbed VOCs was performed with a gas chromatograph (Trace GC Ultra, Thermo Scientific, MA, USA) coupled with quadrupole mass spectrometer (ISQ, Thermo Scientific). A 30 m x0.25 mm i.d., 1.4 μm film thickness, capillary column DB-624 UI (Agilent, CA USA) was utilized with the following oven temperature program: 40 °C for 5 min, then ramped 6 °C min^{-1} to 160 °C, 10 min at 160 °C, ramped 6 °C min^{-1} to 200 °C, 15 min at 200 °C, ramped 6 °C min^{-1} to 220 °C and 5 min at 220 °C.

The temperatures of the transfer line and the ion source of quadrupole were 280 °C and 220 °C, respectively. The mass spectrometry was performed at 70 eV electron impact ionization energy, in full-scan mode, with scan range 40–250 amu.

The XCalibur software (Thermo Scientific) allowed acquisition and elaboration data.

In order to prevent memory effects, after each analysis, two empty ReCIVA steel tubes© (without adsorbent phase) were analyzed to remove eventual residues of the previous sample from the desorber and analysis apparatus.

2.3. Colon tissues sampling and analyses

Samples of fresh cancer tissue and normal colonic mucosa were obtained from the colonic specimen of seven patients (four men and three women, mean age of 65 ± 10 years, cancer located in right colon, stage III) submitted to curative surgery. Immediately after resection, the tissues were cut in weighted biopsies of $0.5 \pm 0.0.5$ g and hermetically sealed in a 7 mL screw top amber glass vial with a PTFE/silicone septum (Sigma-Aldrich), containing 1 mL of sterile culture medium. Within 30 min from the resection, the vial was incubated in a thermostatic bath set at 37 °C and the analyses were performed after 1, 2, 3 and 7 days of incubation.Simultaneously, 1 mL of culture medium was transferred to a similar vial, subjected to the same analysis used as blank.

The VOCs produced by the tissues and accumulated in the vial head space were sampled, extracted and concentrated with a head space solid phase microextraction (HS-SPME), utilizing 1 cm long fibers (divinylbenzene/carboxen/polydimethylsiloxane, 50/30 μm; Sigma–Aldrich) by means of a manual holder (Supelco, PA, USA). The fibers were pre-conditioned according to the manufacturer instructions to avoid memory effects (5 min in the gas chromatograph injector at 280 °C), inserted into the sealed vial, by manually penetrating the septum, and then exposed for 15 min at 37 °C to the head space of the vial containing the incubated tissue. After the sampling, the SPME fiber was analyzed by means of a gas chromatograph (Trace GC Ultra Gas, Thermo Scientific) coupled to an ion trap mass spectrometer (Polaris Q, Thermo Scientific). The SPME fiber was inserted into the GC injector (splitless mode) for 5 min at 200 °C, for the desorption of collected VOCs. The chromatographic separation was performed with a capillary column TRACE TR-5MS (Thermo Scientific), 30 m x0.25 mm i.d., 0.25 μm film thickness. The oven temperature program was40 °C for 5 min, then ramped 3 °C min^{-1} to 240 °C and then 5 min at 240 °C.

Helium was used as carrier gas, with linear velocity of 1 mLmin^{-1}. Transfer line and ion source temperatures were 240 °C and 200 °C, respectively. Mass spectra (40–250 m/z) were acquired at 70 eV electron impact ionization energy.
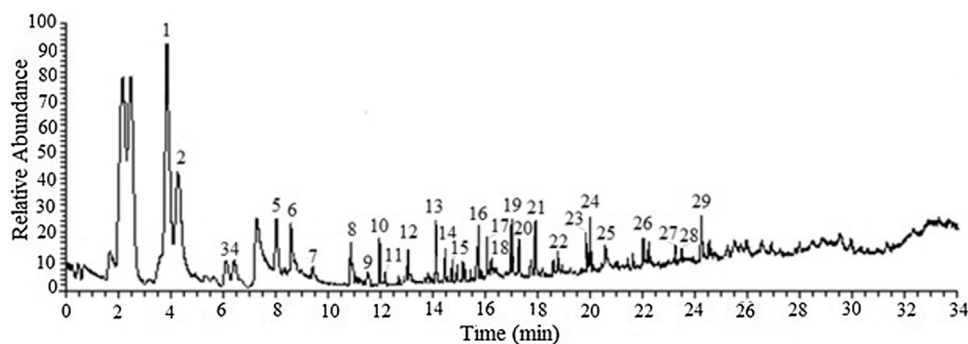
Fig. 1. TIC chromatogram of the VOCs contained in the exhaled breath of CRC patient #5.Peaks relative to identified substances (Table 1S) are numbered.

The XCalibur software, also in this case, permitted acquisition and elaboration data.

