RSC Advances

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Cite this: *RSC Adv.*, 2025, **15**, 36428

# Development of an improved and greener HPLC-DAD method for the determination of fecal sterols in sediment and water samples using ultrasonic-assisted derivatization with benzoyl isocyanate

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Received 19th June 2025
Accepted 21st August 2025

DOI: 10.1039/d5ra04381f

rsc.li/rsc-advances

The analysis of sterols by high-performance liquid chromatography (HPLC) with UV detection presents a challenge, owing to the intrinsically low molar absorptivity of these compounds. In this study, we developed a novel derivatization method for sterols (7-dehydrocholesterol, cholesterol, and coprostanol), which serve as indicators of fecal pollution, for analysis by HPLC-DAD. We proposed benzoyl isocyanate as the derivatization reagent, as isocyanates are characterized by a functional group containing –N═C═O, which reacts with the hydroxyl (–OH) groups present in the structure of sterols, thereby introducing the chromophore group of benzoyl isocyanate to the sterols. This method is more environmentally friendly compared to previously published derivatization techniques. The conditions for sterol derivatization were optimized using chemometric tools, including a $2^2$ factorial design with a central point and central composite design for the variables of molar ratio (sterol/benzoyl isocyanate = 0.046, equivalent to $1.57 \times 10^{-5}$ mols of sterol and $3.39 \times 10^{-4}$ mols of benzoyl isocyanate) and ultrasonic bath time (32.1 minutes). The chromatographic parameters $t'_R$, $k$, $\alpha$, Rs, N, and As were determined. The quantification limits were established at 0.6 mg $L^{-1}$ for 7-dehydrocholesteryl-*N*-benzoylcarbamate and 0.5 mg $L^{-1}$ for cholesteryl-*N*-benzoylcarbamate and coprostanolyl-*N*-benzoylcarbamate. The feasibility of the proposed method was confirmed by analysing water and sediment samples from lakes in Londrina, Paraná, Brazil, yielding recovery values ranging from 91 to 108%.

## 1. Introduction

The contamination of fecal coliforms in domestic sewage, water, and sediment is a public health concern. Typically, microbiological methods have been used for determining bacteria as fecal indicators of the coliform group, but these methods suffer from environmental factors, including effluent characteristics, the presence of contaminants, temperature, and salinity. Another drawback is that these markers cannot differentiate between various sources of fecal contamination. A more reliable way to assess fecal pollution is through chemical indicators by determining fecal sterols.[1–3]

Sterol compounds with chemical stability and specific origins serve as reliable indicators of domestic sewage contamination.[1] Coprostanol has been widely used as a marker of the presence of domestic sewage, more specifically of organic fecal matter, because it originates from the biohydrogenation of cholesterol in the intestines of humans and other higher animals.[4] Moreover, coprostanol is present in human feces, up to 60% of all fecal sterols.[4] For this reason, coprostanol, in association with other sterols (epicoprostanol, cholesterol, cholestanol, β-sitosterol), is applied in sterol ratios to differentiate between sources of fecal matter and identify human fecal contamination.[5–7]

Gas chromatography with flame ionization detection (GC-FID)[8] and gas chromatography coupled with mass spectrometry (GC-MS)[9] are the most widely used techniques to analyse fecal sterols in environmental samples. These methods require derivatization due to the difficulty of volatilizing these compounds. High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is less commonly employed for this purpose and faces challenges because some sterols, such as coprostanol, do not absorb radiation in the UV owing to the absence of a chromophore in their molecular structure. 7-Dehydrocholesterol and cholesterol exhibit a maximum absorption wavelength ($\lambda_{max}$) at 210 nm, which is close to that of most solvents, such as acetonitrile, that have a UV cut-off of 190 nm, as well as to concomitants in the environmental samples. As a result, spectral interference may be observed.

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To improve the selectivity and detectability of HPLC-UV for sterols analysis, derivatization methods have been developed by introducing chromophores into the structure of sterols.

Fitzpatrick and Siggia (1973),[10] proposed the use of a benzoylation reaction using benzoyl chloride in a pyridine medium to form an ultraviolet-absorbing derivative, aiming to improve the detection limit for the analysis of sterols by HPLC-UV in urinary extracts. Piocos and Cruz (2000),[11] suggested the potential application of acylation derivatization of coprostanol with *p*-nitrobenzoyl chloride for the analysis of sterols in various samples. The method was developed by adding a chromophore, *p*-nitrobenzoyl, through a simple acylation of the hydroxyl group of coprostanol. Resende *et al.* (2014),[2] proposed an optimization of the benzoylation reaction using benzoyl chloride to investigate the presence of fecal origin sterols in sediment samples, developing a benzoylation reaction with an ultrasonic bath. In another study, the phytosterol derivatization was accomplished with 4-dimethylaminopyridine (DMAP) as catalyst, dansyl chloride as derivatizing agent, and using dichloromethane as reaction solvent.[12]

In general, the reported sterol derivatization methods make use of toxic reagents, such as benzoyl chloride, pyridine, its derivatives, and highly toxic solvents,[2,10,12], and are also time-consuming, which justifies the search for novel and more environmentally friendly derivatization methods.

Isocyanates are molecules that contain the functional group with the atoms R–N=C=O, and they are extremely reactive towards a variety of nucleophiles, including molecules with hydroxyl (–OH) functional groups. The reactions typically involve an attack on the carbon atom of the isocyanate group by the nucleophile, resulting in the formation of urethanes or carbamates.[13]

The use of isocyanates to derivatize hydroxyl functional groups has been described. Derivatization with phenyl isocyanate (Ph-NCO),[14–18] naphthyl isocyanate,[19,20] 1-(1-naphthyl)ethyl isocyanate,[21] 3-isopropenyl-α,α-dimethylbenzylisocyanate,[22] 2,4-dimethoxyphenylisocyanate[22] and *p*-toluenesulfonyl isocyanate[23] have been reported for molecules of fatty alcohol ethoxylates,[14,15,19] aliphatic *n*-alcohols,[15] polyehyleneglycol,[16] irganox 1076,[17] 1,2-*sn*- and 2,3-*sn*-diacylglycerols,[18] 2-ethoxyethanol,[20] diacyl-*sn*-glycerols,[21] 2-propanol,[22] 1-naphthol,[22] 2-isopropoxyphenol,[22] diethylene glycol[23] and polypropylene glycol.[23] However, to the best of our knowledge, the effectiveness of isocyanates as derivatizing agents for sterol determination using HPLC-DAD has not yet been reported.

Sterols (S–OH) react with benzoyl isocyanate *via* nucleophilic addition of their hydroxyl group to the N=C bond, forming carbamates. The resulting derivative, Ph–CO–NH–CO–OS, contains both an amide and a phenyl group, which enables UV absorption. Thus, this work aimed to develop a more environmentally friendly ultrasonic-assisted derivatization method for the sterols 7-dehydrocholesterol, cholesterol, and coprostanol using benzoyl isocyanate as the derivatizing reagent. Lake water and sediment samples were analyzed by the HPLC-DAD to assess the practical viability of the proposed method.

## 2. Experimental

### 2.1 Chemical and solutions

Cholesterol (CHO), coprostanol (COP), 7-dehydrocholesterol (7-CHO), and benzoyl isocyanate standards (purity 95–99%) were obtained from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), isopropanol (IPA), methanol (MeOH), and acetone are HPLC grade. Chloroform-d ($CDCl_3$) from Sigma Aldrich was used for the analyses of 1H-RMN.

### 2.2 Instruments

A Quimis® 164 ultrasonic bath (Diadema, SP, Brazil) (Frequency 50/60Hz and capacity 2.4 L), and a Fisatom Model 801 rotary evaporator (60Hz) were used. Attenuated total reflection Fourier transform infrared (ATR-FTIR) analysis was carried out on a Bruker Vertex 70 (Bruker Optics, Rheinstetten, Germany), with a platinum ATR reflectance accessory in the range 400–4000 $cm^{-1}$. Spectra were collected with a resolution of 4 $cm^{-1}$ and an accumulation of 16 scans. NMR spectrometer 400 MHz with 5 mm multinuclear probe with field gradient for liquid samples and a 5 mm multichannel probe, using $CDCl_3$ as solvent.

