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Multi-detection of preservatives in cheeses by liquid chromatography–tandem mass spectrometry

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ABSTRACT

The incorrect use of preservatives in cheeses may compromise food safety and damage consumers. According to the law, more than one preservative may be contemporarily used in cheeses. So a method for their contemporary detection may be useful for both manufacturers and control agencies quality control. In this research a liquid chromatography–tandem mass spectrometric with electrospray ionization method for the multi-determination of seven preservatives (benzoic acid, citric acid, hexamethylenetetramine, lysozyme, natamycin, nisin and sorbic acid) in cheese was developed. The preservatives were contemporarily extracted from cheese by a single procedure, and analyzed by RP-LC/ESI-MS/MS (Ion Trap) in positive ionization mode, with single reaction monitoring (SRM) acquisition. Three sample types (hard, pasta filata and fresh cheese) were used for method evaluation. Recoveries were mostly higher than 90%; MDLs ranged from 0.02 to 0.26 mg kg^{-1}, and MQLs were included between 0.07 and 0.88 mg kg^{-1}. Due to matrix effect, quantitation was performed by referring to a matrix matched calibration curve, for each cheese typology. This method was also applied to commercial cheese samples, with good results. It appears fast, reliable and suitable for both screening and confirmation of the presence and quantitation of the preservatives in a single, multi-detection analysis.

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1. Introduction

In order to assure food safety and to protect consumers, the use of additives in foodstuffs is strictly regulated by EU laws [1–4], and recently updated [5–8]. Indeed national and international [9] authorities accurately established the guidelines about additives in food and foodstuffs. The use of additives (conditions, kind of food in which they can be used, maximum quantity allowed, etc.) must comply with these rules.

Manufacturing process protocols of protected designation of origin (PDO) cheese indicate the preservatives permitted, if any, for the production of each PDO cheese, the prescribed conditions of use and/or their maximum concentration.

Effective controls on foodstuffs, with the aim of repressing incorrect or fraudulent use of preservatives, require rapid and reliable methods. Methods developed for a contemporary determination of more than one preservative are preferable, because of their time saving effect, as well as their better impact on lab organization.

Control agencies and, in particular, control labs need sensitive analysis methods for evaluating the presence and/or the quantification of preservatives in food.

In dairy field, during cheese manufacturing, additives, and, among these, preservatives, are commonly used.

In the dairy products commonly used preservatives are:

- Sorbic acid, or its Ca and K salts (E200–203), used for antimicrobial preventing of mould, yeast and fungi growth [10]. These preservatives are allowed in cheeses up to 1000 mg/kg in many kind of non ripened cheeses, up to 2000 mg/kg in processed cheese, and "quantum satis" for only surface treatment of cheeses [4–6]. It is currently available an UV–HPLC based method to determine sorbic acid, or its Ca and K salts, in cheese [11], above a 5 mg/kg concentration;
- Natamycin (also called pimaricin, E235), added to prevent fungal outgrowth on cheese rind. Its use is allowed on cheese at a maximum level of 1 mg/dm^2 surface (not present at a depth of 5 mm) [4–6]. Actually there is an UV–HPLC based method to determine natamycin in cheese [12], with a MQL of 0.5 mg/kg, or a lower limit of the surface-area-related of 0.03 mg/dm^2;

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– Lysozyme (E1105) may be added, “quantum satis”, in long-ripened hard cheeses [4–6,8], to prevent the “late gas blowing”, a phenomenon caused by the growth of *Clostridium tyrobutyricum* [13]. Lysozyme is a potential allergenic agent and it must obey to the specific label rules for allergenic substances in foods [14]. A draft HPLC-fluorimetry method is employed to determine lysozyme in cheese [15], with a MQL of 5 mg/kg, currently under approval as ISO standard, and based on a precedent literature method [16];
– Hexamethylenetetramine (E239), used for the production of the provolone cheese to prevent the gas-producer clostridia bacteria. The legal limit is 25 mg/kg, expressed as formaldehyde concentration [4–6]. A colorimetric official method, approved by Italian government, currently exists for determining its presence in cheese [17], with a MQL of 0.5 mg/kg;
– Nisin (E234) is a ribosome-synthesized peptide with a broad-spectrum Gram-positive spoilage and pathogenic bacteria antibacterial activity dairy products. It is allowed for production of ripened and processed cheeses at a maximum concentration of 12.5 mg/kg [4–6] and it may be naturally present in cheeses due to fermentation processes [6]. No official methods currently exist for determination of nisin in cheese. Literature methods are anyway available. They are based on different techniques, as agar diffusion bioassay [18,19], or LC–ESI-MS [20];
– Citric acid, or its Na, K and Ca salts (E330–333), are commonly used in mozzarella or pasta filata cheese production, as preservative and/or acidity regulator, for the previous acidification of the bulk milk. These additives may be used “quantum satis” in cheeses [4–6]. An international enzymatic method for the determination of the citric acid content of cheese and processed cheese is currently available [20];
– Benzoic acid, or its Na, K, Ca salts, (E210–213) is not permitted in cheese manufacturing, according to the current legislation. Anyway it may be found in cheeses, since its use is allowed in the rennet used during cheese production [4–6] and, moreover, it may be a natural product of microbial metabolism [22]. Literature data [23] show that it may be found in cheeses up to 40 mg/kg, both for technical and microbiological reasons, even if it has not been added to cheese during the production. Currently, benzoic acid, and its salts, may be determined by the previously method for sorbic acid [11], above a 5 mg/kg concentration.

It should be highlighted that all the additives must be declared on the label of foodstuffs [14] and that a general re-evaluation of the rules of use of preservatives is currently on progress [5].

In a precedent work we already developed a method, based on reversed phase high performances liquid chromatography (RP-HPLC) with UV detection, to simultaneously determine sorbic acid, benzoic acid, natamycin and lysozyme [24].