Before new sampling, the fiber was re-conditioned for 5 min in the GC injector port at 280 °C, in order to prevent possible memory effects.

Due to the low available quantity of cancer tissue and normal colonic mucosa, it was not possible to carry out replicate measurements on different fragments of the same piece. For the metabolic evolution of the ex vivo systems, it was not even possible to perform replicate analyses on the same vials to evaluate intra- and inter-day variability. For these reasons, the reproducibility of the analytical procedure was tested through replicated measurements of standard solution, containing selected representative compounds: benzaldehyde, benzene ethyl, butanoic acid, indole, nonanal, octanoic acid, phenol and tetradecane, selected as representative of the major classes of detected compounds. The relative standard deviation (RSD %) of peak areas were always $\leq 6,5$ %, for all analytes and, therefore, differences in peak area were considered significant when higher than 14 % (95 % confidence interval, t-Student = 2,09) for inter-day reproducibility, and 28 % (95 % confidence interval, t-Student = 4,30), in case of intra-day evaluation.

2.4. Qualitative and quantitative analysis

Separated VOCs were identified with the MS database of the National Institute of Standards and Technology (NIST). To confirm the identifications of the VOCs contained in the exhaled breath, 1 μl of working solution, containing authentic standards (50 or 100 μgmL^{-1}), was added into a ReCIVA sorbent tube© which was then analyzed with the procedure described above.

The identification of VOCs produced by the tissues during incubation was performed by adding 1 mL, of a standard compound mixture of suitable concentrations, into 7 mL amber glass vials, which were immediately tight sealed, pre-equilibrated for 24 h at 37 °C, and them extracted and analyzed with the HS-SPME GC–MS procedure.

Limits of detection (LOD) and quantification (LOQ) were considered three and ten times the base noise, respectively.

The reproducibility was calculated on a set of three daily measurements (within day), for seven days (between days). For this purpose, a standard mixture of VOCs (1 mL) were dispensed in 7 ml vials, which were immediately sealed, incubated at 37 °C and analyzed after 1, 2, 3, and 7 days.

2.5. Statistical analysis

The Wilcoxon signed rank test was performed in order to compare the average values of the three selected compounds between the cancer and normal tissues. The p-value < 0.05 was considered

Table 1
List of VOCs identified in the exhaled breath of at least 4 CRC patients and their detection frequency.

N#	Compound	Patients frequency
1	acetone	4/7
2	acetonitrile	4/7
3	benzaldehyde	7/7
4	benzene, ethyl	4/7
5	benzene, methyl	6/7
6	butanoic acid	6/7
7	decanal	4/7
8	**diallyl disulfide**	6/7
9	dodecane	4/7
10	**dodecanoic acid**	5/7
11	eptanal	6/7
12	hexanal	5/7
13	**indole**	4/7
14	nonanal	7/7
15	octanoic acid	7/7
16	pentanal	5/7
17	pentanoic acid	6/7
18	phenol	6/7
19	propanoic acid	6/7
20	**tetradecane**	7/7

In bold the substances not detected, by literature, in the exhaled breath of apparently healthy individuals [26].

statistically significant. The analysis was conducted with the R software (3.5.2 version).

3. Results and discussion

3.1. Exhaled breath analyses

The number of VOCs detected (S/N ≥ 3) in the exhaled breath of the seven CRC patients ranged between 29 and 89. Only species with peak areamore than twice the ambient level was considered, since they probably have endogenous origin.

A typical chromatogram of the exhaled breath is reported in Fig. 1 (CRC patient #5).The peaks below 3 min of retention time (RT) were not considered, since characterized by poor resolution and corresponding to ubiquitarian light compounds.Therefore, 29 VOCs were identified (Table 1S).The breath analysis of the seven CRC patients lead to put in evidence 20 VOCs, present at least in four patients (Table 1). Four of these VOCs, diallyl disulfide, dodecanoic acid, indole and tetradecane, were also detected in the exhaled breath of twenty healthy individuals (negative to colonoscopy) with a very low frequency (< 50 %) (Table 2S). These four molecules, moreover, were not mentioned among the 872 VOCs, reported by de Lacy Costello et al. [27], in the exhaled breath of healthy individuals, suggesting the opportunity of exploring their potential role in the identification of CRC patients.
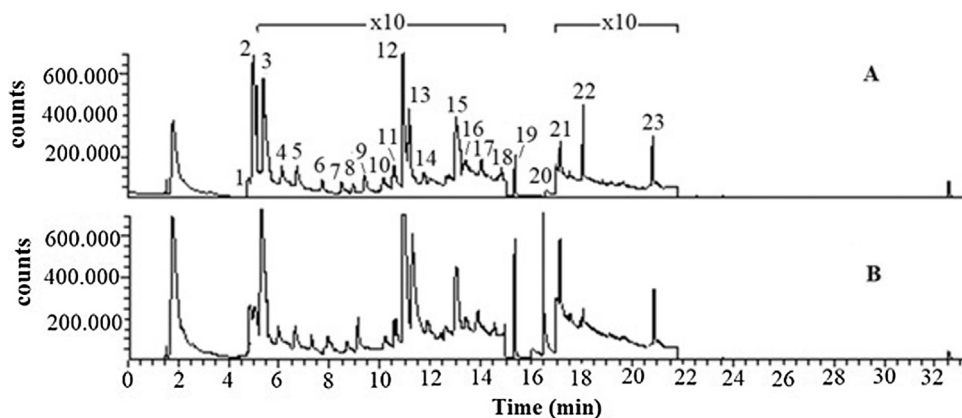
Fig. 2. TIC chromatogram of the VOCs secreted by ex vivo human tissues of patient #5 after 24 h incubation at 37 °C in culture medium. A) normal colonic mucosa; B) cancer tissue. Peaks relative to identified substances (Table 3S) are numbered.

