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From pollen grains to functionalized microcapsules simply using ionic liquids

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Here, we report a simple chemical approach to isolate sporopollenin capsules from pollen grains based on the use of ionic liquids (ILs). Depending on IL structure, it is possible to remove the cellulose-rich cell wall (intine) assuring grain integrity and contemporaneously obtaining a direct functionalization of sporopollein.

Conventional manufacture of polymeric microcontainers with a uniform size distribution and large inner cavity is costly and difficult, especially when the target are microcapsules.[1–3] Thus, the possibility to obtain them from pollen grains or spores has gained increasing attention in the last years. Pollen grains are multicellular male gametophytes of seed plants, that have vegetative and generative cells enclosed in a multilayered cell wall constituted by two components: a cellulose-rich cell wall (intine) and a highly resistant outer wall composed largely of sporopollenin (exine). This latter component can provide microcapsules of different size and shape providing a natural solution to the development of smart drug delivery vehicles, cell encapsulating scaffolds and biotemplates for advanced materials.[4-10] Exine morphology varies indeed significantly between pollen species retaining however highly species peculiar and reproducible 3D architectures and morphologies. It is noteworthy that sporopollenin capsules with a large internal cavity, interconnected pores, uniformity in size, and containing only carbon, hydrogen, and oxygen are highly biocompatible and allergen free and, depending on the source, available in abundant quantities.[11,12] Moreover, they are characterized by an extraordinary resistance to chemical degradation or dissolution, contemporaneously being readily amenable to derivatization. Described as a network of functional groups, such as phenols, polyunsaturated acids, with carotenoid type linkers, the sporopollenin structure is still undetermined, despite the recent research efforts.[13-16] Controversial on structural features are at least in part attributable to the chemical processes applied to remove all interior and surface pollen constituents generally based on harsh and time consuming multi-step procedures, including the use of organic solvents, alkaline-lysis, acido-lysis, and enzymatic processes, which are able to modify sporopollenin structure.[4,5,17] The development of a simple chemical process to purify sporopollenin is therefore highly desirable.

Recently, ionic liquids (ILs) have attracted widespread attention for their ability to dissolve many biopolymers that are insoluble in conventional solvents, such as cellulose, chitin, chitosan, lignin.[18] Constituted exclusively by ions, these salts liquid at/or near room temperature present several unique physico-chemical properties, such as a negligible vapour pressure, a high conductivity and, generally, non-flammability.[19] Additionally, their chemical and physical properties can be fine-tuned through a proper choice of the cation and anion, effectively making them designer solvents. For these reasons, ILs have been intensively researched for the last two decades as supposedly green alternatives to conventional organic solvents able to exert also specific catalytic abilities, when suitable functional groups are included on cation and/or anion.[19,20]

Here, we report for the first time the use of some ionic liquids (ILs) as a simple chemical approach to isolate sporopollenin capsules from pollen grains. Since IL structure could affect the efficiency of the intine-rich cell wall removal as well as the nature and distribution of functional groups on exine surface, we decided to investigate two classes of ILs: *i)* ILs with strong hydrogen bond acceptor anions, capable of solubilising cellulose under mild conditions: tetrabutylphosphonium hydroxide, $[Bu_4P]OH$ (**1**), 1-butyl-3-methylimidazolium chloride, [BMIM]Cl (**2**), 1,3-dimethylimidazolium methylphosphonate, $[DMIM][(MeO)(H)PO_2]$ (**3**), 1,3-dimethylimidazolium methyl methylphosphonate, $[DMIM][(MeO)(Me)PO_2]$ (**4**) and 1,3-dimethylimidazolium dimethylphosphate $[DMIM][(MeO)_2PO_2]$ (**5**); *ii)* Brønsted acidic ILs, eventually capable to modify the external cell wall of the pollen, due to their catalytic properties: 1-(4-sulfonic acid)butyl-3-methylimidazolium hydrogensulfate $[MIMC_4SO_3H][HSO_4]$ (**6**) and

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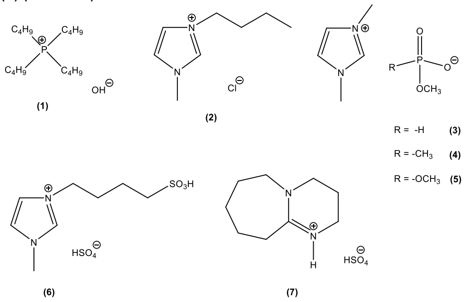
1,8-diazabicycleundec-7-eneninium hydrogensulfate [DBUH][HSO_4] (**7**) (Scheme1).



