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A review on the most frequently used methods to detect antibiotic residues in bovine raw milk

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1 **Review**

2

3 **Methods to identify antibiotic residues in bovine raw milk**

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15

16 **Abstract**

17 Bovine milk is a low cost and high nutritional value product, which is worldwide commercialized.
18 To maintain high standard levels and to preserve consumer's health, tests could be used to identify
19 antimicrobial drug residues, and control procedures must be planned and performed, according to
20 legislative requirements. Ideally, a test should detect a large number of antibiotic molecules, should
21 be rapid in the execution and not expensive. None of the actually available methods possesses the
22 characteristics of the ideal test. Authors decided to write this review to resume the key features of
23 immunoassays, high-performance liquid chromatography, liquid chromatography – tandem mass
24 spectrometry, microbiological test, and biosensor assays, representative of the most used methods to
25 detect antimicrobial residues in bovine raw milk. The final aim was to give essential information to
26 veterinary practitioners and researchers who are engaged in on site screenings, official controls or in
27 research.

28 *Keywords:* Analytical methods, Antimicrobials, Bovine milk, Residues, Screening test

29 **1. Introduction**

30 Bovine milk is one of the most consumed foods in the world and it is important for its high
31 nutritional value and for its key role in the worldwide economy (FAO, 2019). Its composition
32 comprehends proteins (3.0-3.9%), carbohydrates lactose and oligosaccharides (4.4-5.6%), fat (3.3-
33 5.4%), and ash (0.7-0.8%) (Roy, Ye, Moughan, & Singh, 2020). Milk is an important dietary source
34 of a variety of micronutrients, including calcium, phosphorus, magnesium, zinc, iodine, potassium,
35 vitamin A, vitamin D, vitamin B12, and vitamin B2 (Dror & Allen, 2014). This variety of components
36 is responsible for biochemical and nutritional features, and the derived energy is mainly due to the
37 high presence of lactose fraction (Luiz, Bell, Rocha, Leal, & Anjos, 2018; Roy, et al., 2020).

38

39 One of the most important concerns about bovine milk is the contamination with veterinary
40 medicine: antibiotics and anti-inflammatory drugs are administered to dairy cows to treat pathologies
41 such as mastitis, endometritis, bronchopathies, pneumonia and lameness (EMA and EFSA, 2017; Han
42 et al., 2015). The treatment of animals is mandatory to respect animal welfare but must be performed
43 in a rational way: focusing on antibiotics, a targeted therapy with narrow spectrum molecules should
44 be preferred, for the shortest time necessary to achieve a therapeutic outcome (Rajala-Schultz,
45 Nødtvedt, Halasa, & Persson Waller, 2021). In the past, antibiotics were employed not only for the
46 sole therapeutic purpose, but also to promote growth of food-producing animals and in metaphylactic
47 protocols (Lees, Pelligand, Giraud, & Toutain, 2021). Without a stringent regulation, an overuse, or
48 a misuse of antibiotics was diffused and, consequently, foodstuff contamination due to antibiotics and
49 antibiotic residues was not unusual (Lees et al., 2021). The interest of the scientific community
50 pushed several nations to change legislation to restrict antibiotic drug usage and preserve consumers'
51 health (Lees et al., 2021; Luiz et al., 2018). European legislation (Regulation EU 2019/6) confirmed
52 the ban of these substances as growth promoting agents and severely restricted prophylactic and
53 metaphylactic uses. Recently, USA and China banned the use of antimicrobial drugs as growth
54 promoting agents, as well (Lees et al., 2021).

55

56 Antibiotic residues might persist after the treatment and therefore, a withdrawal period is
57 mandatory to ensure that the foodstuff derived from the treated animal do not contain levels of the
58 drug that exceed the maximum residue limit (MRL) (Almashhadany, 2021; Jayalakshmi,
59 Paramasivam, Sasikala, Tamilam, & Sumithra, 2017).

