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REVIEW

CHEMOPREVENTIVE POTENTIAL OF MINOR COMPONENTS OF OLIVE OIL AGAINST CANCER

POTENZIALI CHEMIOPREVENTIVE DI ALCUNI COMPONENTI MINORI DELL'OLIO DI OLIVA CONTRO IL CANCRO

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ABSTRACT

Epidemiological studies have suggested that a diet rich in olive oil is associated with a reduced risk of a number of common cancers. In the last decade significant advances have been made in the understanding of how virgin olive oil may work to prevent cancer. A plethora of olive oil constituents have been identified as effective agents against the initiation, promotion and progression of multistage carcinogenesis. These include minor constituents such as the well-studied tocopherol

RIASSUNTO

Studi epidemiologici hanno suggerito che una dieta ricca di olio di oliva è associata ad un minor rischio di cancro. Negli ultimi decenni sono avvenuti sensibili miglioramenti sulle conoscenze relative alle modalità di azione preventiva dell'olio di oliva sul cancro. Numerosissimi composti presenti nell'olio d'oliva sono stati identificati come efficaci nella prevenzione dell'iniziazione, promozione e progressione delle carcinogenesi. Tra questi composti minori sono elencati i tocoferoli, i ca-

- Key words: cancer, chemoprevention, olive oil -

and carotenoid antioxidants, a number of very efficient antioxidant phenolics (simple phenols-hydroxytyrosol, tyrosol- and linked phenols- secoiridoids, lignans-), the triterpene hydrocarbon squalene and the phytosterol β-sitosterol. Provocative recent studies have revealed that the major antioxidant components of olive oil can also exert important non-antioxidant biological functions. Because the chemopreventive role of olive oil constituents is based on hypotheses and not hard data, it needs to be evaluated and verified in animal models of multistage carcinogenesis and in humans in order to gain a better understanding of the effect of olive oil consumption on human cancer. In this review, the antioxidant activity of a number of minor olive oil components is presented and evidence of their ability to interfere with and modulate cellular pathways important in carcinogenesis is reviewed. The implications of these properties for their cancer chemopreventive potential is discussed.

rotenoidi, numerosi antiossidanti fenolici (idrossitirosolo, secoiridoidi, ecc.), lo squalene ed il β-sitosterolo. Recenti studi hanno dimostrato come i più rappresentativi antiossidanti dell'olio d'oliva esercitano anche un importante ruolo non-ossidante nelle funzioni biologiche. Poiché il ruolo chemiopreventivo dell'olio d'oliva è basato solamente su ipotesi, è necessario testarlo e verificarlo su modelli carcinogenesi multistadio su animali e sull'uomo al fine di migliorare le conoscenze dell'effetto dell'olio d'oliva sul cancro. In questa review è presentata l'attività antiossidante di numerosi componenti minori dell'olio d'oliva, unitamente alla loro capacità di interferire su importanti percorsi chimici della carcinogenesi. Sono infine discusse le implicazioni di queste proprietà sulla loro potenzialità chemiopreventiva.

INTRODUCTION

Diet plays an important role in the pathogenesis of cancer, and it is estimated that about one-third of all human cancers may be attributed to dietary factors (DOLL and PETO, 1981). The traditional Mediterranean diet is a healthy, disease-preventing diet that is characterized by a high consumption of foods of plant origin, a relatively low consumption of red meat, and a high consumption of olive oil. With respect to cancer prevention, it has been suggested that up to 25% of the incidence of colorectal cancer, ~15% of the incidence of breast cancer, and ~10% of the incidence of prostate, pancreas, and endometrial cancer could be prevented if the populations of the highly developed Western countries would shift to the traditional, healthy Mediterranean diet (TRICHOPOULOU *et al.*, 2000).