Scheme 1. ILs used in this study.

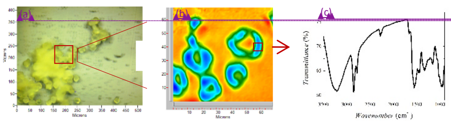


Figure 1. Visible (a) and FPA (b) images of *Populus deltoides* pollen grains.(c) FT-IR spectra of the pristine pollen *Populus deltoides*.

Populus deltoides pollen grains have been dispersed in the selected ILs and heated under stirring at 160°C for 30 min. Afterwards, the residual solid was separated and extensively washed with water and/or organic solvents in order to eliminate any residual trace of IL, and finally dried overnight at 50°C.[21] The recovered pollen grains generally presented a dark-brown colour, losing their bright yellow original aspect. However, when ILs (**1**) and (**2**) were used, no solid was obtained at the end of the treatment: the high solubilisation ability of these ILs towards biopolymers favoured an undesired complete dissolution. Furthermore, by means of comparison, the conventional treatment with Ac_2O in H_2SO_4 at 160 °C for 15 min was also carried out on a sample, with slight modifications with respect to literature procedure.[21]

The ATR-FT IR spectra of the pristine, Ac_2O/H_2SO_4-treated and IL-treated pollens were obtained with a FTIR Cary 620 Microscope spectrometer using a micro-ATR slide accessory with a Germanium crystal. Chemical imaging data were collected with a 64x64 Focal Plane Array (FPA) detector utilizing Agilent's ATR-Imaging technique. In agreement with literature,[22] the IR spectrum of the pristine pollen (Figure 1c) can be divided into three main regions associated to vibrational modes of *i)* lipids, 1722 cm^{-1} (C=O stretching), 1413 cm^{-1} (CH_2 deformation) and 1236 cm^{-1} (C-O stretching); *ii)* proteins, 1633 cm^{-1} (amide I) and 1540 cm^{-1} (amide II); and *iii)* carbohydrates, 1200-1000 cm^{-1} interval (C-O-C and C-OH stretching). Moreover, it presents a broad band at 3300 cm^{-1}, attributable to the O-H groups, as well as the stretching of the aliphatic C-H bonds at *ca.* 2900 cm^{-1}. In contrast, as expected, the IR spectrum of the Ac_2O/H_2SO_4-treated pollen is characterized by the presence of a new band at 1742 cm^{-1} and an increase of the C-O band at 1222 cm^{-1} attributable to the esterification of the O-H groups by Ac_2O, endorsed also by the disappearance of the broad band at 3300 cm^{-1} and by a significant decrease of the band at 1050 cm^{-1} (Figure S1).

Significantly different from each other and with respect to pristine pollen resulted also the IR spectra of the IL-treated pollen, thus suggesting that the structural features of the employed ILs and their catalytic abilities can alter the physico-chemical structure of the resulting microcapsule. In particular, the IR spectra of the pollen grains treated with alkyl-phosphate or alkyl-phosphonate-based ILs (**3-5**) (Figure S.2), point out the practically complete removal of the lipidic component and a drastic reduction of the intine (cellulose). The intensity of the bands at *c.a.* 1050 cm^{-1}, attributed to the vibrations of the C-O bonds of the cellulosic intine, is indeed significantly reduced. The most intense bands of the spectrum remain, however, those at 1640 and 1550 cm^{-1} attributable to amide I and amide II, respectively. In agreement with the results of the elemental analysis (Table S.1), which show a high nitrogen content (apparently increased with respect to the pristine pollen). These data strongly support the hypothesis that proteic component is still present. Finally, no bands attributable to phosphorylation processes are present.[23]