Chromatographic analyses were performed using a liquid chromatograph model LC20AT, Shimadzu Prominence, Tokyo, Japan, operating isocratically elution mode, a manual injection valve with a 20.0 μL sample loop, and a diode array detector (Shimadzu). A stationary phase constituted by a column CLC-ODS C18 Kinetex Core-shell (250 mm × 4.6 mm i.d., 5 μm in particle size) and a guard column Phenomemex (4.0 mm × 3.0 mm i.d., 5.0 μm in particle size) were used.

LC-MS analyses were performed using a liquid chromatograph coupled to a triple quadrupole model (LC-MS-8040, Shimadzu, Tokyo, Japan). A CLC-ODS C18 Kinetex Core–shell column (250 mm × 4.6 mm i.d., 5 μm in particle size) and a Phenomenex guard column (4.0 mm × 3.0 mm i.d., 5.0 μm in particle size) were used as the stationary phase. Acetonitrile and isopropanol (95 : 5, v/v) were used as the mobile phase in isocratic mode and at a flow rate of 1.0 mL $min^{-1}$, with a column oven temperature of 40 °C.

### 2.3 HPLC procedure

Chromatography separation of sterols was carried out using acetonitrile and isopropanol (95 : 5, v/v) as mobile phase at isocratic mode and using a flow rate of 1.0 mL $min^{-1}$. The temperature of chromatographic separation (40 °C) was controlled using a column oven. To choose the appropriate mobile phase for sterol separation, the chromatography parameters, relative retention time ($t'_R$), retention factor ($k$), separation factor ($\alpha$), resolution ($R_s$), theoretical plate (N), and asymmetry factor (As) were determined.[24–26]

### 2.4 Derivatization

The molar ratio plays the most important role in the analytical performance of the method in the derivatization reaction of sterols (7-dehydrocholesterol, cholesterol, and coprostanol) with benzoyl isocyanate.

The first molar ratio[22] investigated was 0.01 ($3.39 \times 10^{-6}$ moles of sterols/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate). The compounds were accurately weighed, dissolved in acetonitrile in a glass flask. The flask was kept in a nitrogen ($N_2$) atmosphere and placed in an ultrasonic bath for 30 min. After the reaction, the solvent was evaporated under vacuum, and the product was obtained. The derivatization reaction products obtained were diluted in the chromatographic mobile phase (acetonitrile and isopropanol, 95 : 5 v/v) and analyzed by HPLC-DAD.

To find the best molar ratio of sterols and benzoyl isocyanate and ultrasonic bath time, firstly, a $2^2$ factorial design with a central point (−1, 0, +1) was performed. The evaluated levels were: 0.04 ($0.04 = 1.35 \times 10^{-5}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate), 0.021 ($0.021 = 7.13 \times 10^{-6}$ moles of sterols/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate) and 0.002 ($0.002 = 6.79 \times 10^{-7}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate) and the ultrasonic bath time: 30, 50 and 70 min.

Afterwards, a new $2^2$ factorial design with a central point considering three levels (−1, 0, +1) for variables molar ratio between sterols and benzoyl isocyanate: 0.021 ($0.021 = 7.13 \times 10^{-6}$ moles of sterols/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate), 0.04 ($0.04 = 1.35 \times 10^{-5}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate) and 0.059 ($0.059 = 2.00 \times 10^{-5}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate) and the ultrasonic bath time (10, 30 and 50 min) was performed.

The final optimization was accomplished using a central composite design (CCD)[27] considering five levels ($-\sqrt{2}$, −1, 0, +1, $\sqrt{2}$) for the variables molar ratio between sterols and benzoyl isocyanate: 0.013 ($0.013 = 4.37 \times 10^{-6}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate), 0.021 ($0.021 = 7.13 \times 10^{-6}$ moles of sterols/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate), 0.04 ($0.04 = 1.35 \times 10^{-5}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate), 0.059 ($0.059 = 2.00 \times 10^{-5}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate) and 0.067 ($0.067 = 2.27 \times 10^{-5}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate), and the ultrasonic bath time: 2.11, 10, 30, 50 and 58.8 min.

Derringer-Suich desirability function[28] was used to obtain the best derivatization condition for multicomponent (all sterols), by converting the chromatographic areas into the individual desirability ($d_i$) function and the global desirability ($D$) (eqn (1) and (2)). The scale of the individual desirability function varies between $d = 0$ for a completely undesirable response and $d = 1$ for a completely desirable response. To convert the individual criteria into desirability values, two types of transformation are possible: a unilateral transformation and a bilateral transformation. Seeking to maximize the response (target value as the most desirable response), the unilateral transformation was applied according to eqn (1), in which $Y_i$ is the chromatographic area, $r$ is the weight, and $L$ and $H$ are the most undesirable and desirable responses of all experiments, respectively.[28] Weight values were the same for all sterols.

$$\begin{aligned} d_i &= 0, \text{if} \quad Y_i \leq L \\ d_y &= \left(\frac{Y_i - L}{H - L}\right)^r \\ d_i &= 1, \text{if} \quad Y_i \geq L \end{aligned} \tag{1}$$

After calculating the desirability for each response obtained, they must be combined into a global desirability ($D$), which expresses the geometric mean of the individual desirability values, using eqn (2).[28]

$$D = \sqrt[n]{d_1^{p1} d_2^{p2} d_3^{p3} \ldots d_m^{p}} \tag{2}$$

where $m$ is the number of responses considered for each experiment during optimization, and $p$ is the weight assigned to each response. Response surface methodology was used to obtain the best condition for the derivatization reaction, attested by analysis of variance (ANOVA) at a confidence level of 95.0%.

### 2.5 Analytical parameters

Analytical parameters, including analytical curve, intra-day and inter-day precision, and limits of detection (LD) and quantification (LQ), were determined under the optimized conditions. Solutions of the sterols (7-dehydrocholesterol, cholesterol, and coprostanol), ranging from 1.0 to 300.0 mg $L^{-1}$ were prepared in acetonitrile, subjected to derivatization reaction, and analyzed by HPLC-DAD.

The LD and LQ were determined as 3std/$m$ and 10std/$m$, respectively, where std is the standard deviation from 10 measurements of the blank and $m$ is the slope of the analytical curve.[29] After determining the LQ value, a solution at the concentration corresponding to the LQ was prepared, and the chromatographic determination was performed to evaluate the effect of the theoretical LQ on the calibration curve. This procedure allowed us to conclude that the LQ is reliable, as the linear model of the analytical curve was preserved. The intra-day and inter-day precision (two consecutive working days) was assessed in terms of repeatability by analyzing ($n = 10$) concentrations of 3.0 and 100.0 mg $L^{-1}$, and the relative standard deviations (RSD) were determined.

### 2.6 Sample collection, preparation, and preservation

Water and sediment samples were collected from Londrina (Igapó Lake, coordinates: 23°19′15.2″S 51°10′54.9″W, Ribeirão Cambé, coordinates: 23°33′78.2″ S 51°15′22.6″ W and Cabrinha Lake, coordinates: 23°26′54,87″ S 51°14′94,68″ W) in Paraná State, Brazil (Fig. 1S).

Superficial water samples (30 cm below the surface) were collected in amber bottles and filtered through a 0.45 μm Nylon® membrane to remove solid impurities. In triplicate, 50.0 mL of water samples were evaporated overnight at 50 °C, taken up in acetonitrile, subjected to the derivatization method, and analyzed by HPLC-DAD. The accuracy of the method was evaluated through spiking and recovery tests, in which samples were fortified with two concentrations (3.0 and 50.0 mg $L^{-1}$) of sterols. Following derivatization, chromatographic analysis was performed on the sterol-*N*-benzoylcarbamate forms.