In this work we successfully developed a method for the contemporary determination of the seven preservatives above cited, by RP-HPLC and tandem mass spectrometry (MS/MS) detection, with electrospray interface (ESI), in order to assure a correct identification according to current UE criteria [25].

Reference preservatives were individually analyzed and MS/MS characterized, with optimization of detection and fragmentation conditions. A mix of these preservatives was then separated, by optimization of RP-HPLC conditions.

Three typologies of cheese were spiked with the preservatives, and then analyzed, to optimize the entire method and determine accuracy (recovery), precision, method detection limit (MDL), method quantification limit (MQL), and linearity range.

The method was finally tested on many commercial cheese samples.

2. Materials and methods

2.1. Reagents, chemicals and working solutions

All the solvents were of HPLC grade. Other reagents were of analytical grade. PTFE syringe filters (0.45 μm pore size, 13 mm diameter) were purchased by Millipore (Billerica, MA, USA).

Standards of benzoic acid, natamycin, lysozyme and sorbic acid, were purchased by Sigma–Aldrich (St. Louis, MO, USA). Standard of nisin A, was purchased by MP Biomedicals Europe (Illkirch, France), standard of hexamethylenetetramine was purchased by Carlo Erba Reagenti (Milan, Italy), standard of citric acid was purchased by Merck KGaA (Darmstadt, Germany).

Standard stock solutions of each preservative were prepared at 1 mg/mL level in ultra-pure water, and stored at 4 °C in glass vials, for maximum one month.

The declared purity of the standard preservatives was considered, when preparing the standard stock solutions.

Standard mix working solution, containing the seven preservatives, was prepared at the day of use by mixing aliquots of each standard stock solution and diluting with 0.1 M acetate buffer (pH = 4.5):methanol (2:1, v/v) to obtain the final concentration of 0.1 mg/mL of the analytes.

2.2. Instrumentation

A linear ion trap-mass spectrometry LXQ, coupled with an ESI interface at an HPLC system, mod. Surveyor, composed of a quaternary pump, a degassing device, a column oven, an autosampler with a 20 μl loop and a DAD detector, from THERMO Fisher Scientific Inc. (Waltham, MA, USA), were used.

X-Calibur 2.0.7 software (THERMO Fisher Scientific Inc., Waltham, MA, USA) was employed for data acquisition and processing.

A chromatographic RP column model Zorbax 300SB-C8 (150 mm × 4.6 mm i.d., 300 Å pore size, 5 μm particle size) from Agilent Technology (Santa Clara, CA, USA), and a security guard column (ODS, 4 mm × 2.1 mm i.d.), from Phenomenex (Torrance, CA, USA), were used.

Ultra-pure water was produced by a PureLab Option system from ELGA LabWater (High Wycombe, UK).

2.3. Samples

Three different classes of cheese were chosen as reference samples for method evaluation: hard ripened, pasta filata, and fresh cheeses.

For each class, Italian cheeses, from retail market or available in our department as a result our control activity, were used. As hard ripened cheeses were used *Parmigiano Reggiano*, *Pecorino* and *Trentingrana*; as pasta filata cheeses were used *Mozzarella* and *Provolone*; as fresh cheeses were used *Crescenza* and *Stracchino*.

All the reference cheeses were declared as preservative-free, as recommended by the law or their respective manufacturing process protocols. The absence of the investigated preservatives was confirmed by HPLC–MS/MS analysis.

Method was finally tested on some commercial cheeses, whether purchased from Italian retail markets or available in our department as a result our control activity.

Each sample was well homogenized, analyzed in triplicate, and data were averaged.

2.4. Extraction procedure

Extraction was performed by mixing, with an Ultraturrax, for 1 min, 2 g of grated, well homogenized cheese sample, with 30 mL

of the extraction buffer solution, consisting in 1 M NaCl in 0.1 M acetate buffer (pH = 4.5):methanol (2:1, v/v).

The suspension was placed in an ultrasonic bath for 10 min and centrifuged for 5 min at 4000 rpm. The supernatant was collected, filtered through a 0.45 μm PTFE membrane syringe filter, and 20 μl were injected into the HPLC–ESI-MS/MS system.

2.5. LC/ESI-MS/MS analysis

Chromatographic elution was performed by a linear gradient of 0.05% TFA in acetonitrile (v/v) (eluent A) and 0.05% TFA in water (v/v) (eluent B) at the flow-rate of 1 mL/min.

Eluent A was linearly increased from 0 to 80% in 40 min, then it was maintained constant for 10 min to rinse the column. Finally, the eluent A content was lowered to 0% in 1 min and the column re-equilibrated for 15 min. Column was maintained at 35 °C.

The eluent from the column was divided with an appropriate split device, so that a 850 μL/min flow was directed to the PDA detector and a 150 μL/min flow was directed to the MS detector.

Ionization was carried out in the positive mode. The spray needle voltage was set to 4000 V, and the capillary voltage at 20 V. The temperature of the heated capillary was 270 °C. The flow rates of the nitrogen sheath gas and the auxiliary gas were set to 30 and 20 arbitrary units, respectively.

An helium flow, as damping gas was introduced according to the manufacturer's recommendations. Ionization and mass spectrometric conditions were optimized for each preservative by direct infusion of a 5 μL/min flow rate of a 0.01 g/L solution in acetonitrile:water 1:1 (v/v), containing 0.05% TFA (v/v).

Analytes were mass-selected and fragmented. For each compound two or three suitable transition pairs were chosen for acquisition in single reaction monitoring (SRM) mode. In order to increase the resolution, seven different scan events, with specific collision energies were applied, one for each analyte. Tuning parameters are summarized in Table 1.

Analytes were also detected on the PDA detector, in the range 200–400 nm.