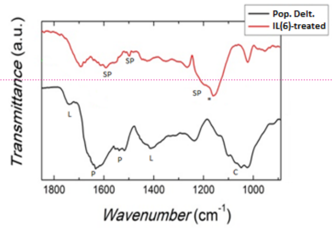


Figure 2. FT-IR spectra of the pristine pollen grains *Populus deltoides* and acidic IL (**6**) treated pollen [$MIMC_4SO_3H$][HSO_4].

On the other hand, when the acidic IL [$MIMC_4SO_3H$][HSO_4] (**6**) has been used, the FT IR spectrum resulted totally different with respect to the pristine pollen (Figure 2).The bands attributable to proteic and carbohydrate components are practically absent, whereas it is possible to observe a small band at 1708 cm^{-1} probably due to the lipidic component. Furthermore, the spectra are characterized by several new bands at 1606, 1514 and 970 cm^{-1}, associates by Zimmermann et al.[22] to the vibrations of the sporopollenin aromatic rings (SP in the spectrum). It is also possible to appreciate the appearance of a new intense band at 1176 cm^{-1}, attributable to the SO_3 asymmetric stretching. In principle, this band could be related either to the IL hydrogensulfate anion, eventually present despite the extensive washing, or to the presence of sulfate monoester groups arising from sulfurylation of the hydroxyl groups on sporopollenin, a process this latter probably catalyzed by the acidic IL (**6**). The presence of a relevant amount of sulphur is also confirmed by elemental analysis: in [$MIMC_4SO_3H$][HSO_4]-treated pollen the percentage of sulphur increases from 0.487% (pristine pollen) to 2.83%. Furthermore, elemental analysis shows a low amount of N (2.25%) confirming that the proteic component has been largely or completely eliminated. It is worth to note that nitrogen is present also in [$MIMC_4SO_3H$], and it should be present

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as counteranion of hydrogensulfate and/or may be responsible in the formation of the sulfate monoester (Figure 3).

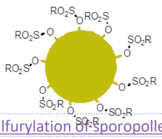

Figure 3. Sulfurylation of sporopollenin hydroxyl groups

Finally, it is to mention that when the less acidic [DBUH][HSO_4] (**7**) was employed, the IR spectra of the recovered treated pollen are quite similar (Figure S.3) to those obtained after the treatment with phosphorous-based ILs. Since both Brønsted acidic ILs have the same anion, the different features evidenced for the treated pollens strongly support the hypothesis that the intense band at 1176 cm^{-1}, peculiar of the [$MIMC_4SO_3H$][HSO_4]-treated pollen grain, has to be attributed to sporopollenin sulfurylation and contemporaneously shows that this reaction requires a strong acid, such as the [$MIMC_4SO_3H$]$^+$ cation, whereas the less acidic [DBUH]$^+$ cation is unable to catalyze this reaction.

It is well known that pollen grains exhibit auto-fluorescence due to their constituents. Therefore fluorescence measurements have been carried out both on pristine and on Brønsted acidic ILs-treated pollens.[23] Figure 4D shows the strong autofluorescence characterizing pollen before the treatment and the merged images (G-I) confirm that the fluorescence is in all samples (treated or untreated) originated from the pollen. Actually, a strong autofluorescence at 535 nm characterizes also the [DBUH][HSO_4] (**7**) treated pollens (E), which maintain also the pristine morphology, confirming the moderate ability of this IL to modify pollen grain structure. Pollen grains still show spherical shape and similar size with respect to the untreated sample (A). This is also evidenced in the case of [$MIMC_4SO_3H$][HSO_4] (**6**) treated pollens (C). In this latter case, however, the autofluorescence at 535 nm (F) generally attributed to carotenoid species,[24] is strongly reduced compared to the pristine pollen (C) suggesting selective removal of other fluorescent components or chemical modification of the exine layer.