Sediment samples (5.0 g), in triplicate, were submitted to ultrasound-assisted extraction using 55.0 mL of a mixture of methanol–acetone (1 : 1 v/v).[30] The sediments were spiked with a mixture of 7-dehydrocholesterol, cholesterol, and coprostanol at a final concentration of 6.0 and 100.0 mg kg$^{-1}$ for each compound. Before HPLC analysis, the slurry samples were centrifuged for 5 min at 4000 rpm, and the supernatant was taken up and evaporated in a rotary evaporator under vacuum. Further, the samples were subjected to the derivatization method and analyzed by HPLC-DAD.

## 3. Results and discussion

### 3.1 Characterization of sterol-*N*-benzoylcarbamate by ATR-FTIR, and 1H-NMR

The feasibility of the reaction of sterols and benzoyl isocyanate to form sterols-*N*-benzoylcarbamate compounds (Fig. 1) was evaluated using ATR-FTIR, whose spectra are depicted in Fig. 2S. The synthesis was carried out using the molar ratio 0.01 ($3.39 \times 10^{-6}$ moles of sterols/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate). The bands at 3400 cm$^{-1}$ are attributed to the hydroxyl group (–OH) of the sterol molecules.[31] The strong intensity band at 2251 cm$^{-1}$ for benzoyl isocyanate is characteristic of the stretching vibration of the isocyanate group, while the band at 1690 cm$^{-1}$ can be attributed to the vibrations of both the –C=N– and C=O bonds, which are present in the isocyanate group.[31,32]

The urethane bond formed between sterols and benzoyl isocyanate can be confirmed by the absence of the isocyanate band at 2251 cm$^{-1}$ in the spectra of sterol-*N*-benzoylcarbamate compounds (Fig. 2S-b). The band at 1777 cm$^{-1}$ is attributed to the diamide (–CO–NH–CO–), and a much lower wavenumber at around 1650 cm$^{-1}$. The NH can form a tautomer with either CO groups, and the C=N can have two different locations (thus a doublet). The bands 1296 cm$^{-1}$ and 1024 cm$^{-1}$ are attributed to the stretching of the C–N and C–O bonds, respectively, confirming the formation of the urethane bond and the carbamate structure.[31,32]

The 1H-NMR spectrum for benzoyl isocyanate (Fig. 3S) shows the corresponding signals to the protons from the aromatic ring, resulting in a multiplet of 3 signals, appearing at 7.40–7.62 $\delta$ (ppm). The 1H-NMR spectra of sterols present similar signs in 0.63–0.69, 0.81, 0.90–0.91, 1.00–1.01, 2.28–2.37, 3.17–3.40, and 3.64–3.76 $\delta$ (ppm), differing by the sign that appears approximately in the region of 5.00 $\delta$ (ppm). The 7-dehydrocholesterol presents a triplet (5.50 $\delta$) and multiplet (5.59 $\delta$) in this region, and the cholesterol presents a triplet (5.31 $\delta$). The coprostanol does not show signs in this region, due to the absence of unsaturation in the molecule.[33]

The 1H-NMR spectra of the sterol-*N*-benzoylcarbamate compounds show the characteristic signals of their precursors. 7-Dehydrocholesteryl-*N*-benzoylcarbamate exhibits multiplets at 4.76, 5.39, and 5.50 $\delta$ (ppm); cholesteryl-*N*-benzoylcarbamate shows multiplets at 4.76 and 5.36 $\delta$ (ppm); and coprostanolyl-*N*-benzoylcarbamate displays a multiplet at 4.73 $\delta$ (ppm).[33]

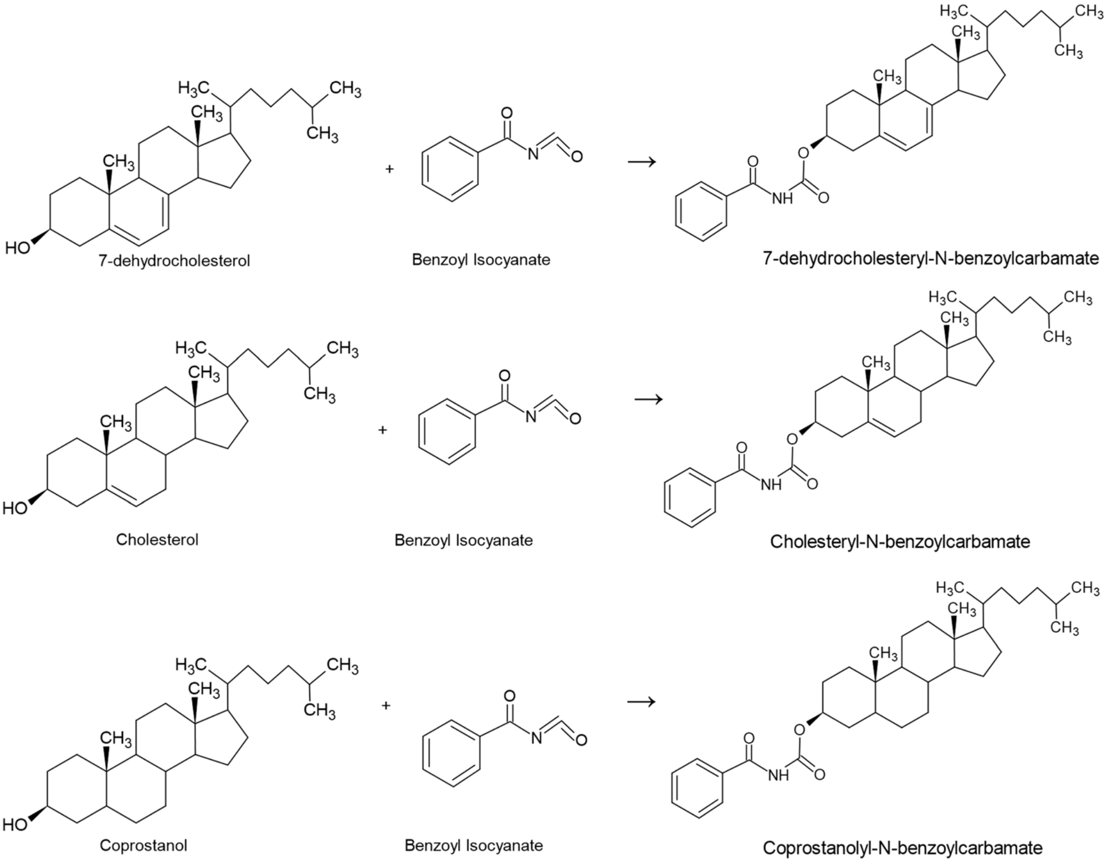


Fig. 1 Derivatization reaction scheme between sterols and benzoyl isocyanate, forming sterol-*N*-benzoylcarbamate.

### 3.2 Development of the HPLC-DAD method

Firstly, a preliminary evaluation of chromatographic conditions for the determination of sterol-*N*-benzoylcarbamate compounds by HPLC-DAD was performed. The molar ratio used in the derivatization reaction was 0.01 ($3.39 \times 10^{-6}$ moles of sterols/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate). The chromatographic conditions were: mobile phase of acetonitrile and isopropanol (80 : 20 v/v) in isocratic mode, sampling loop of 20 µL, and CLC-ODS C18 Kinetex Core-shell column at a temperature of 40 °C.[34] The concentration of the sterols, 7-dehydrocholesterol, cholesterol, and coprostanol was set as 3.0 mg $L^{-1}$.