Identification of the preservatives was performed by comparison, respectively, of the retention time, with that of the corresponding standards, and the MS/MS spectra of the analyte, with that of the standard.

Quantitation was assessed by considering the analyte peak area relating to a matrix matched calibration curve.

2.6. Method validation

2.6.1. Linearity, calibration curves and matrix effect

For each analyte, linearity was evaluated by analyzing solutions at increasing concentration of the analytes, to cover the concentration range usually found, for each preservative, according to the common good manufacturing practices.

A standard calibration line was constructed by analyzing mix solutions at five concentration levels in the ranges of 5–500 mg/kg.

Three different matrix matched calibration curves were also performed by spiking the extracts of hard ripened, pasta filata, and fresh cheeses, respectively, in order to cover three main typologies of cheese, giving the lack of an accepted and complete cheese classification.

A linearity *t*-test and a linearity *F*-test were performed, related to each analyte, both for the standard calibration curve and the matrix matched calibration curves. The tests were performed with Data Analysis tool of MS Excel (Microsoft Corp., USA).

In order to assure a representative matrix matched curve for each kind of cheese, the different cheeses previously reported were chosen as reference samples.

Each curve was constructed by addition of appropriate volumes of the standard mix working solution at blank cheese sample extracts in order to have the same concentration levels of the standard working solution.

The preservative peak area versus preservative concentration in cheese samples were plotted to get the calibration curves.

Standard and matrix matched solutions were prepared three times for each level; each solution was once injected and the results were averaged. For each preservative, unweighted regression curves both for standard and matrix-matched solution were calculated and compared.

Signal suppression on ESI-MS/MS response due to matrix effects was evaluated, for each analyte, by comparing the slope of the standard calibration curve with the slope of the matrix matched calibration curve.

2.6.2. Accuracy and precision

Accuracy was evaluated in terms of percentage of recovery on the three cheese typologies earlier described. For recovery studies different blank cheeses, reported in Section 2.3, were spiked prior to the extraction step. A weighted aliquot of the homogenized or grated sample was added of a small and suitable volume of working solutions of the analyte. After a few minutes extraction was carried out, as previously described.

For each analyte, three levels of concentration, corresponding to 10, 250, and 500 mg/kg, were investigated. Each experiment was conducted three times and data were averaged. The averaged recovery, for each cheese typology, and the relative standard deviations (RSD) were calculated.

2.6.3. MDL and MQL

For each analyte MDL and MQL were estimated from the MS analysis by the SRM LC/MS/MS chromatograms, as follows. A 5 mg/kg of each analyte standard solution was injected and the resulting trace was smoothed by the Gaussian smoothing algorithm (X-Calibur software). The peak height-to-averaged-background-noise ratio (S/N) was then measured for each analyte. MDL was established as the injected amount of analyte corresponding to a

Table 1
Retention time and instrumental parameter settings under single reaction monitoring (SRM) conditions of preservatives.

Preservative	Retention time (min)	Precursor ion (m/z)	RCE (%)[a]	Product ions (m/z)
Benzoic acid	15.32	122	47	105; 131; 140
Citric acid	3.14	210	63	147; 175; 193
Hexamethylenetetramine	3.18	141	37	98; 112
Lysozyme	21.42	1590[b]	47	1352; 1570
Natamycin	21.54	665	41	629; 633; 647
Nisin	2.57	567	24	373; 431
Sorbic acid	15.45	113	21	93; 98

[a] Relative collision energy expressed as %.
[b] The precursor ion of lysozyme corresponds to its ninth charged ion.

S/N = 3. MQL was established as the injected amount of analyte corresponding to a S/N = 10.

3. Results and discussion

3.1. General remarks

A single method able to determine in a single analysis the main preservatives used, fraudulently or not, during cheesemaking, is currently lacked. This is probably due to the strong differences of the most common preservatives in term of structure (organic acids, aromatic or not, polyene macrolide antibiotic, protein), as shown in Fig. 1, as well as to the different usual ranges of concentration. For instance, as previously mentioned, nisin is an antibiotic and is permitted up to 12.5 mg/kg, whereas sorbic acid is a linear organic acid and may be used up to 1000 mg/kg and benzoic acid is an aromatic organic acid, which is currently forbidden in cheeses. For this reason the development of a simple, accurate and fast method allowing to extract, identify and quantify all these compounds is in high demand.

3.2. Extraction

The extraction procedure is a really critical step, because it must be able to get a good recovery of several compound having different chemical–physical properties. In a precedent work [24] we proposed a fast and simple extraction procedure to extract four preservatives (benzoic acid, natamycin, lysozyme and sorbic acid), with a good recovery.

A similar approach was developed in this work showing to extract all the preservatives of this work with a good recovery. The procedure was not changed, except for the ratio “cheese sample weight: extraction solution volume”. It was optimized to achieve the best recovery for all the analytes.

The proposed extraction procedure, described in the Section 2, was applied to the three different kinds of cheeses (hard ripened, fresh and pasta filata) in order to verify its suitability, with satisfying results in terms of recovery (Table 3). All the % recovery were above 80%, many of these were above 90%. Recoveries were just a little under 80%, at 10 mg/kg, only for natamycin in hard ripened cheese (74%) and hexamethylenetetramine in pasta filata cheese (79%). The averaged recovery showed good results, ranging from 82% for natamycin in fresh cheese to 103% for benzoic acid in pasta filata cheese.

Sorbic acid recovery was over 91% for each kind of cheese and spiking level. The same was for benzoic acid, except for hard ripened cheese at 10 mg kg^{-1}. The literature data available for sorbic acid and benzoic acid [26,27] are in agreement with the recovery obtained.

The averaged recovery for each preservative, at each concentration level, for the different cheese typologies, was calculated, showing a good RDS.

3.3. Optimization of the LC/ESI-MS/MS conditions

3.3.1. Optimization of the chromatographic separation conditions

The multi-detection of analytes, with different chemical physical properties, is still a drawback that may be resolved by optimizing a proper separation through a single chromatographic separation step.