Olive oil, one of the oldest known vegetable oils, is extracted from the fruit of the olive tree, *Olea europea*, and is almost unique among the vegetable oils because it can be consumed without any refining (OWEN *et al.*, 2000 b; DE LA LASTRA *et al.* 2001). Circumstantial evidence derived from epidemiological studies suggests that olive oil may play an important role in the cancer-protective activity of this type of diet (TRICHOPOULOU *et al.*, 2000; OWEN *et al.*, 2000b; WCRF, 1997). For example, although Greek women consume signifi-

cantly more total fat than Israeli women, they have a substantially lower risk of breast cancer, possibly because olive oil makes up a much larger proportion (57%) of their fat intake (ROSE *et al.*, 1986) or because of a higher intake of ω-3 polyunsaturated fatty acids and monounsaturated fatty acids (BARTSCH *et al.*, 1999). Furthermore, the risk of breast cancer is reduced by 25% in Greek women who consume olive oil more than once a day (TRICHOPOULOU *et al.*, 1995). Additional supporting evidence comes from epidemiological studies in Italy (LA VECCHIA *et al.*, 1998; BRAGA *et al.*, 1998) and Spain (MARTIN-MORENO *et al.*, 1994). After reviewing the available studies, LIPWORTH *et al.* (1997) concluded that the evidence for a protective effect of olive oil was strong (though not conclusive) for breast cancer and promising for other cancers.

The study of the chemoprevention of cancer is a new discipline founded upon epidemiological evidence which suggests that dietary components may be used to prevent or halt carcinogenesis (BRENNER, 2000). Over the last decade, as our understanding of the molecular and biochemical mechanisms of the carcinogenesis process has improved, chemoprevention researchers have been trying to discover food components which, by themselves or in combination, may inhibit carcinogenesis. Food components with a cancer chemopreventive potential may act in any one of a number of ways, including (a) modification of carcinogen activation through the inhibition of Phase 1 enzymes, (b) modification of carcinogen detoxification through Phase 2 pathways, (c) scavenging of DNA reactive agents, (d) suppression of the abnormal proliferation of early neoplastic lesions, and (e) inhibition of certain properties of the cancer cell (WARGOVICH, 1997). Promising chemopreventive drugs are being developed from dietary substances (e.g. green and black tea polyphenols, soy isoflavones, curcumin, lycopene, perillyl alcohol). Many of these dietary constituents have shown chemopreventive activity in animal models, while experiments with cultured cells reveal various potential mechanisms of action. Compounds, classified as blocking agents, prevent or reduce the initiation of carcinogenesis, while suppressing agents affect later stages of the process by reducing cell proliferation. Many comounds express both of these types of activity (KELLOFF *et al.*, 1999; MANSON *et al.*, 2000).

Although epidemiological studies support a cancer-preventive activity of olive oil, they cannot, by themselves, be used to establish which constituents of this vegetable oil contribute to its protective action. On the other hand, while direct evidence of a cancer-protective effect of specific olive oil components is also limited, in recent years significant data have been reported which demonstrate effects of such agents on molecular pathways critical to carcinogenesis and strongly imply an anticarcinogenic potential.

The aim of this review is to present an overview of the most recent data concerning the cancer-preventive potential of specific olive oil components. Two reviews have appeared recently which deal with the biological activity of olive oil phenols (MANNA *et al.*, 1999; VISIOLI *et al.*, 2002). The elucidation of the anticarcinogenic potential of olive oil may lead to the development of new strategies of intervention, including improvements in the production process of olive oil to yield a more enriched product (some of the chemopreventive components are abundant in the oil mill waste water). Mechanistic information yet to be obtained may permit the discovery of novel targets for chemoprevention and the development of novel chemopreventive agents.

CHEMICAL COMPONENTS OF OLIVE OIL

Olive oil is mainly composed of triglycerides (~95%). In addition, it contains a certain amount of minor components which can be divided into two groups. The first group includes fatty acid derivatives (mono- and diacylglycerols, phosphatides, waxes and esters of sterols), while the second group consists of classes of compounds that are chemically unrelated to fatty acids (hydrocarbons, aliphatic alcohols, free sterols, tocopherols, chlorophylls, carotenoids and polar phenolic compounds). Most of the fatty acids present as glycerides are unsaturated. The major fatty acids are oleic (18:1), linoleic (18:2), palmitoleic (16:1), palmitic (16:0) and stearic (18:0) acids. Oleic acid is present in much higher concentrations (70-80% in weight) than the other acids. Furthermore, olive oil constituents can be divided into the saponifiable fraction (triglycerides, free fatty acids, phosphatides, etc.) and the unsaponifiable fraction containing lipids of natural origin such as sterols, higher aliphatic alcohols, pigments and hydrocarbons as well as any foreign organic matter nonvolatile at 103°C. Polar components, such as phenols, which are water soluble and are removed during solvent extraction after saponification, should not be listed as part of the unsaponifiable fraction. Olive oil contains many volatile compounds, including hydrocarbons, alcohols, aldehydes, esters, phenols and phenol derivatives, oxygenated terpenes, and furan derivatives A small fraction of this complex mixture of volatiles causes the characteristic aroma of olive oil (KIRITSAKIS, 1990; BOSKOU, 1996). Freshly prepared virgin olive oil has also been shown to contain small amounts of significant biological phospholipids (ALTER and GUTFINGER, 1982), proteins and enzymes (GEORGALAKI *et al.*, 1998 a; b; HIDALGO *et al.*, 2001).