Under these conditions, chromatographic peaks were observed: the first at a retention time of 2.38 min, attributed to benzoyl isocyanate, and at 5.38 min, 4.89 min, and 5.76 min corresponding to cholesteryl-*N*-benzoylcarbamate, 7-dehydrocholesteryl-*N*-benzoylcarbamate, and coprostanolyl-*N*-benzoylcarbamate, respectively (Fig. 4Sa–c). The chromatographic peaks were monitored at $\lambda = 229$ nm. Afterward, the derivatization reaction was accomplished using the three sterol compounds, cholesterol, 7-dehydrocholesterol, and coprostanol, at the same molar ratio of 0.01. Fig. 4Sd shows the coelution of cholesteryl-*N*-benzoylcarbamate and coprostanolyl-*N*-benzoylcarbamate; thus, a new separation chromatographic condition was evaluated using acetonitrile and isopropanol (95 : 5, v/v) at a flow rate of 1.0 mL $\text{min}^{-1}$, with the column temperature set at 40 °C. Fig. 2 shows the chromatogram with well-resolved peaks of sterol-*N*-benzoylcarbamate compounds.

Table 1 shows the obtained chromatographic parameters under optimized conditions, and based on the values, effective separation of the compounds can be attested.[24–26]

Apart from FT-IR and H1 NMR spectra, LC-MS was also used to confirm the formation of products. Thus, sterol standards were derivatized to their sterol-*N*-benzoylcarbamate forms to investigate their ionization behaviour and fragmentation pathways using LC-MS. Under optimized chromatographic separation conditions, the sterol-*N*-benzoylcarbamate forms were readily detected as the protonated molecular ion $[M + H]^+$. The mass spectra (Fig. 5S) of the chromatographic peak attributed to benzoyl isocyanate showed a molecular ion at *m*/*z* 149.15, a fragmentation at *m*/*z* 105.1, and a characteristic peak at *m*/*z* 77.15, all of which are typical of aromatic isocyanates.[35,36]

The ion molecular of the sterol-*N*-benzoylcarbamate was detected at *m*/*z* 536.85 for the 7-dehydrocholesteryl-*N*-benzoylcarbamate (Fig. 5Sa), *m*/*z* 538.64 for the cholesteryl-*N*-benzoylcarbamate (Fig. 5Sb), and *m*/*z* 539.90 for the coprostanolyl-*N*-benzoylcarbamate (Fig. 5Sc). The peak at *m*/*z* 384.90, 388.39, and 389.98 corresponds to the elimination of benzoyl isocyanate from the structure of sterol-*N*-benzoylcarbamate. The peak at *m*/*z* 149.95 corresponds to benzoyl isocyanate.[37,38]

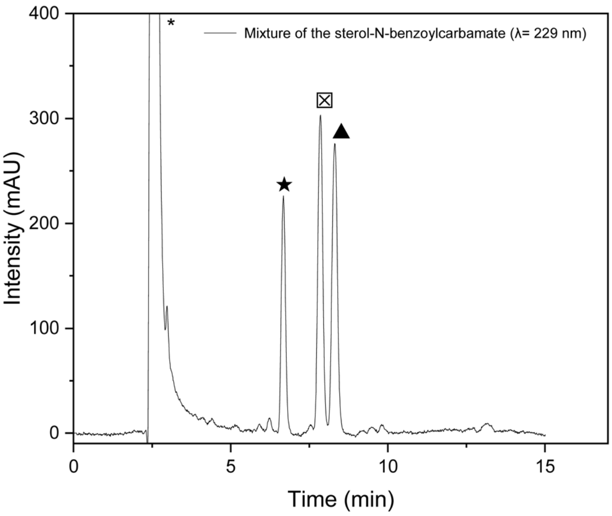


Fig. 2 Chromatogram of the sterols at 3.0 mg $L^{-1}$ after the derivatization reaction, using ACN: ISO (95 : 5, v/v) at 1.0 mL $\text{min}^{-1}$, 40 °C, $\lambda = 229$ nm (*– benzoyl isocyanate; ★-7-dehydrocholesteryl-*N*-benzoylcarbamate; ☒-cholesteryl-*N*-benzoylcarbamate, and ▲-coprostanolyl-*N*-benzoylcarbamate).

### 3.3 Optimization of derivatization reaction using factorial design and central composite design (CCD)

After establishing the best chromatographic separation condition, the optimum condition for the derivatization reaction was evaluated by investigating the molar ratio of sterols and benzoyl isocyanate and ultrasonic bath time. This optimization is crucial for enhancing the detectability of chromatographic separation and reducing the time required for the ultrasonic bath. Fig. 3 depicts the graphical representation of the $2^2$ factorial design with the central point (−1, 0, +1), using the global desirability as the response. As one can see, the more favorable condition was obtained in the levels +1 and −1 for the molar ratio (0.04) and ultrasonic bath time (30 min), respectively. Thus, a steepest descent was carried out to move the variables toward the region of maximum response (Fig. 4, in red). This approach revealed a maximum response at the central point along the path.

From the results, and to obtain a quadratic model with more statistical parameters, the levels -$\sqrt{2}$ and $\sqrt{2}$ from the central composite design (CCD) were inserted in the experiment for final optimization using response surface. Table 2 shows the CCD design containing the chromatographic areas converted into individual desirability ($d_i$) and global desirability ($D$). The relationship between the independent variables and the response was calculated using a second-order polynomial equation (eqn (3)). The feasibility of the polynomial equation was evaluated using analysis of variance (ANOVA). The ANOVA, presented in Table 2S, showed good determination ($R^2 = 0.9609$ and adjusted $R^2 = 0.9217$). Furthermore, the model's fit to the experimental values was verified according to the F-test. No lack of fit was observed, as the $\text{MQ}_{\text{lack of fit}}/\text{MQ}_{\text{pure error}}$ ratio of 2.13 was lower than the critical $F_{3.2}$ at 95% confidence ($F_{3.2} = 19.16$).

The statistical significance of the linear and quadratic terms of the quadratic model was verified by evaluating the probability level values ($p < 0.05$) using a 95% confidence interval. Values of $p < 0.05$ were obtained for the variables ratio ($L$) and ($Q$), and time ($Q$) (marked with * in Table 2S). Thus, eqn (3) was reduced to eqn (4) for building the response surface (Fig. 5).

Table 1 Chromatographic parameters for optimized separation by HPLC-DAD[a]

| Parameters | 7-dehydrocholesteryl-*N*-benzoylcarbamate | Cholesteryl-*N*-benzoylcarbamate | Coprostanolyl-*N*-benzoylcarbamate |
|---|---|---|---|
| $T'_r$ | 6.70 | 7.89 | 8.38 |
| $k$ | 2.01 | 2.51 | 2.78 |
| $\alpha$ | — | 1.24 | 1.11 |
| Area (%RSD) | 1.36 | 1.73 | 1.10 |
| N | 3136 | 3997 | 6767 |
| Rs | — | 3.55 | 1.45 |
| As | 1.08 | 1.00 | 1.00 |

[a] $T'_r$ = retention time; $k$ = retention factor (where $T'_M = 2.2$ is defined as the first significant baseline disturbance, corresponding to the retention time of an unretained solution); $\alpha$ = separation factor; area (%RSD) = relative standard deviation for the retention time of the analyte, expressed as a percentage; N = theoretical plates; Rs = resolution; As = asymmetry factor.

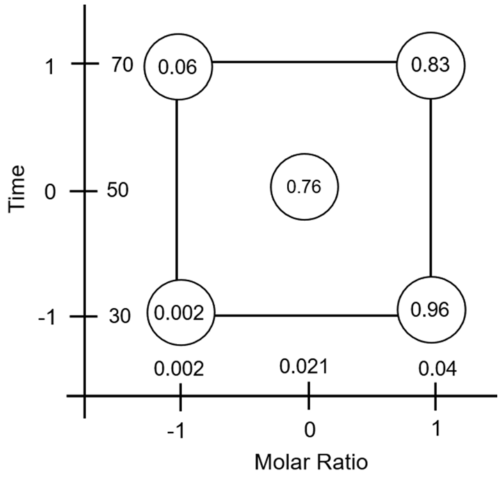


Fig. 3 Graphical representation of $2^2$ factorial design with the central point, using global desirability as the response.