Previous works report separation based on liquid chromatography and UV detection [28], in different conditions, according to the structure of the preservatives: each preservative need a specific and different mobile phase pH. For instance organic acids, such as sorbic and benzoic acids, were well separated by reversed phase chromatography using a linear gradient of 0.1 M acetate buffer at pH 4.5 and methanol [25]; lysozyme [16] and nisin [21] were eluted with a linear gradient of 0.1% TFA in acetonitrile (v/v); natamycin

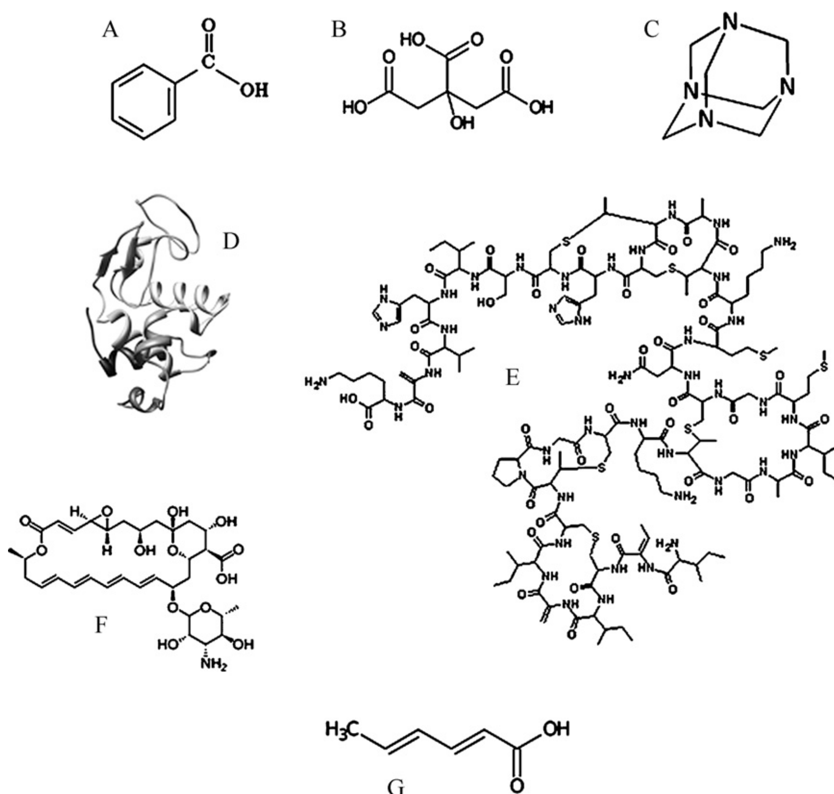

Fig. 1. Molecular structures of benzoic acid (A), citric acid (B), hexamethylenetetramine (C), lysozyme (D), natamycin (E), nisin (F), and sorbic acid (G).

was determined in reversed phase liquid chromatography, with an isocratic elution of methanol, water and acetic acid [29]; citric acid was eluted with 0.01 M potassium dihydrogen phosphate (pH 2.25) on C18 column [30]. A single and reliable method, able to separate these preservatives in a single chromatographic run, is thus strongly required.

In our research we considered the references for each preservative and found out a good compromise, in terms of method performances, analytical conditions, analysis time, and easiness of application. A linear gradient of 0.05% TFA in acetonitrile was select as better mobile phase to separate all the analytes, as described in the Section 2.5. Addition of the acid to the eluent system is effective for enhancement the formation of $[M-H]^+$ of acidic compounds such as sorbic acid under the positive ion mode, because H^+ adheres to the molecular form.

Since the preservatives have different ultraviolet (UV) spectra, the wavelength program of the variable wavelength detector was used in this study to detect all the compounds at their most sensitive wavelength.

The UV chromatograms are reported in Fig. 3, showing an overall good "class" separation. Sorbic and benzoic acids resulted roughly co-eluted, as well as lysozyme and natamycin, and citric acid and hexamethylenetetramine. The UV separation is thus not satisfying by itself.

This issue was overtaken by the mass spectrometric detection, that allows a good discrimination between the single analytes, even with a slight co-elution.

3.3.2. Optimization of the mass spectrometric revelation conditions

A drawback of protein revelation by MS is that it is dependent by protein structure. When modifications in their structures occur and the modification, or degradation, involve the fragment of the analyte, revealed by the MS, the analyte may be not revealed. This may be true for lysozyme and nisin, as already shown in literature [31,32]. Anyway, it should be also considered that the current limits for preservatives in food have to be applied for the native preservative in the final product, so that degradation or other modifications are not relevant for control purposes.

As previously highlighted, preservatives with different structures and chemical physical properties, may behave very differently in the ESI ionization source. For this reason, tuning conditions were individually optimized for each of them.

The optimization of the tuning conditions was reached by carrying out individual analysis, in the optimized separation conditions, of the seven preservatives. The instrument was programmed, through a specific function of the software, to perform, in the time window of the single analyte, the optimization of the tuning conditions, in order to get the most reliable signal.

After the definition of the optimized tuning condition of each preservative, the MS acquisition program was sub-divided in different time windows segments. Each segment was thus set with the specific tuning condition for each preservative.

This allowed enhancing the S/N ratio of some compounds, as natamycin and lysozyme, which had really different ionization conditions, compared to the other analytes.

An issue was the identical mass of sorbic acid and the sodium lactate, a compound usually found and/or added in cheese. According to the Commission Decision 2002/657/EC, Annex, Table 6 concerning the performance of analytical methods and the interpretation of results, two different transitions for each analyte were considered in order to assure their identification. In particular by modulating the collision energy (CE) reported in Table 1, two diagnostic fragments for sorbic acid (m/z 93 and 98) were identified: these fragments were not found in the fragmentation pattern of sodium lactate.