OLIVE OIL COMPONENTS AND CHEMOPREVENTION

The question of which component (or components) of olive oil is responsible for the cancer protective effect is of major interest. Although olive oil consists mainly of mono-unsaturated fatty acids (MUFA), as indicated above it is more than a mixture of mono-unsaturated fats. While current evidence from experimental and human studies suggests that saturated fats and ω-6 polyunsaturated fatty acids (linoleic acid in olive oil) (PUFA) have tumor-enhancing effects, ω-3 PUFA and MUFA, such as oleic acid (ω-9), are tumour-protective (BARTSCH *et al.*, 1999). It is likely that the MUFA play a central role in the cancer-protective effect, but it has been difficult to demonstrate this effect in epidemiological studies (TRICHOPOULOU *et al.*, 2000). In northern Europe and the United Stated most of the human intake of MUFA is derived from the consumption of animal products, i.e. their intake is accompanied by a high intake of saturated fat (SAFA), and consequently the epidemiological evaluation of the independent health effects of SAFA and MUFA is difficult to achieve. The fact that MUFA are also found in relatively high amounts in other kinds of fat, that are not associated with cancer protection, suggests that other olive oil components (minor components) may also be important.

To date no epidemiological study has precisely discriminated between the effects of different types of fatty acids, while at the same time adequately controlling the influence of other confounding dietary factors. Thus no significant effect, either protecting against or promoting the pathogenesis of cancer, has been proven for either oleic acid or MUFA as a whole. It is possible that olive oil components other than MUFA (e.g. micronutrients) may be important mediators of its cancer-protective effect. Although the evidence is limited, and no conclu-

sive statements can be made at present, some experimental evidence concerning the anticarcinogenic potential of specific minor components of olive oil is presented, including (A) components with antioxidant action and (B) constituents which mainly express non-antioxidative biological action, namely squalene and phytosterols.

ANTIOXIDATIVE CONSTITUENTS OF OLIVE OIL AND CANCER

Dietary antioxidants, ubiquitous in plant foods may be protective and reduce the risk of cancer in humans. Reactive oxygen species (ROS) appear to be involved in all stages of cancer development, including (i) the induction of DNA mutations in somatic cells (initiation), (ii) the stimulation of tumorigenic expansion of initiated cell clones (promotion) and (iii) the malignant conversion of tumours into cancer (progression) (reviewed in DREHER and JUNOD, 1996). Consequently, dietary antioxidants could be beneficial throughout the carcinogenic process (DIPLOCK, 1996; COZZI *et al.*, 1997). Epidemiological data provide strong evidence of a cancer-protective effect for high intakes of vegetables, fruits and whole grains. The cancer-inhibitory effects reported for these plant foods may be attributed to various antioxidant constituents (e.g. β-carotene – provitamin A, vitamins E and C, selenium) as well as other phytochemicals (e.g. polyphenols, carotenoids). It is likely that numerous constituents contribute to the overall protective effect. The Chemoprevention Program developed at the U.S. National Cancer Institute (NCI) is systematically carrying out preclinical and clinical studies on numerous potential chemopreventive agents, including naturally-occurring antioxidant micronutrients and phytochemicals, as well as synthetic antioxidants. Based on Phase I pharmacokinetic and clinical safety trials, the most promising agents have progressed to Phase II and Phase III clinical trials (reviewed in GREENWALD and McDONALD, 1999).