Table 2
List of VOCs, secreted by normal colonic mucosa and cancer tissues of seven CRC patients, also present in the relative exhaled breaths. The detection frequency is also reported.

N#	compound	Patient							Detection frequency
		1	2	3	4	5	6	7	
1	benzaldehyde	*	*			*	*	*	5/7
2	benzene ethyl	*	*		*	*			4/7
3	benzene methyl	*	*	*		*	*	*	6/7
4	butanoic acid		*		*		*	*	4/7
5	dodecanoic acid				*	*	*	*	4/7
6	indole	*	*	*		*			4/7
7	nonanal	*	*		*		*		4/7
8	octanoic acid		*	*		*	*	*	5/7
9	pentanoic acid	*	*		*		*		4/7
10	phenol			*	*	*	*		5/7
11	tetradecane	*	*		*	*		*	5/7

3.2. Tissues analysis

The chromatograms of the VOCs produced by the incubated cancer tissue and normal colonic mucosa of CRC patient N#5 are reported in Fig. 2. Both chromatograms contain the same 27 peaks but with different intensities, showing different fingerprints, as also occurs for the other six patients. Only peaks with intensity more than twice the "blank" sample level were further considered. Table 3S lists the 23 VOCs identified with the NIST library and authentic standards; in particular, the presence of esters of primary alcohols with branched-chain fatty acids, and putrefactive substances can be noticed.

Branched-chain fatty acids, such as butanoic acid 2-methyl, butanoic acid 3-methyl, indole, phenols and propanoic acid 2-methylare not produced by human enzymes, but they are unique colonic bacterial metabolites [28]. For instance, indoles and phenols are generated during the bacterial degradation of tyrosine and tryptophan, respectively, while the sulfur containing species form for the fermentation of sulphur amino acids by sulfate-reducing bacteria. These findings are intriguing since the role of the gut microbiota, a group of 10^{14} bacteria, eukaryotes and virus living in gastrointestinal tract, is nowadays believed of great relevance in the carcinogenesis of human colorectal cancer [29].

The direct role of some of these VOCs in human cancer has been also suggested by other studies. For instance, it has been shown that changes to cell walls influence the action of tetradecane, which can destroy the integrity and functional properties of cells, promoting the incorporation of substantial amounts of equivalent-chain-length fatty acids into cell membrane lipids [30]. Dodecanoic acid, on the other hand, a saturated equivalent-chain-length fatty acid, has been shown to trigger apoptosis in colon cancer cells, through oxidative stress, promoting inflammatory processes, activating the nuclear factor-κB transcription factor, as well as stimulating the expression of cyclooxygenase-2 and pro-inflammatory cytokines CRC cells [30].

Eleven out of the twenty-seven volatile compounds identified in the tissues of the CRC patients were also present in their exhaled breaths (Table 2). Replicate measurements of the same tissue samples after 48, 72 h and after seven days of incubation from surgery, confirmed the constant production of these VOCs. Analytical reproducibility data of eight of the early cited eleven compounds, selected as representative of the different classes of substances, are reported in Table 3.

The chromatograms of the cancer tissue and normal colonic mucosa for each patient were examined in order to find significant increments/decreases of the VOCs peak area (percent difference higher than 14 %, intra-day variability) passing from the normal colonic mucosa to the cancer tissue. Three compounds, benzaldehyde, benzene ethyl and indole, showed relevant difference of concentration. In particular, the production of benzaldehyde and indole was found significantly higher for the cancer tissue respect to the normal colonic mucosa (p-value 0.016 and 0.022, respectively) Benzene ethyl, on the contrary, appeared reduced in presence of cancer but no statistical significance was reached.