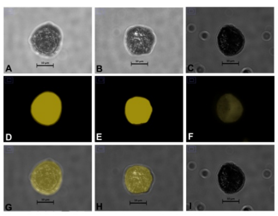

Figure 4. Effects of ILs on pollen autofluorescence at 535 nm. A,B,C) Bright-field images of pristine (A), IL(**7**)-treated (B) and IL(**6**)-treated (C) pollen. D,E,F) Autofluorescence at 535 nm in response to 420 nm excitation light of pristine (D), IL(**7**)-treated (E) and IL(**6**)-treated (F) pollen. G,H,I) Merged bright-field and fluorescence images of pristine (G), IL(**7**)-treated (H) and IL(**6**)-treated (I) pollen.

In order to collect further information about the nature of the sulfur on the surface of [$MIMC_4SO_3H$][HSO_4] (**6**)-treated pollen grains, XPS measurements were also carried out. The high resolution 2p XPS peak of sulphur is displayed in Figure 5. It appears as a large broad peak which can be fitted by two doublets ($2p_{1/2}$ and $2p_{3/2}$ due to spin orbit coupling) representative of sulphur in two different chemical environments. On the base of the relatively high value of binding energy ($2p_{3/2}$ 168.0 and 169.4 eV respectively) and in accordance with literature data[25,26] both the components can be however, attributed to S(VI); probably, sulfate monoester and hydrogensulfate. In particular, it is reasonable to think that the larger one, located at lower binding energy value (168.0 eV red line in Figure 5) is due to sulphate organic monoester. In this case indeed the inductive effect of organic substituent reliefs the electron depleted sulphur atom reducing its BE value. Vice versa the component at higher BE value (169.4 eV, blue line Figure 3) can be attributed to hydrogensulfate anion.

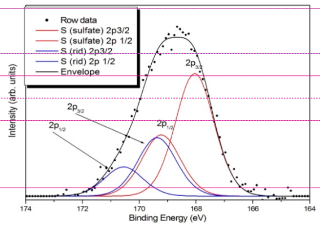

Figure 5. Sulphur XPS peaks (S 2p doublet) of the IL(**6**)-treated pollen grains. Row data are represented by dots while two doublets are displayed as blue and red lines respectively. Envelope curve is displayed as black line.

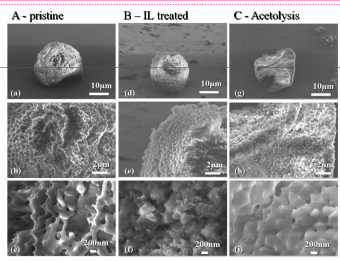

Figure 6. Scanning electron microscopy (SEM) images. SEM micrographs of (a-c) a pristine pollen, (d-f) a pollen grain (treated with the IL [$MIMC_4SO_3H$][HSO_4] (**6**), and (g-i) a pollen grain after acetolysis treatment. Different morphologies were observed between the single pristine pollen and the single pollen grains (on the ten-microns scale, see panels a, d and g) and between the surrounding shells (on the micron scale - see panels b, e and h – and on the sub-micron scale – see panels c, f and i).

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Finally, shape and morphology of the [MIMC$_4$SO$_3$H][HSO$_4$] treated pollen grains have been addressed -at level of individual grains– using scanning electron microscopy (SEM). In particular, in Figure 6, we report representative SEM micrographs of a single pristine pollen (a-c), the images of a single pollen grain after treatment with the IL [MIMC$_4$SO$_3$H][HSO$_4$] (**6**) (d-f) and the images of a single pollen grain after Ac$_2$O/H$_2$SO$_4$-treatment (g-i). For each sample, 5 single grains have been characterized using a wide magnification range from 250X to 64000X. The images reported in Figure 6 allow to appreciate the relevant features of each sample (pristine, IL-treated and after acetolysis treatment), being the single grain shown representative within the three different groups studied. The overall spherical 3D structure of the single grain of the pristine pollen is evident in Figure 6a. Increasing the magnification, the presence of a surrounding shell becomes apparent (6.b), characterized by a well-defined sub-micron structure. Indeed, punched saw-teeth-like nanostructures are clearly observed (6c). Treating the pollen grains with the IL (**6**), SEM images (6d) indicate that the pollen grains still retain the characteristic spherical 3D structure of the original grains. However, the morphology of the surrounding shell is markedly changed, as is evident in Figure 6e. The initial perforated nanostructure is replaced by a dense, full covering completely different arrangement (6f) not uniform in size nor in shape. Finally, we can observe that acetolysis treatment is detrimental to the morphology of the single grains, as is evident in Figure 6g where it is possible to appreciate that the pollen grain is heavily damaged, losing its peculiar 3D structure and causing the rupture and the flattening of the grains. More in detail (h,i), it is clearly evident that the morphology of the outer shell structure is different to the IL-treated pollen grains and to the pristine pollen, showing a full-covering uniform spiky nanostructure all over the pollen grain surface.