The major antioxidant components of virgin olive oil which provide oxidative stability to the oil, are polar and non-polar phenols (polyphenols, tocopherols) and carotenoids (BOSKOU, 1996; APARICIO *et al.*, 1999; PSOMIADOU and TSIMIDOU, 2002). Polyphenols seem to represent the most important constituents of olive oil from the point of view of its antioxidant/anticarcinogenic potential. The prominent position of polyphenols among the other olive oil constituents with similar action stems from their strong antioxidant potency and their ability to act in both hydrophilic and lipophilic cellular environments. The concentrations of tocopherols and carotenoids are significantly lower in olive oil than in other foods, but their antioxidant properties have been well studied (see below). Polyphenols (e.g. hydroxytyrosol, tyrosol) are characteristic constituents of olive oil which help oil stability and provide health protection. Today the olive oil industry is encouraged to produce polyphenol-enriched olive oil products. Moreover, in various lipid systems, the antioxidant activity of virgin olive oil components may be strengthened through synergistic action (MEDINA *et al.*, 2002; FUHRMAN *et al.*, 2000).

In the following section recent experimental evidence concerning the chemical/biological action of olive oil antioxidants is summarized, with emphasis given to:

(I) the polyphenol action related to: (i) their antioxidant potential, (ii) their putative non-antioxidant biological function, and (iii) some key pathways in carcinogenesis which may be modulated by these olive oil constituents; and

(II) the non-antioxidant biological action of tocopherols and carotenoids.

Phenolic compounds

The phenolic compounds found in olive oil are conventionally characterized as "polyphenols" (not all of them are polyhydroxy derivatives) and are part of the polar fraction which is usually obtained from the oil by extraction with methanol-water (BOSKOU, 1996). HPLC chromatography of the methanol extract of virgin olive oil reveals seven major peaks corresponding to hydroxytyrosol, tyrosol, two secoiridoids (dialdehydes related to oleuropein and lingstroside, but lacking the carboxymethyl group at C4), oleuropein (the aglycone of lingstroside) and a peak containing the lignans (+)-1-acetoxypinoresinol and (+)-pinoresinol (OWEN *et al.*, 2000 a; b; c; d). The structures of the phenolic compounds and other minor components discussed in the text have been presented by BOSKOU (1996) and OWEN *et al.* (2000a). Different samples of olive oil have been found to contain on average about 200 mg/kg total phenolics. Secoiridoids and lignans were the major linked phenols identified (OWEN *et al.*, 2000 d). Other simple phenols detected included vanillic acid, p-hydroxybenzoic acid, p-coumaric acid and o-coumaric acid (OWEN *et al.*, 2000 a). A number of other phenolic compounds have been reported to be present in small concentrations in virgin olive oil (BOSKOU, 1996). Recently, tannin-like polymeric polyphenols (including epigallocatechin) have been identified in virgin olive oil and in olive mill waste waters (our unpublished results). The concentrations of these phenolics in extra virgin olive oils and in refined virgin olive oils were significantly different (OWEN *et al.*, 2000 d). Refined olive oil does not contain phenols because the phenols are polar compounds which are completely removed by the water during refining (BOSKOU, 1996).

Antioxidant potential

All three classes of phenolic compounds (simple phenols, secoiridoids, lignans) have potent antioxidant properties that are substantially greater than those of the classical *in-vivo* and *in-vitro* free radical scavenger, vitamin E. It seems possible that these properties could be related to their anticancer potential. In this respect, hydroxytyrosol, secoiridoids and lignans had the most prominent inhibitory action in an experimental system involving an attack of reactive oxygen species on salicylic acid in a hypoxanthine/xanthine oxidase assay (OWEN *et al.*, 2000 b; c; d). Olive oil extracts containing a mixture of known and unknown phenolics were effective at far lower concentrations than the individual component (OWEN *et al.*, 2000 c). In addition to their direct antioxidant capacity, olive oil extracts were also potent inhibitors of xanthine oxidase activity, although similar concentrations of individual simple phenols had no effect (OWEN *et al.*, 2000 c). It should be emphasized that the antioxidant activity of each compound examined greatly depended on the oxidation assay system used. Thus, an evaluation of the protective action of hydroxytyrosol (3,4-DHPEA) and 3,4-DHPEA-EA (the dialdehydic form of elenolic acid linked with 3,4-DHPEA) against linoleic acid peroxidation in a micellar system showed that 3,4-DHPEA-EA was more efficient due to its greater lipophilicity (FOGLIANO *et al.*, 1999). Recently, hydroxytyrosol acetate has been synthesized and its antioxidant activity assessed and compared with that of other olive oil components in bulk oil and oil-in-water emulsions (GORDON *et al.*, 2001). It was revealed that in oil the antioxidant activity of hydroxytyrosol acetate was similar to that of 3,4-DHPEA-EA but much higher than that of α-tocopherol or oleuropein, while in an emulsion 3,4-DHPEA-EA and α-toco-

pherol were more effective antioxidants than hydroxytyrosol acetate.