60

61 As defined by the European Union, a residue is a pharmacologically active substance, the
62 parental drug, or its metabolites, which remains in foodstuff obtained from animals to which a
63 medicinal product was administered (Regulation EC 470/2009; Regulation EU 37/2010). For each
64 active substance, a MRL value has been established and is a precautionary value, which does not
65 guarantee the absence of drug residues in milk or dairy products, thus adverse reactions in consumers
66 are not excluded and might occur (Treiber & Beranek-Knauer, 2021). The MRLs established for
67 antibiotics frequently used in dairy cows are summarized in Table 1.

68

69 Antibiotic residues from food can impair human health causing allergic reactions, mutations
70 in cells, development of intestinal microbiome imbalances, and permitting to bacteria to get used to
71 subtherapeutic concentrations of antibiotic drug and leading to the development and spread of AMR
72 (Sachi, Ferdous, Sikder, & Azizul Karim Hussani, 2019; Treiber & Beranek-Knauer, 2021;
73 Yazdanpanah et al., 2021). The direct consequences are complicated therapeutic outcomes, treatment
74 failure, or the possibility to share and diffuse resistant pathogen microorganisms among animals and
75 humans (Vercelli, Gambino, Amadori, & Re, 2022).

76

77 The control of milk to adhere to law requirements, the risks to share AMR through the
78 worldwide consumption of milk and the preservation of consumers' health, are elements of a
79 complicated network and they are all related each other (Ortelli, Cognard, Jan, & Edder, 2009).
80 Ideally, a screening test should detect a large number of molecules in the same assay, should not be

81 time- and money-consuming and should be easy in the execution, even if performed by not
82 specialized personnel. Nowadays several tests are commercially available or are under investigation
83 to evaluate their possible use on site or in laboratory conditions, as confirmatory tests or for research
84 purposes. Unfortunately, none of them possesses the characteristics of the ideal test. Thus, prior to
85 use a test to detect the presence of antibiotic residues, advantages and disadvantages should be
86 carefully evaluated to reach the most reliable result. Moreover, milk is a complex matrix, composed
87 by several elements that may interfere with residues identification: purification, dilution and
88 preparation of samples or specific storage conditions might be required (Serraino et al., 2013).
89 Additionally, the purposes of the tests might be different: some tests are easy and fast in their
90 execution and suitable for screening purposes but necessitate confirmation methods in case of
91 positivity, leading to delay in obtaining result and to increased costs (Bilandžić et al., 2011; Burke &
92 Adley, 2021). Regardless of the final use, test validation is mandatory accordingly to Commission
93 Decision 2002/657/EC, concerning the performances of analytical methods and interpretation of
94 results, thus establishing the criteria of linearity, sensitivity, specificity, intra- and inter-assay
95 precision, and accuracy (2002/657/EC).

96

97 Considering all aforementioned factors, authors would like to summarize in the present review
98 the most important methods that are currently used to identify antibiotic residues in bovine raw milk.

99

100 **2. Current available methods to detect antibiotics residues in milk**

101 *2.1 Immunoassays*

102 Enzyme-Linked Immunosorbent Assay (ELISA) technique was first described in 1971 by
103 Engvall et al. (1971) and found multiple applications ranging from medical to biotechnological
104 purposes. The method is based on an antigen-antibody linkage that produces a colorimetric reaction
105 due to the presence of a chromophore linked to the antigen. The ELISA technique may be designed
106 as qualitative (positive/negative result) or quantitative (result as measurement or concentration). Also,

107 a semiquantitative test exists, giving different levels of positivity and negativity that must always be
108 compared to a reference scale (Belmar, Aly, Karle, & Pereira, 2019). Several ELISA-based kits are
109 commercially available and used to rapidly detect a few families of antibiotics in bovine milk: due to
110 the easy execution and the low cost, they can be performed as screening tests by veterinarians and
111 dairy industry personnel (Belmar et al., 2019) (Table 2).