In conclusion, some ILs can represent a simple chemical approach to isolate sporopollenin capsules from pollen grains avoiding ruptures and flatting of grains. ILs structure significantly affect the efficiency of the process. Moreover, functionalized ILs having reactive anions, such as [MIMC$_4$SO$_3$H][HSO$_4$], can act as catalysts, allowing a direct functionalization of the sporopollenin surface.

Acknowledgements

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21. Ionic liquid treatment: *typical procedure.* 200 mg of pollen grains from *Populus deltoides* were suspended in 2.0 g of ionic liquid in a glass reactor (30 mL) equipped with a PTFE cap and placed in a carrousel with 12 entries (Radleys Discovery Technologies, United Kingdom). The glass reactors were heated at 160°C during 30 minutes. Then, the grains were collected by filtration and washed extensively with distilled water and/or dichloromethane in order to eliminate any residual trace of IL retained in the pollen structure and finally dried in an oven 12 hours at 50°C (30-50% wt yield). Conventional treatment; *typical procedure.* 200 mg of pollen grains (*Populus deltoides*) were placed in a 10 mL round-bottom flask. 540 µL of Ac$_2$O and 60 µL of H$_2$SO$_4$ were added into the flask and heated in an oil bath at 160°C for 15 minutes. Then, the resulting mixture was cooled up to room temperature and the resulting grains were collected by filtration and washed with a series of solvents (3x0.2 mL) of AcOH, EtOH, H$_2$O, CH$_2$Cl$_2$, in order to eliminate any residual product and dried in an oven 12 hours at 50°C. Finally, 20 mg of pollen grains were obtained (10% wt yield).
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Formattato: Giustificato, Rientro: Sinistro: 0 cm, Sporgente 0.5 cm, Numerazione automatica + Livello:1 + Stile numerazione: 1, 2, 3, … + Comincia da:1 + Allineamento: A sinistra + Allinea a: 0.63 cm + Imposta un rientro di: 1.27 cm
ha eliminato: the corresponding
ha formattato: Tipo di carattere: Calibri
ha formattato: Tipo di carattere: Calibri
ha eliminato: s
ha formattato: Tipo di carattere: Calibri, Inglese americano
ha formattato ... [34]
ha formattato: Inglese americano
ha formattato ... [35]
ha formattato: Tipo di carattere: Calibri, Inglese americano
ha eliminato: ¶
ha formattato ... [36]
ha formattato ... [37]
ha formattato: Tipo di carattere: Calibri, 9 pt, Grassetto, Proporzioni car 105%, Non Evidenziato
ha formattato ... [38]
ha formattato ... [39]