For lysozyme, a multiply charged ion was chosen as the precursor ion, due to its structure. In particular its ninth charged ion was chosen because its formation was found constant in different samples. In Fig. 2 is reported the mass chromatograms and the extract ion chromatograms (XICs) of a standard mix at 100 mg/kg concentration level, showing that a good chromatography separation and an accurate choice of both CE and product ions may be really useful for a proper determination of all these compounds.

3.4. Method validation

3.4.1. Calibration curves, linearity, and matrix effect

As illustrated in Section 2.6, linear calibration curves were obtained both by standard calibration and by matrix matched procedures. The linearity ranges of all the analytes, in the three different cheese typologies, were evaluated. Linearity t-test and F-test for the curves were also performed. For each analyte the calibration curves, their linear regression analysis and the results of statistical tests are shown in Table 2.

The response was linear in a wide range of concentration, including the concentrations of preservatives commonly used for cheese making, and the levels of interest of the EU limits. t-Test and F-test fully confirm the linearity in the ranges under evaluation.

It should be emphasized that the control of the presence of a preservative in cheeses may be, in some cases, simply reduced to check if the preservative is present, or not, in the cheese (presence/absence), since there are no limits established ("*quantum satis*"), or the limits are really high.

The matrix effect was calculated as reported in Section 2.6 and shown in Table 2. The average ratio between slopes ($b_{matrix}/b_{standard}$) is strongly dependent both on cheese typology and on the preservative. For some preservatives it was more than 0.60, showing a considerable matrix effect. Due to these high differences between standard and matrix matched calibration, we chose to carry out the evaluation of method performances on the matrix curve, in order to improve the accuracy of the evaluation.

The matrix effect seems to be related both to the cheese typology and the preservatives. Differences in the chemical nature and structure of the preservatives, in the composition of the cheese matrices, due to the ripening, different microbial fermentations, different chemical-physical structures, different chemical conditions (water activity, pH, salt concentration,. . .) may give rise to really different chemical behaviours, that explain the observed differences between standard and matrix matched calibration curves, and among the matrix matched calibration curves.

In this study we used several blank samples for each cheese typology, different for manufacturing process and curing times. For example, in the case of hard cheese, we used Parmigiano Reggiano, Pecorino and Trentingrana, as already described. By comparing results (data not shown) we confirmed that matrix effect was the same for different cheeses, belonging to the same typology.

3.4.2. Accuracy

The evaluation of accuracy, expressed as percentage of recovery, was carried out on blank sample extracts, spiked with a known amount of the analytes. In order to test the method suitability, accuracy was investigated in the three cheese typologies (hard ripened, pasta filata and fresh cheese). Recoveries (Table 3) were evaluated at three different levels of concentration for each analyte, corresponding to the minimum, the maximum and an intermediate value of the evaluated range.

For the results, see Section 3.2. Experimental data showed the overall good accuracy of the method for the seven preservatives. Accuracy data need to be used to correct the concentration level experimentally found in the real samples.