112
113 Anyway, in case of positivity and official controls, a confirmation test is necessary, using
114 more specific and sensitive tests, such as high-performance liquid chromatography (HPLC) or Liquid
115 Chromatography-Tandem Mass Spectrometry - LC-MS/MS) (Belmar et al., 2019). These two
116 methods will be described in further paragraphs. In fact, the limit of detection (LOD – the lowest
117 amount of analyte that can be identify in a sample) and the limit of quantitation (LOQ) (lowest amount
118 of analyte in a sample that can be quantitatively determined) of immunoassay – based techniques are
119 proven to be higher of that of analytical methods, such as HPLC or LC-MS/MS (Ahmed et al., 2020;
120 Moudgil, Bedi, Aulakh, Gill, & Kumar, 2019).

121
122 The ELISA method was a milestone to develop new detection techniques such as a biochip-
123 based test, which has been recently validated and seems to represent a significant improvement of the
124 actual screening methods, considering the relative low-cost and rapid execution, even if it is able to
125 detect only β -lactams (Yazdanpanah et al., 2021). Taking inspiration from antigen-antibody
126 interaction, a new technique based on a magnetic immunoreaction has been developed to detect
127 kanamycin and penicillin in dairy milk, demonstrating an extremely low LOD and a high sensitivity,
128 but pretreatment and dilution steps of samples are necessary. This method is a proof-of-concept, not
129 yet on the market (Pietschmann, Dittmann, Spiegel, Krause, & Schröper, 2020). A microarray assay
130 ELISA- based has been recently validated: it can detect simultaneously norfloxacin, tetracycline,
131 lincomycin and streptomycin in milk samples, reaching a remarkably high accuracy rate (ranging

132 from 77,6% to 116,4% of the different antibiotics), but having the disadvantage to require specialized
133 and trained personnel. So far, this method is suggested for control programs in the dairy industry (Du
134 et al., 2019).

135
136 A commercially available immunoassay method is Lateral Flow Immunoassay (LFIA), that
137 compared to ELISA is less time – consuming (easier samples preparation), with fast results
138 acquisition, permits to analyze a large quantity of samples, and it is less expensive (Ahmed et al.,
139 2020, Jiang et al., 2022). In the last few years, this method has been widely used to detect antibiotics
140 in milk, such as β -lactams, tetracyclines, streptomycin and chloramphenicol and it has been recently
141 implemented also for other matrices, such as honey, muscle, and liver (Ahmed et al., 2020).

142
143 Among immunoassays also radioimmunoassay (RIA), fluorescence immunoassay (FIA) and
144 colloidal gold immune-chromatographic assay (CGIA) must be listed (Ahmed et al., 2020).
145 Radioimmunoassays have been widely used in the past to detect antibiotic residues in foodstuff due
146 to its fast execution and low LOD (Yang & Carlson, 2004), but the short half-life of the radioisotopes
147 used to label the analyte, and the possible risks for the personnel and the environment connected to
148 the use of radioisotopes limited its application to a few clinical purposes (Ahmed et al., 2020).
149 Fluorescence immunoassay is based on the linkage of a fluorophore to the antigen, giving a precise
150 and well visualized detection, but background signal can interfere with the emission thus giving
151 ambiguous results (Ahmed et al., 2020). This specific immunoassay has been extensively used to
152 detect fluoroquinolones (Hu, Sheng, Zhang, Wu, & Wang, 2015), beta lactamases (Benito-Peña,
153 Moreno-Bondi, Orellana, Maquieira, & van Amerongen, 2005) and tetracyclines (Song et al., 2015)
154 in milk. Colloidal gold immune-chromatographic assay has been validated and used to detect
155 tetracycline, sulphonamides, and fluoroquinolones residues rapidly and simultaneously in milk
156 (Wang et al., 2017) but the scarce precision of the method leads in some cases to unclear results that
157 limited its application (Zhou et al., 2018).