Please do not adjust margins

Pagina 2: [1] ha eliminato	**Cinzia**	**13/09/16 11:06:00**
Pagina 2: [1] ha eliminato	**Cinzia**	**13/09/16 11:06:00**
Pagina 2: [2] ha eliminato	**Cinzia**	**13/09/16 16:34:00**
Pagina 2: [2] ha eliminato	**Cinzia**	**13/09/16 16:34:00**
Pagina 2: [3] ha eliminato	**Cinzia**	**13/09/16 11:07:00**
Pagina 2: [3] ha eliminato	**Cinzia**	**13/09/16 11:07:00**
Pagina 2: [3] ha eliminato	**Cinzia**	**13/09/16 11:07:00**
Pagina 2: [3] ha eliminato	**Cinzia**	**13/09/16 11:07:00**
Pagina 2: [4] ha eliminato	**Stefania**	**15/09/16 11:46:00**
Pagina 2: [4] ha eliminato	**Stefania**	**15/09/16 11:46:00**
Pagina 2: [5] ha eliminato	**Stefania**	**15/09/16 11:49:00**
Pagina 2: [5] ha eliminato	**Stefania**	**15/09/16 11:49:00**
Pagina 2: [6] ha eliminato	**Stefania**	**15/09/16 11:52:00**
Pagina 2: [6] ha eliminato	**Stefania**	**15/09/16 11:52:00**
Pagina 2: [7] ha eliminato	**Cinzia**	**13/09/16 16:42:00**
Pagina 2: [7] ha eliminato	**Cinzia**	**13/09/16 16:42:00**
Pagina 2: [7] ha eliminato	**Cinzia**	**13/09/16 16:42:00**
Pagina 2: [7] ha eliminato	**Cinzia**	**13/09/16 16:42:00**
Pagina 2: [7] ha eliminato	**Cinzia**	**13/09/16 16:42:00**

Pagina 2: [7] ha eliminato	**Cinzia**	**13/09/16 16:42:00**

Pagina 2: [8] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 2: [8] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 2: [9] ha eliminato	**Cinzia**	**13/09/16 16:44:00**

Pagina 2: [9] ha eliminato	**Cinzia**	**13/09/16 16:44:00**

Pagina 2: [10] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 2: [10] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 2: [10] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 2: [11] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 2: [11] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 2: [11] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 2: [12] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 2: [12] ha formattato	**stefania**	**13/09/16 18:20:00**

Tipo di carattere: 6 pt

Pagina 3: [13] ha eliminato	**Stefania**	**15/09/16 12:04:00**

Pagina 3: [13] ha eliminato	**Stefania**	**15/09/16 12:04:00**

Pagina 3: [14] ha eliminato	**Cinzia**	**13/09/16 11:37:00**

Pagina 3: [14] ha eliminato	**Cinzia**	**13/09/16 11:37:00**

Pagina 3: [15] ha eliminato	**Cinzia**	**13/09/16 11:12:00**

Pagina 3: [15] ha eliminato	**Cinzia**	**13/09/16 11:12:00**

Pagina 3: [15] ha eliminato	**Cinzia**	**13/09/16 11:12:00**

Pagina 3: [16] ha eliminato	**Stefania**	**15/09/16 12:17:00**

Pagina 3: [16] ha eliminato	**Stefania**	**15/09/16 12:17:00**

Pagina 3: [17] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 3: [17] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 3: [18] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 3: [18] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 3: [19] ha eliminato	**Cinzia**	**13/09/16 11:18:00**

Pagina 3: [19] ha eliminato	**Cinzia**	**13/09/16 11:18:00**

Pagina 3: [20] ha eliminato	**Cinzia**	**13/09/16 11:31:00**

Pagina 3: [20] ha eliminato	**Cinzia**	**13/09/16 11:31:00**

Pagina 3: [21] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 3: [21] ha formattato	**Cinzia**	**15/09/16 16:56:00**

Non Evidenziato

Pagina 3: [22] ha eliminato	**Stefania**	**15/09/16 12:23:00**

Pagina 3: [22] ha eliminato	**Stefania**	**15/09/16 12:23:00**

Pagina 3: [23] ha eliminato	**Cinzia**	**13/09/16 11:42:00**

Pagina 3: [23] ha eliminato	**Cinzia**	**13/09/16 11:42:00**

Pagina 3: [24] ha eliminato	**Cinzia**	**13/09/16 11:43:00**

Pagina 3: [24] ha eliminato	**Cinzia**	**13/09/16 11:43:00**

Pagina 3: [24] ha eliminato	**Cinzia**	**13/09/16 11:43:00**

Pagina 3: [24] ha eliminato	**Cinzia**	**13/09/16 11:43:00**

Pagina 3: [24] ha eliminato	**Cinzia**	**13/09/16 11:43:00**

Pagina 3: [25] Formattato	**Cinzia**	**09/09/16 12:20:00**

Regola lo spazio tra testo asiatico e in alfabeto latino, Regola lo spazio tra caratteri asiatici e numeri