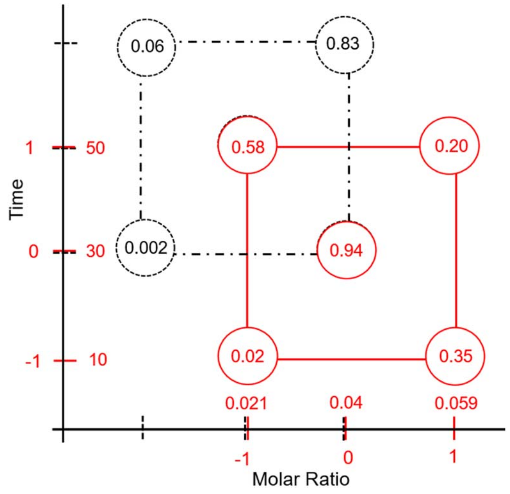


Fig. 4 Graphical representation of steepest descent of $2^2$ factorial design with the central point (in red), using global desirability as the response.

$$D = 0.9041_{\pm 0.0446} + 0.1947_{\pm 0.0273}\text{molar ratio}^{*} - 0.2917_{\pm 0.0326}\text{molar ratio}^{2*} + 0.08421_{\pm 0.0273}\text{time} - 0.3382_{\pm 0.0326}\text{time}^{2*} -{}^{*}0.0353_{\pm 0.0386}\text{molar ratioxTime} \quad (3)$$

Considering the significant terms that affect the derivatization reaction, eqn (4) was used for building the response surface.

$$D = 0.9041_{\pm 0.0446} + 0.1947_{\pm 0.0273}\text{molar ratio}^{*} - 0.2917_{\pm 0.0326}\text{molar ratio}^{2*} - 0.3382_{\pm 0.0326}\text{time}^{2*} \quad (4)$$

The surface response shows within the experimental domain, the critical points as being a molar ratio of 0.046 ($0.046 = 1.57 \times 10^{-5}$ moles of sterol/$3.39 \times 10^{-4}$ moles of benzoyl isocyanate) and 32.1 min of the ultrasonic bath. Thus, this optimized condition was selected for the derivatization synthesis.

Fig. 6 shows the chromatograms obtained in the absence and presence of the derivatization reaction. More intense chromatographic peaks for derivatized sterols were observed. Additionally, coprostanolyl-*N*-benzoylcarbamate, the main chemical marker for fecal pollution, showed a higher intensity signal, contrasting with the absence of signal for coprostanol in the absence of the derivatization reaction.

### 3.4 Analytical parameters of the derivatization method

The analytical parameters obtained under optimized conditions are presented in Table 3, while the graphs of analytical curves are shown in Fig. 7.

The quality of the adjustment of the experimental data obtained from the analytical curves was assessed by analysis of variance (ANOVA) at a 95% confidence level, allowing us to infer that there is no lack of adjustment between the values predicted by the model in terms of concentrations and the observed values, since in all curves, the $F_{\text{calculated}}$ ($\text{MQ}_{\text{lack of adjustment}}/\text{MQ}_{\text{pure error}}$) (Table 3) was lower than the $F_{\text{tabled}} = 2.75$. The obtained RSD values for interday and intraday analysis of sterol-*N*-benzoylcarbamate forms are satisfactory according to AOAC.[39]

The derivatization of fecal sterols using benzoyl isocyanate demonstrated superior analytical performance compared to the

Table 2 Central composite design (decoded and coded variables) and responses obtained (area for each derivatization product), and desirability calculated

| | Molar ratio | Time ultrasonic bath (min) | 7-dehydrocholesteryl-*N*-benzoylcarbamate | Cholesteryl-*N*-benzoylcarbamate | Coprostanolyl-*N*-benzoylcarbamate | Global desirability (D) |
|---|---|---|---|---|---|---|
| 1 | 0.021<br>(−1) | 10<br>(−1) | 2107<br>0.0559 | 3070<br>0.0202 | 4675<br>0.0228 | 0.0295 |
| 2 | 0.021<br>(−1) | 50<br>(+1) | 4580<br>0.3484 | 5134<br>0.1662 | 6518<br>0.0867 | 0.1712 |
| 3 | 0.059<br>(+1) | 10<br>(−1) | 7831<br>0.7329 | 10280<br>0.5301 | 15351<br>0.3930 | 0.5345 |
| 4 | 0.059<br>(+1) | 50<br>+1 | 8653<br>0.8301 | 12055<br>0.6557 | 12121<br>0.2810 | 0.5348 |
| 5 | 0.04<br>(0) | 30<br>(0) | 8892<br>0.8702 | 12452<br>0.6837 | 31745<br>0.9615 | 0.8301 |
| 6 | 0.04<br>(0) | 30<br>(0) | 9957<br>0.9843 | 16923<br>1 | 31961<br>0.9690 | 0.9844 |
| 7 | 0.04<br>(0) | 30<br>(0) | 10089<br>1 | 13048<br>0.7259 | 32853<br>1 | 0.8987 |
| 8 | 0.067<br>(√2) | 30<br>(0) | 7048<br>0.6403 | 11309<br>0.6029 | 14 717<br>0.3710 | 0.5232 |
| 9 | 0.04<br>(0) | 58.28<br>(√2) | 7173<br>0.6551 | 7724<br>0.3493 | 10697<br>0.2316 | 0.3756 |
| 10 | 0.013<br>(−√2) | 30<br>(0) | 2634<br>0.1182 | 3331<br>0.0386 | 4352<br>0.0116 | 0.0376 |
| 11 | 0.04<br>(0) | 2.11<br>(−√2) | 1634<br>0.0000 | 2784<br>0.0000 | 4017<br>0.0000 | 0.0000 |

method proposed in the literature. In the study reported by Resende *et al.* (2014),[2] the acylation was carried out with benzoyl chloride in pyridine, and HPLC-DAD was used as an analytical technique. In the earlier study, the LQ for cholesterol and coprostanol were 10.82 mg L$^{-1}$ and 7.28 mg L$^{-1}$, respectively, values considerably higher than the 0.5 mg L$^{-1}$ achieved for both sterols in the present work.

In another study, Dubber *et al.* (2024),[40] determined fecal sterols by GC-MS analysis and derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (1 h at 100 °C) to form trimethylsilyl derivatives (TMCS). The LD for coprostanol was 5 mg L$^{-1}$, and for cholesterol it was 10 mg L$^{-1}$.

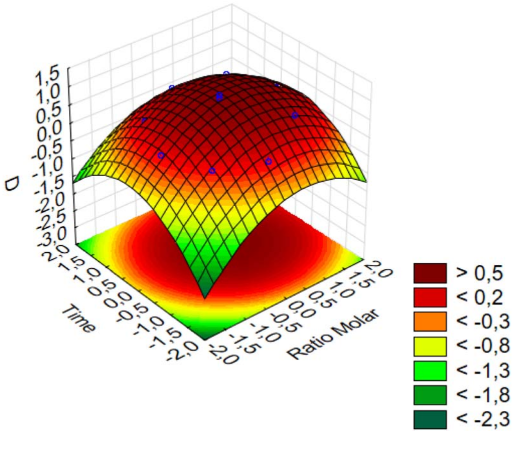


Fig. 5 Response surface obtained from central composite design.

In the study reported by Keller and Jahreis (2004),[41] the LQ obtained was also higher (1.19–1.35 mg L$^{-1}$ for cholesterol and coprostanol), analysed by GC-MS, when compared to the proposed method.

Overall, these findings indicate that benzoyl isocyanate provides a more efficient and sensitive alternative for the analysis of fecal sterols.