158

159 *2.2 High-Performance Liquid Chromatography (HPLC)*

160 As previously explained, rapid tests are such as ELISA and other immunoassays are used as
161 first line, in-field screening methods and in case of positivity, the results must be confirmed using
162 more accurate methods such as HPLC or LC-MS/MS (Bilandžić et al., 2011).

163

164 The HPLC is a chromatographic technique that requires specific and expensive instruments,
165 time-consuming procedures and must be performed by trained personnel (Table 2). It is considered a
166 gold standard method to detect antibiotic residues due to the high sensitivity, specificity, and
167 quantification capability (Parmar, Chaubey, Gupta, & Bharath, 2021). The HPLC instrument is
168 composed of 5 major components: mobile phase, detector, pump, column, and sampler (manual or
169 automatic) (Fig. 1). Samples are injected and carried by the mobile phase flow through the column
170 where separation of compounds occurs according to their differential affinity for the mobile phase.
171 The pump generates optimal flow and pressure and pushes the mobile phase through the column that
172 reaches the detector: a signal is generated, and it is proportional to the amount of the compounds in
173 the sample (Parmar et al., 2021). Comparing the peaks resulting from the analysis to the calibration
174 curve (that is the reference), it is possible to identify the compound that has been isolated and to
175 calculate its concentration in the sample.

176

177 It has been validated the use of HPLC to detect tetracycline, sulfonamides and amphenicols
178 in milk (Darko, Borquaye, Acheampong, & Opong, 2017) but it is sometimes very challenging to
179 adequately prepare and purify milk sample prior performing the procedure: fat, protein and sugars
180 present in milk can compromise the correct identification of residues (Luiz et al., 2018; Roy et al.,
181 2020; Yang, Guo, Liang, Zhou, & Zhu, 2022). The problems due to the matrix effect are reported in
182 the most recent literature related to innovative technique based on ultraviolet/visible spectroscopy
183 that has been validated to simultaneously identify multiple residues in milk (Parmar et al., 2021).

184 Moreover, results can vary according to pH and temperature, leading to alteration of some analytes
185 that are not stable, such as tetracycline, leading to an incorrect or missing identification of
186 antimicrobial residues in milk (Kurjogi et al., 2019).

187

188 A recent paper (Kumar, Panda, & Sharma, 2022) described the optimization and validation
189 of HPLC-diode array detection (HPLC-DAD) method for the simultaneous determination of seven
190 antimicrobial drugs: sulphadiazine, sulphamethoxazole, oxytetracycline, doxycycline, tetracycline,
191 enrofloxacin and chloramphenicol residues in bovine milk. The final aim of this advances HPLC
192 technique is to improve the features of the method, allowing multiple identifications and saving time.
193 Samples preparation is a fundamental step and authors explained that solid-phase extraction (SPE)
194 was performed to eliminate proteins from milk samples. This highlights once again the importance
195 of a careful sample preparation prior the execution of analysis. This advanced technique was
196 successfully validated in accordance with European Commission Decision 2002/657/EC
197 (2002/657/EC) and applied for the analysis of antibiotic residues in 21 raw milk samples obtaining
198 recovery values ranging from 83.3 to 111.8% (3.5-16.2% standard deviation). The LOQ values
199 relative to all tested antibiotics, except for chloramphenicol, were below the MRLs, making the
200 method dependable and susceptible for further implementation for routine application (Kumar et al.,
201 2022).

202

203 *2.3 Liquid Chromatography -Tandem Mass Spectrometry (LC-MS/MS)*

204 Liquid Chromatography-Tandem Mass Spectrometry is an advanced analytical technique
205 characterized by a high performance in identifying and quantifying antimicrobials, their metabolites
206 or residues present in various foodstuff, also in milk, with remarkably high accuracy and precision
207 (Parmar et al., 2021). Sample constituents eluted from the column are ionized and vaporized,
208 subjected to fragmentation, and separated according to the mass-to-charge ratio (M/Z) in the mass

209 analyzer. The abundance or intensity of each ion with a different M/Z value is measured by the
210 detector and it is proportional to the concentration of the analyte in the sample (Fig. 2).