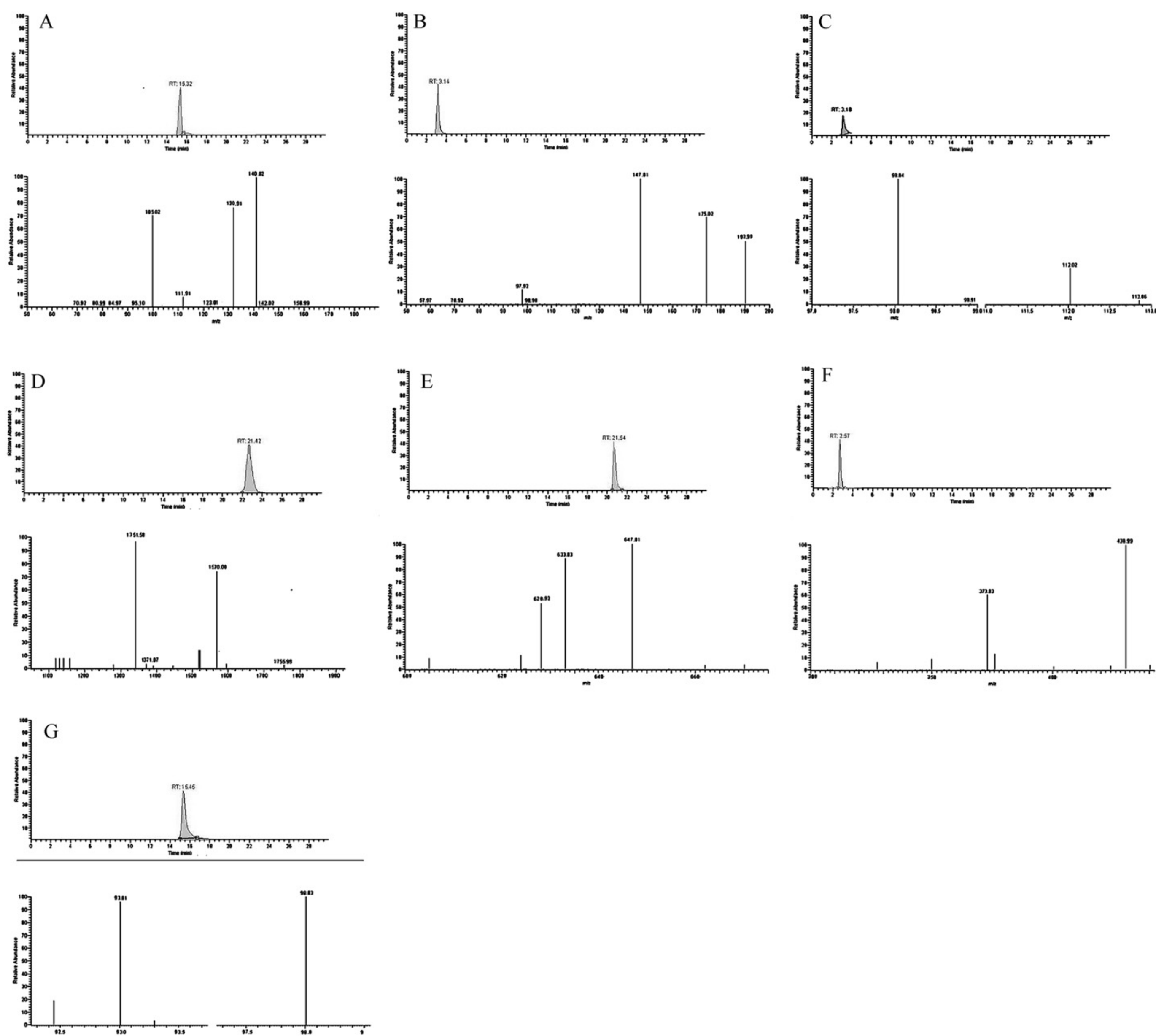

Fig. 2. Mass chromatograms and extract ion chromatograms (XICs) of benzoic acid (A), citric acid (B), hexamethylenetetramine (C), lysozyme (D), natamycin (E), nisin (F), and sorbic acid (G) obtained by injecting a standard mix solution at 100 mg/kg concentration level. MS conditions common to all the analytes: positive mode, spray needle voltage 4000 V, capillary voltage 20 V, capillary temperature 270 °C, nitrogen sheath gas flow rate 30 arbitrary units, auxiliary gas 20 arbitrary units.

3.4.3. MDL and MQL

MDL and MQL were evaluated as described in Section 2.6 and data are listed in Table 4. Results showed that this method allows to detect concentrations of preservatives under 1 mg/kg. That corresponds to concentration values that are widely coherent with the control purposes, according to both EU legislation [4–6] and good manufactory practice.

MDL and MQL values are similar, and even better, to those reported in the introduction paragraph.

3.5. Real sample analysis

The method was finally tested on several commercial cheeses and data, expressed as mg/kg, are shown in Table 5. The seven preservatives were analyzed in commercial samples that were chosen to cover the main cheese types. Each sample was three times analyzed as usually recommended in the main official methods and performed in the routine analysis. So data were averaged and the relative standard deviations (RSD) were calculated.

In order to assure an accurate determination, quantitation was calculated by using the specific matrix matched calibration curve depending on the cheese typology.

Qualitative results always agreed with the preservatives declared on the label. No other preservative, among those under investigation, was detected. Quantitative analysis was carried out without reference values, since no concentration level of the preservatives was declared on the label of the samples.

Anyway, all the preservatives declared on the label were found at a lower concentration than the legal upper limit and

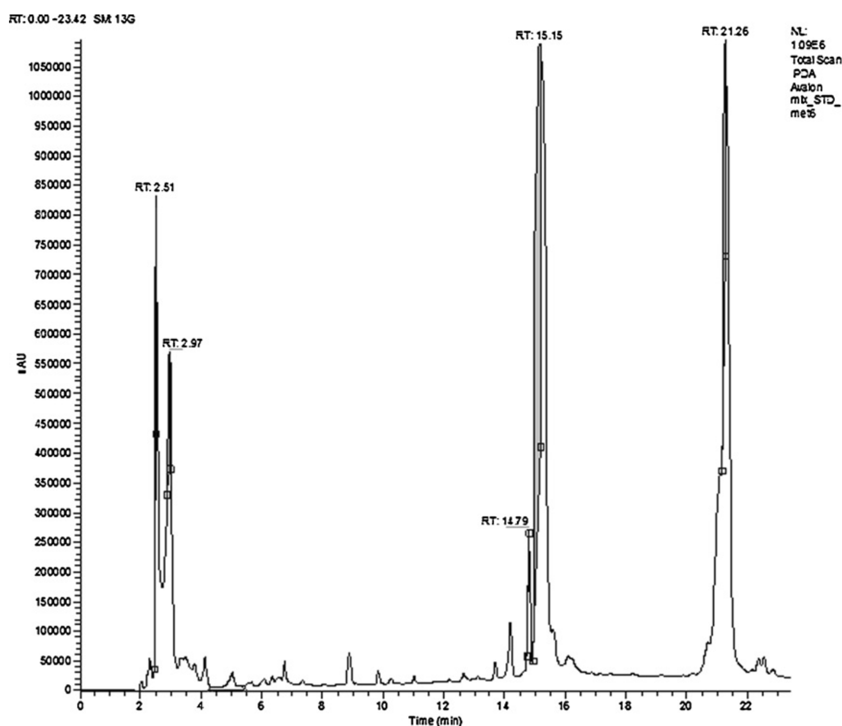

Fig. 3. Total scan (200–400 nm) separation chromatogram of the seven preservatives. Retention times (min): nisin (2.51), citric acid and hexamethylenetetramine (2.97), benzoic and sorbic acid (15.15), lysozyme and natamycin (21.26). Co-elutions are resolved with MS/MS analysis.

Table 2
Calibration curves.