Another important role of the phenolic components of olive oil, in relation to the formation of reactive oxygen species (ROS) of polyunsaturated fatty acids and the development of human cancer, concerns their inhibitory action on the production of eicosanoids. Substantial evidence supports a causal relationship between cancer development and an abnormal over expression of eicosanoid-forming enzymes, i.e. lipoxygenases (LOX) and cyclooxygenases (COX). Moreover, experimental results suggest that arachidonic acid metabolites play an important role in growth-related signal transduction, implying that intervention through these pathways may help arrest cancer progression. Many members of the LOX family are known, including 5-, 8-,12- and 15-LOX, whose main products are 5(S)-, 8(S)-, 12(S)- and 15(S)-hydroxyeicosatetraenoic acids (HETEs), respectively. Furthermore, two closely related forms of COX have been identified (COX-1 and COX-2), which transform arachidonic acid into prostaglandins; the distribution and physiological roles of these two forms differ (reviewed in CUENDET and PEZZUTO, 2000; NIE and HONN, 2002; MARKS *et al.*, 2000). Thus, intervening with specific LOX and COX inhibitors at the level of these pathways could be useful for arresting cancer progression. In this respect, it has been shown that hydroxytyrosol inhibited platelet 12-LOX activity (IC_{50}, 4.2 μM) and polymorphonuclear leukocyte 5-LOX activity (IC_{50} 13 μM) but not cyclooxygenase activity. Hydroxytyrosol inhibited both types of LOX activity more than oleuropein, caffeic acid, or seven other related phenolic compounds (KOHYAMA *et al.*, 1997). DE LA PUERTA *et al.* (1999) also showed that several principal phenolic compounds from the polar fraction of virgin olive oil efficiently inhibited the generation of leukotriene B_4 at the 5-LOX level in the order hydroxytyrosol> oleuropein> caffeic acid> tyrosol. In contrast, none of these compounds substantially inhibited the generation of thromboxane via the cyclooxygenase pathway. While the above results did not provide evidence of a direct inhibition of COXs by the specific olive oil phenolics examined, in a recent publication (MORENO *et al.*, 2001), it was reported that dietary olive oil decreased both oxidative stress and the production of prostaglandin E2, without affecting the expression of prostaglandin G/H synthase-2. These findings suggest that some component(s) of olive oil inhibited COX/prostaglandin synthase activity. Similarly, according to another report (BARTOLI *et al.*, 2000), dietary olive oil prevented the development of aberrant crypti foci and colon carcinomas in rats by modulating the metabolism of arachidonic acid and the synthesis of local prostaglandin E(2).

Recent theoretical and structural studies have revealed the mode of binding of two plant polyphenols, curcumin, a major yellow pigment with anti-inflammatory and anti-tumor activities, and 4-nitrocatechol, to some LOX isoforms. These studies have shown that these polyphenols bind to the central cavity of LOX, close to but at a non-bonding distance from the non-heme iron, and promote conformational changes which modify its coordination (SKRZYPCZAK-JANKUN *et al.*, 2000; NELSON *et al.*, 1995). It is also known that curcumin can directly inhibit COX-2 activity (ZHANG *et al.*, 1999). Since curcumin has some structural features that are similar to olive oil lignans, these oil phenolics might also be able to act as COX inhibitors.