211

212 When an MS/MS is used the “first MS” is set to select a parent ion (the M/Z value is most
213 often the molecular ion of the substance), which then is passed into the “second MS” where it
214 undergoes further fragmentation (Parmar et al., 2021). Ions are usually extremely specific to a given
215 substance, and therefore their analysis provides an exceptionally reliable identification and
216 quantification (Cronly et al., 2010; Zhao, Zulkoski, & Mastovska, 2017). This technique is extremely
217 accurate (more than HPLC), and it is intended as high standard and confirmation tool. The main
218 disadvantages of this technique are the high cost, the long execution, and the need to work in
219 standardized experimental conditions (i.e., control ionization, pH, analyte stability) (Zhao et al.,
220 2017). Also in this case, the complexity of milk composition may lead to misleading results and recent
221 papers underlined that a reliable sample preparation is fundamental before LC-MS/MS analysis
222 (Meklati et al., 2022; Zhao et al., 2017). The undoubted advantages are the simultaneous detection of
223 multiple residues of different antibiotic classes and the very high analytical accuracy: this is ensured
224 by the fact that structural information of the analyte is gained permitting the identification and
225 quantification of very small amounts, having lower LOD and LOQ compared to HPLC (Parmar et
226 al., 2021).

227

228 An advanced technique that has been successfully applied to detect antimicrobial residues is
229 ultra-high performance liquid chromatography (UPLC), used alone (Castillo-Aguirre, Cañas, Honda,
230 & Richter, 2021; Rahman, Hassan, & Chowdhury, 2021) or coupled with electrospray ionization and
231 tandem mass spectrometry (UPLC–ESI–MS/MS) (Castillo-Aguirre et al., 2021; Luiz et al., 2018;
232 Meklati et al., 2022). The method proposed by Igualada, Giraldo, Font, & Yusà (2022) aimed to
233 identify 255 veterinary drug residues and contaminants in bovine raw milk. The procedure included
234 two-step precipitation and ultra-performance liquid chromatography, operating both in positive and

235 negative multiple reaction mode (MRM). For most of the analytes, pretreatment process was a crucial
236 step to ensure a successful identification.

237

238 A study specifically focused to simultaneously detect 38 veterinary antibiotic residues in raw
239 milk was recently performed using UPLC–MS/MS (Han et al., 2015). The methodology differs from
240 others present in literature due to a purification of samples by Oasis HLB cartridge and a dilution
241 with water and acetonitrile (1mL sample; 0.5 mL water; 3 mL acetonitrile), before the injection into
242 the UPLC–MS/MS system. The results indicated variable recoveries of 68–118% for drugs belonging
243 to β -lactams groups, 79–118% for quinolones, 71–106% for sulfonamides, 76–116% for
244 tetracyclines, 78–106% for macrolides, and 88–103% for lincosamides, with coefficients of variation
245 less than 15% (Han et al., 2015).

246

247 *2.4 Microbiological tests*

248 Microbiological tests have been used for a long time as screening tests to detect residues in
249 milk: they are still used with significant improvements (Sachi et al., 2019). No sample preparation is
250 needed, and a milk sample is considered positive when an inhibition zone appears in the reference
251 bacterial cultures. The diameters of the inhibition zone must be measured to interpret the results
252 (Gaudin et al., 2004). This technique has a low specificity that might lead to incorrect results: high
253 somatic cells or pH variations in case of mastitis, may lead to false negative results (Wu et al., 2019).
254 Nevertheless, this kind of method continues to be the focus of several investigations to improve the
255 current method due to the brief time of execution (Table 2) and to the versatility with various products
256 of animal origin, i.e., eggs and honey and raw, pasteurized, and bulk milk (Wu et al., 2019).