Preservative	Standard equation[a]	Matrix-matched equation[b]		Matrix effect[c]
Benzoic acid	$y = 0.6747x - 13.333$	Hard ripened	$y = 0.3933x + 7.1667$ ($R^2 = 0.9930$)	0.58
	($R^2 = 0.9917$)	Fresh	$y = 0.3493x + 1.3333$ ($R^2 = 0.9999$)	0.52
		Pasta filata	$y = 0.5091x + 6.2368$ ($R^2 = 0.9912$)	0.75
Citric acid	$y = 806.59x - 2755.7$	Hard ripened	$y = 527.13x - 2955.9$($R^2 = 0.9999$)	0.65
	($R^2 = 0.9999$)	Fresh	$y = 150.76x + 3767.5$ ($R^2 = 0.9932$)	0.29
		Pasta filata	$y = 334.38x + 3185.3$ ($R^2 = 0.9998$)	0.41
Hexamethylenetetramine	$y = 11.679x + 148.75$	Hard ripened	$y = 2.4667x - 0.8333$($R^2 = 0.9998$)	0.21
	($R^2 = 0.9957$)	Fresh	$y = 2.2858x - 7.3307$($R^2 = 0.9994$)	0.20
		Pasta filata	$y = 4.44x - 1.8333$ ($R^2 = 0.9996$)	0.38
Lysozyme	$y = 0.7867x + 2.000$	Hard ripened	$y = 0.462x - 1.5618$ ($R^2 = 0.9998$)	0.59
	($R^2 = 0.9999$)	Fresh	$y = 0.7693x - 8.1667$($R^2 = 0.9976$)	0.98
		Pasta filata	$y = 0.774x - 2.4435$ ($R^2 = 0.9989$)	0.98
Natamycin	$y = 6.2866x + 8.5657$	Hard ripened	$y = 420.61x + 118953$($R^2 = 0.9987$)	0.66
	($R^2 = 0.9987$)	Fresh	$y = 384.91x + 166790$($R^2 = 0.9950$)	0.60
		Pasta filata	$y = 410.43x + 134103$($R^2 = 0.9976$)	0.64
Nisin	$y = 641.61x + 229429$	Hard ripened	$y = 420.61x + 118953$($R^2 = 0.9987$)	0.66
	($R^2 = 0.9924$)	Fresh	$y = 384.91x + 166790$($R^2 = 0.9950$)	0.60
		Pasta filata	$y = 410.43x + 134103$($R^2 = 0.9976$)	0.64
		Hard ripened	$y = 3.6294x - 49.666$($R^2 = 0.9946$)	0.90
Sorbic acid	$y = 4.0396x + 13.92$	Fresh	$y = 1.1859x - 9.1024$($R^2 = 0.9987$)	0.29
	($R^2 = 0.9909$)	Pasta filata	$y = 2.3929x - 45.231$($R^2 = 0.9975$)	0.59

[a] Unweighted regression lines; y = preservative peak area, and x = concentration of preservative expressed as mg/kg. Standard calibration lines were constructed by analyzing mix standard solutions at five concentration levels in the ranges of 5–500 mg/kg. All the solutions were prepared three times for each level, once injected and the results were averaged.

[b] Unweighted regression lines; y = preservative peak area, and x = concentration of preservative expressed as mg/kg. Matrix matched calibration lines were constructed by addiction of appropriate volumes of the standard mix working solution at blank cheese sample extracts of hard ripened cheeses, pasta filata cheeses and fresh cheeses, respectively, in order to have the same concentration levels of the standard working solution. Each sample was three times analyzed, and data were averaged in order to assure a representative matrix matched curve.

[c] Matrix effect was evaluated for each analyte by comparing the slope of the standard calibration curve with the slope of the matrix-matched calibration curve.

Table 3
Accuracy and precision.

Preservative	Cheese typology	Spiking level	Rec. (%)	Av. Rec (%)	RSD
Benzoic acid	Hard ripened	10 mg/kg	84	92	8
		250 mg/kg	95		
		500 mg/kg	98		
	Fresh	10 mg/kg	91	93	2
		250 mg/kg	94		
		500 mg/kg	95		
	Pasta filata	10 mg/kg	97	103	5
		250 mg/kg	103		
		500 mg/kg	108		
Citric acid	Hard ripened	10 mg/kg	89	88	3
		250 mg/kg	85		
		500 mg/kg	91		
	Fresh	10 mg/kg	86	92	5
		250 mg/kg	96		
		500 mg/kg	93		
	Pasta filata	10 mg/kg	90	91	3
		250 mg/kg	94		
		500 mg/kg	88		
Hexamethylenetetramine	Hard ripened	10 mg/kg	84	83	3
		250 mg/kg	80		
		500 mg/kg	85		
	Fresh	10 mg/kg	93	94	2
		250 mg/kg	97		
		500 mg/kg	93		
	Pasta filata	10 mg/kg	79	88	12
		250 mg/kg	100		
		500 mg/kg	85		
Lysozyme	Hard ripened	10 mg/kg	98	98	2
		250 mg/kg	95		
		500 mg/kg	100		
	Fresh	10 mg/kg	104	101	5
		250 mg/kg	95		
		500 mg/kg	105		
	Pasta filata	10 mg/kg	94	95	6
		250 mg/kg	89		
		500 mg/kg	101		
Natamycin	Hard ripened	10 mg/kg	74	83	12
		250 mg/kg	93		
		500 mg/kg	81		
	Fresh	10 mg/kg	80	82	2
		250 mg/kg	82		
		500 mg/kg	83		
	Pasta filata	10 mg/kg	103	95	8
		250 mg/kg	96		
		500 mg/kg	87		
Nisin	Hard ripened	10 mg/kg	92	93	3
		250 mg/kg	97		
		500 mg/kg	91		
	Fresh	10 mg/kg	93	93	3
		250 mg/kg	90		
		500 mg/kg	95		
	Pasta filata	10 mg/kg	100	102	2
		250 mg/kg	104		
		500 mg/kg	102		
Sorbic acid	Hard ripened	10 mg/kg	98	96	8
		250 mg/kg	103		
		500 mg/kg	96		
	Fresh	10 mg/kg	95	93	2
		250 mg/kg	91		
		500 mg/kg	93		
	Pasta filata	10 mg/kg	99	95	4
		250 mg/kg	92		
		500 mg/kg	95		

Rec., recovery expressed in percentage; Av. Rec, average of recoveries; RSD, relative standard deviation expressed in percentage.

in a range of concentration coherent to the good manufacturer practices.