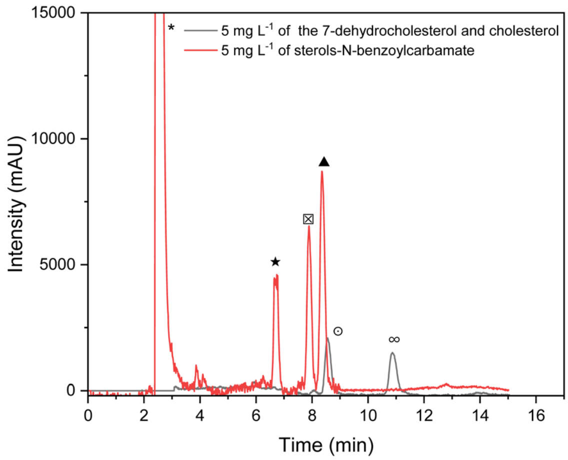


Fig. 6 Chromatograms of sterols in the absence and the presence of the derivatization reaction (*– benzoyl isocyanate; ★-7-dehydrocholesteryl-*N*-benzoylcarbamate; ⊠-cholesteryl-*N*-benzoylcarbamate, and ▲-coprostanolyl-*N*-benzoylcarbamate ⊙ – 7-dehydrocholesterol and ∞ – cholesterol).

Table 3 Analytical parameters of the method for the derivation of products

| | Linear range (mg L$^{-1}$) | LQ (mg L$^{-1}$) | LD (mg L$^{-1}$) | RSD intraday (%) | | RSD interday (%) | | F$_{calculated}$ |
|---|---|---|---|---|---|---|---|---|
| | | | | 3 mg L$^{-1}$ | 100 mg L$^{-1}$ | 3 mg L$^{-1}$ | 100 mg L$^{-1}$ | |
| 7-dehydrocholesteryl-*N*-benzoylcarbamate | 0.6–300.0 | 0.6 | 0.2 | 4.36 | 2.14 | 4.36 | 2.07 | 2.66 |
| Cholesteryl-*N*-benzoylcarbamate | 0.5–300.0 | 0.5 | 0.2 | 3.75 | 2.28 | 3.75 | 2.17 | 0.83 |
| Coprostanolyl-*N*-benzoylcarbamate | 0.5–300.0 | 0.5 | 0.2 | 4.57 | 3.82 | 4.57 | 3.53 | 2.47 |

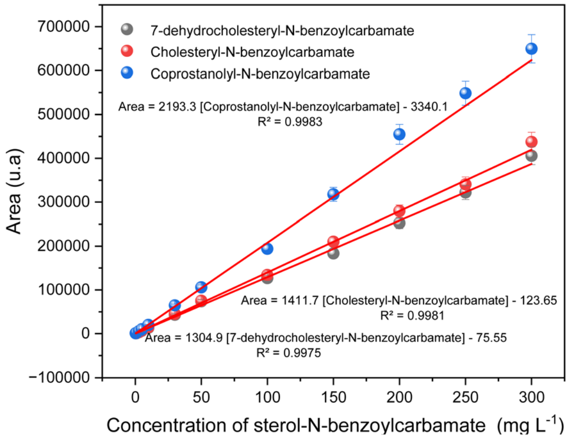


Fig. 7 Analytical curves of the method for the derivatization of sterols analysed in sterol-*N*-benzoylcarbamate forms.

### 3.5 Analysis of lake surface water and sediment samples

The interference-free determination of sterols, as well as the efficiency of the derivatization reaction was evaluated using the proposed method through the analysis of real samples, including lake surface water and sediment. The samples were spiked with known concentrations of sterols, and recovery values were calculated using the external calibration curve (Fig. 7). As shown in Table 4, recoveries ranging from 91 to 108% were obtained, within acceptable recovery according to AOAC,[40] attesting to the feasibility of the proposed method. The chromatograms of the samples are depicted in Fig. 8, which also demonstrates the efficient chromatographic separation.

Previous studies have reported the presence of coprostanol and cholesterol in surface lake sediments at concentrations of 9.24 and 63.64 mg kg$^{-1}$,[42] respectively. Other studies reported concentrations of 1.94 mg kg$^{-1}$ of coprostanol and 1.54 mg kg$^{-1}$ of cholesterol in sediment samples.[43] Coprostanol alone was detected at 13.8 mg kg$^{-1}$ in surface sediments.[44] These findings

Table 4 Analysis of lake water and sediment samples and recovery values ($n = 3$)

| | Concentration added | Lake Igapó | | Lake Cabrinha | | Ribeirão Cambé | |
|---|---|---|---|---|---|---|---|
| | | Concentration found | Recovery (%) | Concentration found | Recovery (%) | Concentration found | Recovery (%) |
| **7-dehydrocholesteryl-*N*-benzoylcarbamate** | | | | | | | |
| Water (mg L$^{-1}$) | — | <LD | — | <LD | — | <LD | — |
| | 3.0 | 3.1 ± 0.1 | 103 | 2.8 ± 0.1 | 92 | 2.9 ± 0.1 | 95 |
| | 50.0 | 48.8 ± 0.1 | 97 | 47.8 ± 0.1 | 95 | 51.1 ± 0.1 | 102 |
| Sediments (mg kg$^{-1}$) | — | <LD | — | <LD | — | <LD | — |
| | 6.0 | 5.6 ± 0.1 | 93 | 5.5 ± 0.1 | 92 | 6.5 ± 0.1 | 108 |
| | 100.0 | 93.7 ± 0.1 | 93 | 95.5 ± 0.1 | 95 | 101.3 ± 0.1 | 101 |
| **Cholesteryl-*N*-benzoylcarbamate** | | | | | | | |
| Water (mg L$^{-1}$) | — | <LD | — | <LD | — | <LD | — |
| | 3.0 | 2.9 ± 0.1 | 97 | 3.2 ± 0.1 | 105 | 3.2 ± 0.1 | 105 |
| | 50.0 | 54.2 ± 0.2 | 108 | 48.7 ± 0.1 | 97 | 45.9 ± 0.1 | 91 |
| Sediments (mg kg$^{-1}$) | — | <LD | — | <LD | — | <LD | — |
| | 6.0 | 5.4 ± 0.1 | 91 | 5.5 ± 0.1 | 92 | 6.4 ± 0.1 | 106 |
| | 100.0 | 101.7 ± 0.1 | 101 | 102.2 ± 0.1 | 102 | 91.9 ± 0.1 | 91 |
| **Coprostanolyl-*N*-benzoylcarbamate** | | | | | | | |
| Water (mg L$^{-1}$) | — | <LD | — | <LD | — | <LD | — |
| | 3.0 | 3.1 ± 0.1 | 102 | 3.0 ± 0.1 | 100 | 3.0 ± 0.1 | 101 |
| | 50.0 | 49.9 ± 0.1 | 99 | 51.9 ± 0.2 | 103 | 52.8 ± 0.1 | 105 |
| Sediments (mg kg$^{-1}$) | — | <LD | — | <LD | — | <LD | — |
| | 6.0 | 6.4 ± 0.1 | 107 | 6.1 ± 0.1 | 98 | 6.1 ± 0.1 | 101 |
| | 100.0 | 108.7 ± 0.1 | 108 | 107.6 ± 0.1 | 107 | 105.7 ± 0.1 | 105 |