257

258 Advancement of this technique permitted to design the use of specific bacteria, sensitive to
259 particular antibiotics on agar gel including nutrients for bacterial growth and a pH indicator (Tumini,
260 Nagel, & Althaus, 2019). All elements are set up in a test tube where milk is added and then the tube

261 is incubated at the appropriate temperature to allow bacteria grow. Without antibiotic residues, the
262 normal growth of bacteria is unaltered and visually detectable, appreciating a change of opacity of
263 the medium and of the color due to acid pH. Otherwise, the growth of bacteria is inhibited, without
264 observable changes (Nagel, Molina, & Althaus, 2013). Nowadays several kits based on this technique
265 are commercially available. Anyway, few families of antibiotics can be identified using this kind of
266 methods, and it is not possible to obtain a quantification of residues amount. Considering two of the
267 most popular kits, Delvotest ST-NP can detect β -lactams, aminoglycosides, macrolides,
268 sulphonamides, tetracyclines and diamino pyrimidine, while Charm Quad 1 test identifies only β -
269 lactams, quinolones, sulfonamides and tetracyclines. Both tests claim in their datasheet the possibility
270 to identify the presence of residues corresponding at least to MRL values, and both declare 95% of
271 sensitivity (Charm QUAD1 datasheet; Delvotest datasheet;).

272

273 Low costs and simple execution are the main pros of this technique, while limitations could
274 be the relative long incubation period (3-24 hours), the possibility of acquisition of antimicrobial
275 resistance in the bacteria used for the detection, the subjective interpretation, the presence of
276 inhibitors in abnormal milk (e.g., mastitis milk or colostrum) leading to misinterpretations (Tumini
277 et al., 2019; Wu et al., 2019). The interest of the scientific community is to develop and validate new
278 kits based on this method that can permit a qualitative identification of antibiotic residues even at
279 concentrations lower than MRL (Gaudin et al., 2004; Tumini et al., 2019; Wu et al., 2019).

280

281 *2.5 Other methods*

282 A methodology based on Fourier transform near-infrared (FT-NIR) spectroscopy associated
283 with principal component analysis (PCA) permitted to develop a portable prototype to detect ceftiofur
284 hydrochloride traces in bovine milk in a fast and accurate way (Luiz et al., 2018). The method
285 permitted to identify the parental molecule and the metabolites (Luiz et al., 2018).

286

287 Another promising technique that has been validated in the last years and that is continuously
288 improved is the method based on the biosensors (Kivirand, Kagan, & Rinke, 2015). Several current
289 methods of analysis are based on this technique, which does not require preliminary preparation of
290 samples, enabling a selective detection for the on-site assessment of milk quality (Martins et al., 2019;
291 Tumini et al., 2019). Biosensors are compact and usually portable devices, able to transfer the
292 selective biochemical recognition into a measurable physical signal for real-time analysis, user-
293 friendly, not requiring specific skills (Kivirand et al., 2015). Five distinct types of biosensor-based
294 techniques are nowadays available: microbial biosensor, immunosensor, receptor and enzyme-based
295 biosensors, aptasensors and molecularly imprinted polymer (MIP) sensors (Babington, Matas, Marco,
296 & Galve, 2012; Beltrán, Berruga, Molina, Althaus, & Molina, 2015; Rebe Raz, Bremer, Haasnoot, &
297 Norde, 2009). The most diffuse are immunosensors and aptasensors, that allow to perform the
298 analysis without an extensive pretreatment of samples and in a brief time (30-40 minutes) (McGrath,
299 Elliott, & Fodey, 2012; Reder-Christ & Bendas, 2011).

300

301 **3. Discussion**

302 In this review, authors aimed to summarize the current available methods to identify antibiotic
303 residues in bovine raw milk, giving some simple explanations about the most notable features, a brief
304 description of how they work, highlighting advantages and disadvantages. Authors are veterinarians
305 having clear in mind the necessity to treat diseased animals, to apply reliable methods to correctly
306 identify antimicrobial residues in milk, to preserve consumers' health, to limit AMR spread and to
307 avoid alterations of dairy products (Burke & Adley, 2021; Kneebone, Tsang, & Townson, 2010).