Pagina 3: [26] ha eliminato	**Cinzia**	**13/09/16 11:48:00**

Pagina 3: [27] ha eliminato	**Cinzia**	**13/09/16 11:52:00**

Pagina 3: [27] ha eliminato	**Cinzia**	**13/09/16 11:52:00**

Pagina 4: [28] ha eliminato	**Stefania**	**15/09/16 12:27:00**

Pagina 4: [28] ha eliminato	**Stefania**	**15/09/16 12:27:00**

Pagina 4: [29] ha eliminato	**Stefania**	**15/09/16 12:28:00**

Pagina 4: [29] ha eliminato	**Stefania**	**15/09/16 12:28:00**

Pagina 4: [29] ha eliminato	**Stefania**	**15/09/16 12:28:00**

Pagina 4: [29] ha eliminato	**Stefania**	**15/09/16 12:28:00**

Pagina 4: [29] ha eliminato	**Stefania**	**15/09/16 12:28:00**

Pagina 4: [29] ha eliminato	**Stefania**	**15/09/16 12:28:00**

Pagina 4: [29] ha eliminato **Stefania** **15/09/16 12:28:00**

Pagina 4: [29] ha eliminato **Stefania** **15/09/16 12:28:00**

Pagina 4: [30] ha formattato **Chus** **05/09/16 12:47:00**

Tipo di carattere: (Predefinito) Calibri, 9 pt, Inglese americano, Proporzioni car 105%

Pagina 4: [30] ha formattato **Chus** **05/09/16 12:47:00**

Tipo di carattere: (Predefinito) Calibri, 9 pt, Inglese americano, Proporzioni car 105%

Pagina 4: [31] ha formattato **Cinzia** **09/09/16 12:57:00**

Tipo di carattere: Corsivo

Pagina 4: [31] ha formattato **Cinzia** **09/09/16 12:57:00**

Tipo di carattere: Corsivo

Pagina 4: [32] ha formattato **Chus** **12/09/16 17:10:00**

Tipo di carattere: Corsivo, Italiano

Pagina 4: [32] ha formattato **Chus** **12/09/16 17:10:00**

Tipo di carattere: Corsivo, Italiano

Pagina 4: [32] ha formattato **Chus** **12/09/16 17:10:00**

Tipo di carattere: Corsivo, Italiano

Pagina 4: [32] ha formattato **Chus** **12/09/16 17:10:00**

Tipo di carattere: Corsivo, Italiano

Pagina 4: [33] ha formattato **Cinzia** **09/09/16 13:16:00**

Tipo di carattere: Corsivo

Pagina 4: [33] ha formattato **Cinzia** **09/09/16 13:16:00**

Tipo di carattere: Corsivo

Pagina 4: [34] ha formattato **Chus** **06/09/16 17:14:00**

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [34] ha formattato **Chus** **06/09/16 17:14:00**

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [34] ha formattato **Chus** **06/09/16 17:14:00**

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [34] ha formattato **Chus** **06/09/16 17:14:00**

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [34] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [34] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [35] ha formattato	Cinzia	09/09/16 13:18:00

Tipo di carattere: Corsivo

Pagina 4: [35] ha formattato	Cinzia	09/09/16 13:18:00

Tipo di carattere: Corsivo

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [36] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%

Pagina 4: [37] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [37] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [37] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [38] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [38] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato

Pagina 4: [39] ha formattato	Chus	06/09/16 17:14:00

Tipo di carattere: Calibri, 9 pt, Proporzioni car 105%, Non Evidenziato