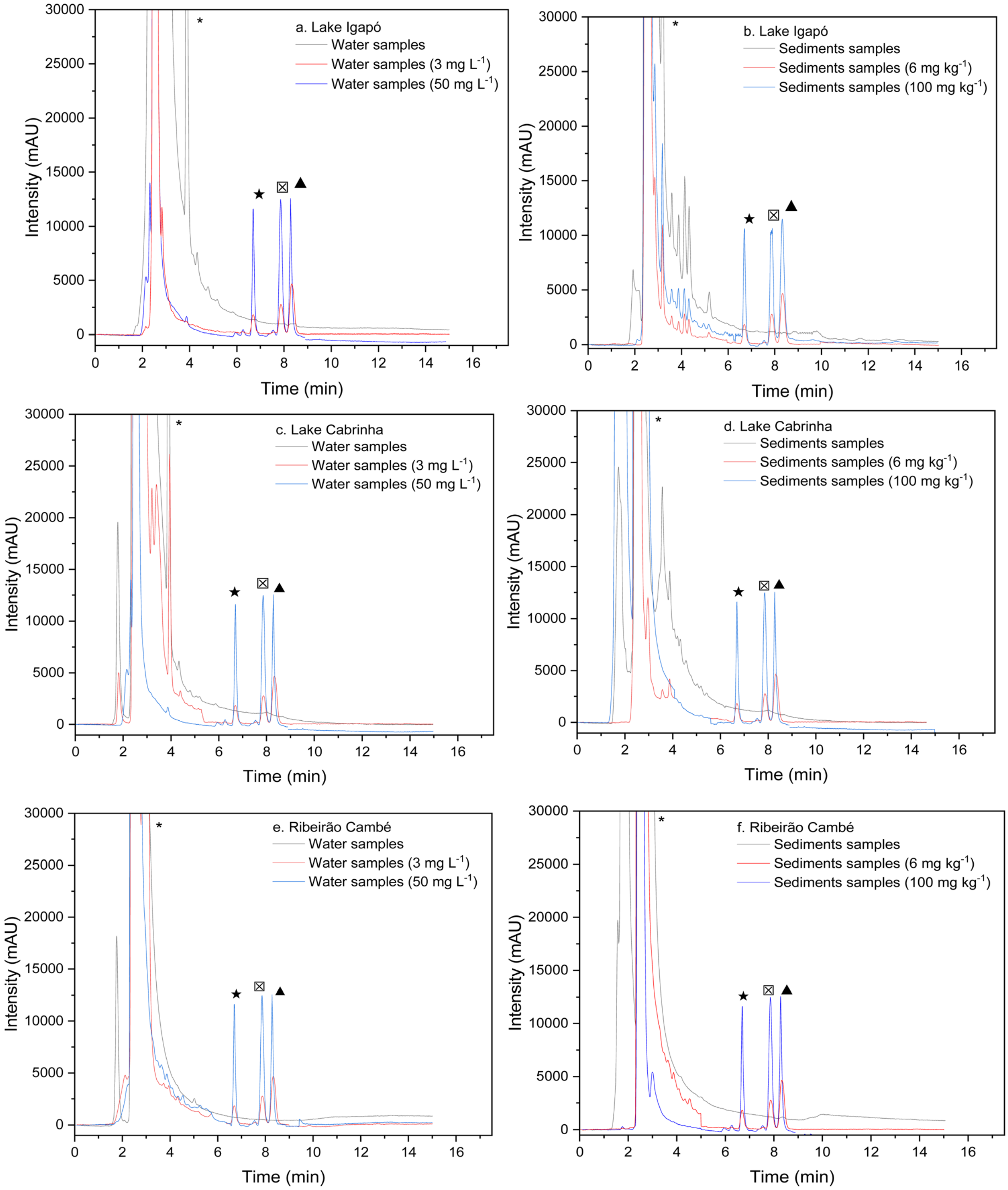


Fig. 8 Chromatograms from the analysis of surface water sample and sediments (*– benzoyl isocyanate; ★-7-dehydrocholesteryl-*N*-benzoylcarbamate; ☒-cholesteryl-*N*-benzoylcarbamate, and ▲-coprostanolyl-*N*-benzoylcarbamate).

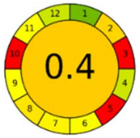
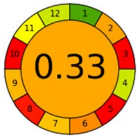
confirm the method's suitability for detecting fecal contamination based on the analytical parameters obtained.

### 3.6 Assessment of methods' greenness profile

Analytical Greenness (AGREE)[45] and Blue Applicability Grade Index (BAGI)[46] were employed to assess the greenness of the developed method. AGREE's measuring instrument rates the 12 GAC (green analytical chemistry) principles on a standard scale from 0 to 1, with 1 representing the greatest result and 0 representing the poorest. The different stages are represented by a color scale of green, yellow, and red. The result is a clock-like graph, with the total score and corresponding color displayed at the center. A score that is closer to one indicates greener conditions. Therefore, the sample pretreatment procedure that scores higher is more ecologically friendly.[45]

Blue Applicability Grade Index (BAGI) is software that assesses the practicality of analytical procedures. It evaluates

Table 5 AGREE and BAGI evaluation for the derivatization methods[a]

| Technique | Reagents | Analytes | Reaction time | AGREE | BAGI | References |
|---|---|---|---|---|---|---|
| GC-FID | BSTFA with 1% TMCS | Coprostanol, epicoprostanol, coprostanone, colestanone, cholesterol, cholestanol, stigmasterol, Β−sitosterol | 90 min at 65 °C |  |  | 7 |
| GC-MS | BSTFA with 1% TMCS | Coprostanol, epicoprostanol, 5a-androstanol-3b-Ol, cholesterol, cholestane, cholestanol, campesterol, stigmasterol, sitosterol, stigmastanol | 1 h at 100 °C |  |  | 8 and 40 |
| HPLC-UV | Benzoyl chloride in a pyridine | Cholesterol | — |  |  | 9 |
| HPLC-UV | *P*-nitrobenzoyl chloride in pyridine | Coprostanol | — |  |  | 10 |
| HPLC-UV | Benzoyl chloride in a pyridine | Coprostanol, epicoprostanol, cholesterol, cholestanol and B-sitosterol | 45 min in ultrasonic bath |  |  | 2 |
| HPLC-DAD | Benzoyl isocyanate in acetonitrile | 7-Dehydrocholesterol, cholesterol, and coprostanol | 32.1 min in ultrasonic bath |  |  | This work |

[a] BSTFA: bis(trimethylsilyl)trifluoroacetamide); TMCS: Trimethylsilyl.

various metrics, including the type of analysis, sample preparation techniques, number of steps involved, samples analysed per hour, sample volume, required preconcentration, instruments needed, and the level of automation.[46] In the present method, a score of 0.54 and 70 from the AGREE and BAGI methods, respectively, were obtained (Table 5).

The obtained result using the AGREE method is satisfactory, especially considering the use of HPLC-DAD, which involves organic solvents and is time-consuming. According to the BAGI data, the score of 70 falls within the accepted range of applicability, which typically spans from 25 to 100. Scores below 25 suggest limited applicability, whereas a score of 100 indicates excellent applicability.[43]

The proposed method, in comparison with other derivatization methods toward sterols determination by GC-FID, GC-MS and HPLC-UV, presents higher greenness and is less-time consuming.

## 4. Conclusions

This study demonstrated a novel and successful derivatization reaction method using benzoyl isocyanate as a derivatizing reagent toward sterol determination by HPLC-DAD. Chemometric tools were critical in optimizing the derivatization reaction, emphasizing low-reagent consumption and low reaction time. Furthermore, the greenness profile of the method was superior to other previously published methods, highlighting its more environmentally friendly appeal. The method demonstrated satisfactory figures of merit, and it was able to determine sterols in lake surface water and sediments without matrix effects. These results underscore the potential of the innovative method for assessing fecal pollution in environmental samples.

## Author contributions

Letícia Maria Effting: conceptualization, methodology, validation, investigation, formal analysis, writing – original draft. César Ricardo Teixeira Tarley: conceptualization, resources, methodology, validation, investigation, formal analysis, writing – review & editing, supervision, funding acquisition.

## Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

The data that has been used is confidential.

Supplementary information is available. See DOI: **https://doi.org/10.1039/d5ra04381f**.