Natamycin was analyzed in the rind of all samples. Values reported in the Table are expressed as mg/kg. When converted in mg/dm^2, they meet the legal limit.

The only "anomalous" value was for nisin in the N. 5 hard ripened cheese sample: we assume that it may be due to an irregular or fraudulent addition.

Literature data for benzoic acid, sorbic acid, natamycin and lysozyme [24,33,34] further confirm the experimental data.

Table 4
Method detection limit (MDL) and method quantitation limit (MQL).

Preservative	MDL (mg/kg)[a]	MQL (mg/kg)[b]
Benzoic acid	0.26	0.88
Citric acid	0.09	0.28
Hexamethylenetetramine	0.07	0.25
Lysozyme	0.18	0.62
Nisin	0.02	0.07
Natamycin	0.03	0.11
Sorbic acid	0.06	0.20

[a] Method detection limit estimated by the SRM LC/MS/MS chromatogram, for an injection of 20 mg/kg of preservative from a standard solution (S/N = 3).
[b] Method quantification limit estimated by the SRM LC/MS/MS chromatogram, for an injection of 20 mg/kg of preservative from a standard solution (S/N = 10).

In Table 6 are resumed, if available, the statistical parameters (MQL, repeatability, reproducibility, matrix effect) of the reference methods, as cited in Section 1, for each preservative, compared with those experimentally determined for this method.

The comparison shows that this method has almost better MQL than the reference methods, except for the nisin. It may be simply explained because we assumed as the MQL for the reference methods the lowest value of the declared application range of the methods. As we also experienced, this value is usually above the real MQL, as previously defined. The value of MQL for nisin of the reference method is quite below that we found, because it is based on a specific, thus more sensitive, bioassay.

The repeatability is almost of the same level both for the reference methods and for this method. No information are obviously available for reproducibility of this method, nor for the matrix effect of the reference methods.

Table 5
Quantitation of the preservatives in commercial cheeses.

Cheese samples		Preservative content (RSD)						
		A	B	C	D	E	F	G
Hard ripened	N. 1	–	–	–	–	–	11 (3)	–
	N. 2	–	–	53 (8)	–	88 (3)	–	–
	N. 3	–	–	–	64 (5)	34 (6)	8 (2)	64 (8)
	N. 4	–	–	–	–	–	–	–
	N. 5	–	–	–	176 (2)	–	41 (5)	270 (3)
Fresh	N. 1	–	–	–	–	–		20 (6)
	N. 2	–	–	–	–	51 (7)	–	57 (6)
	N. 3	–	–	–	–	41 (5)		103(4)
	N. 4	–	–	–	–	–	–	–
Pasta filata	N. 1	–	–	–	–	18 (3)	–	–
	N. 2	–	–	–	–	–	–	–
	N. 3	–	–	–	–	–	–	–
	N. 4	–	–	–	–	–	–	–
	N. 5	–	–	–	–	–	–	56 (5)

A, B, C, D, E, F, and G correspond to benzoic acid, citric acid, hexamethylenetetramine, lysozyme, natamycin, nisin, and sorbic acid, respectively.
Each sample was three times analyzed, data were averaged, and the relative standard deviations (RSD) were calculated. Amount of preservatives was calculated using matrix matched calibration, corrected for recovery, and expressed in mg/kg. Dash stands for preservatives concentration below the MDL.

Table 6
Comparison with parameters of the reference methods.

Preservative		Parameter			
		MDL (mg/kg)	Repeatability	Reproducibility	Matrix effect
Benzoic acid	Ref. method	5[a]	2.235 + 0.031 μ	8.987 + 0.130 μ	
	This method	0.88	2–8[b]		0.58–0.75
Natamycin	Ref. method	0.5[a]	From 0.093 μ (at 60 mg/kg) to 0.29 μ (at 0.3 mg/kg)	From 0.206 μ (at 60 mg/kg) to 0.39 μ (at 0.3 mg/kg)	
	This method	0.11	2–12[b]		0.72–0.97
Lysozyme	Ref. method	5[a]			
	This method	0.62	2–6[b]		0.59–0.98
Hexamethylene tetramine	Ref. method	0.5 (MDL)			
	This method	0.25	2–12[b]		0.20–0.38
Nisin	Ref. method	2 (μg/kg)			
	This method	0.07	2–3[b]		0.60–0.66
Citric acid	Ref. method		0.05 μ	0.08 μ	
	This method	0.28	3–5[b]		0.29–0.65
Sorbic acid	Ref. method	5[a]	2.235 + 0.031 μ	8.987 + 0.130 μ	
	This method	0.20	2–8[b]		0.29–0.90

μ = mean value of two repetitions in routine.
[a] MQL assumed as the lowest concentration of the application range, validated for the method.
[b] Expressed as CV%.

4. Conclusions

This work was intended to develop an analytical method to contemporarily detect many preservatives, commonly used during cheese-making, mainly for quality control and fraud repression purposes.

An RP-LC/ESI-MS/MS (Ion Trap) method was developed for the contemporary detection of seven preservatives (benzoic acid, citric acid, hexamethylenetetramine, lysozyme, natamycin, nisin and sorbic acid) in cheese, really different for nature, function and structure. The method consists in a simple extraction procedure of the preservatives from the cheese, an RP-HPLC separation of the preservatives, an ESI-MS/MS revelation.

The method was developed by using three different typologies of cheese (fresh, hard ripened and pasta filata). A relevant matrix effect in all the three typologies was observed. By applying the matrix matched calibration curves, the method showed good averaged recoveries, always above 80%. MDL and MQL were always below 1 mg/kg, widely consistent with the operative range and the legal limits for the use of the preservatives.

The method was tested against commercial samples, to confirm its reliability, with results in line with their respective labels.

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