The proposed HS-SPME–GC–MS method was tested with a linear regression analysis of the peak area versus the analyte concentration, utilizing standard solutions of suitable concentration. The linearity, the limit of detection and limit of quantification for benzaldehyde, benzene ethyl and indole are reported in Table 4. Finally, Table 5 resumes the average concentration for each of the three selected VOCs and the relative standard deviation.

Table 3
Analytical reproducibility data.

N#	Compound	Concentration (μg/mL)	RSD % intra-day (n = 3)	RSD % inter-days (n = 21)
1	benzaldehyde	0.6	5.9	6.5
2	benzene ethyl	1.5	3.8	6.0
3	butanoic acid	0.06	4.1	5.9
4	indole	0.5	4	5.8
5	nonanal	0.5	3.6	6.5
6	phenol	1.5	6	6.5
7	octanoic acid	0.06	4.8	6.4
8	tetradecane	0.5	3.5	6.5

Table 4
Linearity, limit of detection (LOD) and limit of quantification (LOQ) for benzaldehyde, benzene ethyl and indole, analyzed with HS-SPME and GC–MS. Y is the peak area in a.u., x, the concentration in mg of VOC per mL of culture medium.

Compound	Equation	R2	Linear range (μg/mL)	LOD (S/N = 3)		LOQ (S/N = 10)	
				μg/mL (medium)	μg/g (tissue)	μg/mL (medium)	μg/g (tissue)
benzaldehyde	$Y = 5*10^4 x + 3091$	0.9957	0.090-9.00	0.030	0.06	0.090	0.18
benzene ethyl	$Y = 3*10^5 x - 2233$	0.9348	0.140-14.00	0.040	0.08	0.140	0.28
indole	$Y = 2*10^4 x + 36$	0.9993	0.03-3.00	0.01	0.02	0.03	0.06

Table 5
Average concentration and p-values for benzaldehyde, benzene ethyl and indole, secreted by normal colonic mucosa and cancer tissues and analyzed with HS-SPME and GC–MS.

Compound	Average concentration normal colonic mucosa (μg/g)	Average concentration cancer tissue (μg/g)	p-value
benzaldehyde	0.24 ± 0.08	0.61 ± 0.13	0.016
benzene ethyl	0.21 ± 0.06	0.13 ± 0.05	0.078
indole	0.08 ± 0.03	0.55 ± 0.20	0.022

The data clearly show that, respect to the normal colonic mucosa, cancer tissue is characterized by higher release of benzaldehyde and indole; this could be probably due to the increase of the metabolic processes rate resulting in their formation, catalysed by the cancer disease which, on the contrary, slows down production of benzene ethyl.

Limitations of the reliability of the data, described in this study, include the small sample size and the high biological variations of the metabolic processes *in vivo*, which can influence the pattern of VOCs detected in exhaled breath. Furthermore, the different pattern found in exhaled breath and tissues headspace can reflect different ability of these molecules to pass through the alveolar-capillary membranes.

4. Conclusions

In this paper it was tried to find a correlation between the fingerprint VOCs identified in the exhaled breath of people affected by CRC and the molecules secreted by their normal colonic mucosa and cancer tissue.

At the end of a transversal qualitative and quantitative study, it was found thatbenzaldehyde, benzene ethyl and indole were detected, at the same time, in the exhaled breath and in the head spaceof the tissues, of 57 % at least, of the patients considered. Benzaldehyde and indole concentration, moreover, rose in the pathological tissue, suggesting an increase of the rate of the metabolic processes which led to their formation catalysed by disease. The high level of indole suggests a possible speculation on the role of gut microbiota in human cancerogenesis.

Future studies will be focused on establishing the possible role of the three substances highlighted as CRC biomarkers, in order to a non-invasive diagnosis trough the simple human breath analysis.

Authors contributions

This study was designed, directory and coordinated by Professor D.F. Altomare. D.F. Altomare, together Dr. N. De Vietro and Dr. A. Aresta, as the principal investigators, provided conceptual and technical guidance for all aspects of the project. D.F. Altomare performed surgeries and take the histological samples. Dr. M.T. Rotelli and Dr. C. Lippolis provided to catalogue the resected cancer and normal colonic mucosa tissues and to store them in the culture medium. A. Aresta and N. De Vietro realized the sampling of the VOCs secreted by human tissues by SPME and effected the subsequent GC–MS analysis. N. De Vietro and A. Aresta, moreover, realized the GC–MS analyses of the sorbent tubes containing the expired breath of the patients, sampled by Catia Lippolis, in collaboration with Dr. A. Picciariello, employing the ReCIVA© device. N. De Vietro and A. Aresta provide also to validate the analytical methods pointed out and elaborated all the experimental data.

The manuscript was written by N. De Vietro, A. Aresta, C. Zambonin and M.T. Rotelli and commented on by all the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.113055.

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