## Acknowledgements

The authors acknowledge the financial support and fellowships of Coordenação de Aperfeiçoamento de Nível Superior (CAPES) (Project Pró-Forenses 3353/2014 Grant 23038.007082/2014-03), Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant 307432/2017-3, 307505/2021-9, 420097/2021-0, 190434/2017-1, 311113/2019-2, 408782/2024-2) Fundação Araucária do Paraná (163/2014), SETI do Paraná, and Instituto Nacional de Ciência e Tecnologia de Bioanalítica (INCT) (FAPESP Grant No. 2014/50867-3 and CNPq Grant No. 465389/2014-7).

## References

1 M. G. de Melo, O. C. dos Anjos, A. P. Nunes, M. A. dos, S. Farias, A. L. V. da S. Chaar and G. A. Bataglion, *Chemosphere*, 2023, **326**, 138365.
2 M. F. Resende, M. D. R. Santos, R. C. Matos and A. C. Matos, *Anal. Methods*, 2014, **6**, 9581–9587.
3 M. Alidoust, Y. Saito, H. Takada, K. Mizukawa, T. Karlsson, S. Brosché, B. Beeler and H. K. Karapanagioti, *Environ. Sci. Technol.*, 2024, **58**, 4761–4771.
4 M. P. Araújo, C. Hmacher, C. O. Farias and M. L. G. Soares, *Mar. Pollut. Bull.*, 2021, **165**, 112149.
5 M. S. Islam, K. Nakagawa, Z. Q. Yu, Y. Takao and R. Berndtsson, *J. Environ. Chem. Eng.*, 2023, **11**, 110029.
6 K. Nakagawa, M. S. Islam, S. S. H. Shah, Z. Li, Y. Takao and R. Berndtsson, *Chemosphere*, 2024, **361**, 142335.
7 R. S. Carreira, A. L. R. Wagener and J. W. Readman, *Estuarine, Coastal Shelf Sci.*, 2004, **60**, 587–598.
8 S. Froehner, R. F. Martins, M. R. Errera, *Environmental Monitoring and Assessment, Paraná*, Brazil, 2009, vol. 157, pp. 591–600.
9 L. F. Costa, M. P. Araújo, B. A. Knoppers and R. S. Aquatic, *Geochemestry*, 2011, **17**(1), 1–19.
10 A. Francis and S. Siggia, *Anal. Chem.*, 1973, **45**, 14.
11 E. A. Piocos and A. A. de la Cruz, *J. Liq. Chromatogr. Relat. Technol.*, 2000, **23**, 1281–1291.
12 F. K. Nzekoue, G. Caprioli, M. Ricciutellia, M. Cortese, A. Alesi, S. Vittori and G. Sagratini, *Food Res. Int.*, 2020, **131**, 108998.
13 A. Escrig-Doménech, E. F. Simó-Alfonso, J. M. Herrero-Martínez and G. Ramis-Ramos, *J. Chromatogr. A*, 2013, **1296**, 140–156.
14 D. Steen and J. R. Parsons, *Anal. Chem.*, 1995, **67**, 4409–4415.
15 G. Chávez, B. Bravo, N. Piña, M. Arias, E. Vivas, F. Ysambertt, N. Márquez and A. Cáceres, *Talanta*, 2004, **64**, 1323–1328.
16 T. Grzes, A. Zgoła-grzes, J. Zembrzuska and Z. Łukaszewski, *Chemosphere*, 2006, **64**, 803–809.
17 A. Baillet, S. Rakotomanga, D. Ferrier and F. Pellerin, *J. Chromatogr.*, 1990, **519**, 337–347.
18 J. A. Rodriguez, L. D. Mendoza, F. Pezzotti, N. Vanthuyne, J. Leclaire, R. Verger, G. Buono, F. Carriere and F. Fotiadu, *Anal. Biochem.*, 2008, **375**, 196–208.
19 G. Czichocki, P. Miiller, D. Vollhardt and M. Kriiger, *J. Chromatogr.*, 1992, **604**, 213–218.
20 C. Neuhoff, S. Dorato, E. Mariani and C. Villa, *Int. J. Cosmet. Sci.*, 1999, **21**, 199–205.
21 H. L. P. Laakso and W. W. Christie, *Lipids*, 1990, **25**, 349–353.

22 O. Vandenabeele-Trambouze and L. Garrelly, *Adv. Environ. Res.*, 2001, **6**, 45–55.
23 T. Zhou, H. Zhang and G. Duan, *J. Sep. Sci.*, 2007, **30**, 2620–2627.
24 C. H. Collins, G. L. Braga and P. S. Bonato, *Introdução a Métodos Cromatográficos*, 1997.
25 M. Ribani, C. Beatriz, G. Bottoli, C. H. Collins, I. C. Sales and F. Jardim, *Quim. Nova*, 2004, **27**, 771–780.
26 J. Augusto, R. Paschoal, S. Rath, F. Pereira, S. Airoldi and F. G. R. Reyes, *Quim. Nova*, 2008, **31**, 1190–1198.
27 C. R. T. Tarley, G. Silveira, W. N. L. dos Santos, G. D. Matos, E. G. P. da Silva, M. A. Bezerra, M. Miró and S. L. C. Ferreira, *Microchem. J.*, 2009, **92**, 58–67.
28 G. Derringer and R. Suich, *J. Qual. Technol.*, 1980, **12**, 214–219.
29 G. L. Long and J. D. Winefordner, *Anal. Chem.*, 1983, **55**, 712.
30 M. J. López De Alda and D. Barceló, *J. Chromatogr. A*, 2001, **938**, 145–153.
31 L. Effting, A. Bail, M. G. Segatelli and C. R. T. Tarley, 00, *J. Braz. Chem. Soc.*, 2023, 1–10.
32 S. Güney and O. Güney, *Electroanalysis*, 2017, **29**, 2579–2590.
33 *NMR Data Base*, **https://sciencesolutions.wiley.com/solutions/technique/nmr/**, Accessed January 2025.
34 J. de S. Aquino and J. A. da Silva, *Rev. Inst. Adolfo*, 2010, **69**, 588–594.
35 NIST Mass Spectrometry Data Center, *NIST Mass Spectrometry Data Center*, 1994, Nist Number: 136345, **https://webbook.nist.gov/cgi/inchi?ID=C4461330&Mask=200#Refs**, acessed July 2025.
36 F. G. Kitson, B. S. Larsen, C. N. McEwen, *Chapter 19 - Isocyanates, Gas Chromatography and Mass Spectrometry*, Academic Press, 1996, pp. 157–159.
37 J. Qiu, T. Li and Z. Zhu, *Anal. Chim. Acta*, 2021, **1142**, 108–117.
38 M. Kuo, J. M. Kalbfleisch, P. Rutherford, D. Gifford-Moore, X. Huang, R. Christie, K. Hui, K. Gould and M. Rekhter, *J. Lipid Res.*, 2008, **49**, 1353–1363.
39 AOAC, *Guidelines for Standard Method Performance Requirements*, AOAC International, Rockville, MD, USA, 2016.
40 D. Dubber, L. Brophy, D. O'Connel, P. Behan, M. Danaher, C. Evans, P. Geary, B. Misstear and L. Gill, *Environ. Pollut.*, 2024, **341**, 122884.
41 S. Keller and G. Jahreis, *J. Chromatogr. B*, 2004, **813**, 199–207.
42 C. Li, L. Zhu, J. Ju, Q. Ma and Q. Wang, *Ecol. Indic.*, 2024, **158**, 111487.
43 I. M. Bujagić, S. Grujić, Z. Jauković and M. Laušević, *Environ. Pollut.*, 2016, **213**, 76–83.
44 H. L. Hadlich, N. Venturini, C. C. Martins, V. Hatje, P. Tinelli, L. E. O. Gomes and A. F. Bernardino, *Ecol. Indic.*, 2018, **95**, 21–31.
45 F. Pena-Pereira, W. Wojnowski and M. Tobiszewski, *Anal. Chem.*, 2020, **92**, 10076–10082.
46 N. Manousi, W. Wojnowski, J. Płotka-Wasylka and V. Samanidou, *Green Chem.*, 2023, **25**, 7598–7604.