308

309 The necessity to correctly identify antibiotic residues is only the end of a long story. Among
310 the different pathologies that can affect dairy cows, mastitis are surely the most painful and
311 debilitating conditions, impairing animal welfare and requiring immediate cure using anti-
312 inflammatory drugs and antibiotics. The administration of a therapy is necessary to limit economic

313 losses due to the reduction in milk production and the discard of milk (Alves et al., 2020). Diagnosis
314 of causative agent is often missing, and therapeutic protocols are applied in an empiric way leading
315 to an increased risk to induce antibiotic resistance in bacteria (Vercelli et al., 2022). The presence of
316 small amounts of antibiotic or residue in milk could play a significant role in the outbreak of allergic
317 phenomena and in the dissemination of resistant bacteria or genes (Alves et al., 2020; Treiber &
318 Beranek-Knauer, 2021).

319

320 The different methods differ each other for the expensiveness and the time-consuming
321 procedure, the necessity to prepare or purify the samples, and the interference of matrix effect: all
322 these parameters can affect specificity, sensitivity, and accuracy of each method (Alves et al., 2020;
323 Kurjogi et al., 2019). Along the manuscript, authors highlighted that despite many advances on the
324 detection techniques, sample preparation remains a problem influencing the determination of
325 antibiotics in milk and interfering in analytical procedures (Aguilera-Luiz, Martínez Vidal, Romero-
326 González, & Garrido Frenich, 2012). For the majority of the aforementioned techniques, the high
327 protein and fat content in milk are the main responsible of this interference. The extraction methods
328 are often based on long and tedious procedures, involving liquid-liquid extraction (García-Mayor,
329 Garcinuño, Fernández-Hernando, & Durand-Alegría, 2006) or SPE (Darko et al., 2017) which also
330 include a previous step to precipitate proteins.

331

332 In recent years, the method named QuEChERS (quick, easy, cheap, effective, rugged and safe)
333 has been investigated and now it is commercially available (Zhang et al., 2019). The method is based
334 on an acetonitrile extraction/partitioning of various compounds, while water and proteins are removed
335 from the sample using sodium chloride and magnesium sulphate. Then, SPE clean up permit the
336 extraction of contaminants and antimicrobials residues that remain in the supernatant part after the
337 centrifugation (Zhang et al., 2019). It is a rapid and reliable extraction system, flexible to different

338 situations and that can permit to achieve optimal results also in official controls for milk quality
339 (Zhang et al., 2019).

340

341 Misleading results can occur with every methodology presented in the present review and this
342 is the reason authors stressed the explanations about the rigorous preparation of the samples, the
343 accuracy of the methods and the complexity of milk as matrix. There, the importance to use reliable
344 tests and to obtain relevant results and to preserve consumers' health, especially fragile people, to
345 maintain high standard levels in milk production and in dairy industry, limiting AMR spread.

346

347 **4. Conclusions**

348 Prior to write this review, Authors had to face the vast panorama of different methods designed
349 to identify antimicrobial residues in bovine raw milk. After a careful consultation and selection of the
350 most recent and relevant literature, they were able to summarize the advantages and disadvantages of
351 the most important, dependable, and frequently used methods. It might be concluded that screening
352 tests are mainly represented by microbiological, immuno- and biosensors assays. Among these three
353 categories, several tools are available and significant differences of cost exist while all are
354 characterized by a short time of execution. All of them require a confirmation test in case of positivity
355 using a validated gold standard method (i.e., HPLC or LC-MS/MS).

356

357 Authors hope to have clearly exposed and successfully resumed such a complex topic and that
358 this review will help other veterinarians and researchers dealing with antibiotic residues in bovine
359 raw milk.

360

361 **Declarations of interest**

362 None.

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365

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