The ability of certain polyphenols to chelate metal cations is very important for their antioxidant activity, because "site-specific scavenging" may occur (VAN ACKER *et al.*, 1998). It has been pointed out, however, that polyphenols can have pro-oxidant effects under some reaction conditions, such as in the presence of

ferric chelates or depending on the metal ion concentration (PUPPO, 1992; SUGIHARA *et al.*, 1999). Accordingly, olive oil o-diphenols chelate different metal cations and, under certain conditions, become prooxidants (hydroxytyrosol promoted deoxyribose damage in a deoxyribose assay and also promoted DNA damage in a bleomycin-Fe^{3+} system) (GARCIA *et al.*, 1996; AESCHBACH *et al.*, 1994). The rapid formation of the chelation complexes is usually accompanied by a change in the absorption spectrum of the polyphenol and a slow chemical oxidation of the antioxidant molecule (GARCIA *et al.*, 1996; AFANAS'EV *et al.*, 1989). While the final expression of a pro-oxidant effect of a polyphenol in the presence of a metal cation depends on various parameters of the *in vitro* systems, this does not necessarily apply to *in vivo* conditions (ARUOMA *et al.*, 1998; DEIANA *et al.*, 1999).

Additional studies on olive oil polyphenols, in a variety of *in vivo* and *in vitro* experimental systems, have revealed the following biological effects that are most likely related to their antioxidant potential:

(i) The oxidative stress induced by hydrogen peroxide and xanthine oxidase in Caco-2 human cells was completely inhibited in the presence of 100-500 μmol/L concentrations of hydroxytyrosol. In the same system, tyrosol was ineffective at concentrations below 500 μmol/L (MANNA *et al.*, 1997).

(ii) Hydroxytyrosol and oleuropein were found to be potent scavengers of superoxide radicals and inhibitors of the neutrophil respiratory burst (VISIOLI *et al.*, 1998).

(iii) Oleuropein and hydroxytyrosol were efficient scavengers of reactive nitrogen species, but tyrosol, which does not possess the catechol moiety, was less active (DE LA PUERTA *et al.*, 2001).

(iv) Human hepatic androstenedione 6β-hydroxylase activity and reductive 17β-hydroxysteroid dehydrogenase activity were inhibited by oleuropein glycoside, hydroxytyrosol and gallic acid. Analysis of the structural features of inhibitory compounds established that a 3,4-dihydroxyphenyl ethanol structure was required to inhibit androstenedione 6 β-hydroxylase by this group of compounds (STUPANS *et al.*, 2000). In general, to evaluate the efficacy of the chemopreventive-antioxidant effect of olive oil constituents a suitable animal model and an oxidative biomarker model are needed. In this respect, the profile of polyunsaturated fatty acids in various rat organs has been proposed as an efficient oxidative biomarker model (DEIANA *et al.*, 2001).

Non-antioxidant biological function

Phytoestrogens are of biological interest because of their oestrogenic activity, both *in vitro* and *in vivo*, which is the result of their weak binding to oestrogen receptors. Phytoestrogens also inhibit tyrosine kinase, epidermal growth factor, malignant cell proliferation, differentiation and angiogenesis. These properties make them strong candidates for a role as natural protective compounds against cancer (ADLERCREUTZ, 1990; SETCHELL *et al.*, 1981). The lignans recently identified as major components in the phenolic fraction of olive oil may constitute potentially important contributors to the chemopreventive action of olive oil if they are sufficiently bioavailable. Lignans which belong to the class of phytoestrogens exert antiestrogenic effects and have been shown to inhibit cell growth in cancers of the skin, breast, colon and lung (OWEN *et al.*, 2000 b). Moreover, hydroxytyrosol has been reported to possess estrogenic activity as evaluated using a yeast estrogen screen containing human estrogen receptor (AGRADI *et al.*, 2001).

Today it is well known that oxidation-reduction reactions regulate signal transduction and that ROS may be sec-

ond messengers for transcriptional factor activation, apoptosis and cell growth (SUZUKI *et al.,* 1997). Identifying the upstream targets of oxidants is important for understanding the mechanism of antioxidant biological actions. In this respect, protein kinase C (PKC) is considered a candidate for redox modification by oxidants and antioxidants that may, in part, determine their cancer-promoting and anticancer activities, respectively (GOPALAKRISHNA *et al.,* 2000). Accordingly, oxidized curcumin (as stated above, this polyphenol bears structural similarities to olive oil lignans), is a potent PKC inhibitor which interacts with reactive cysteines of the C-terminal catalytic domain of kinase (GOPALAKRISHNA *et al.,* 2000). Several olive polyphenols bearing the galloyl moiety may also interfere with carcinogenesis by directly interacting with signalling protein kinases (see next section).

Key pathways in carcinogenesis

Mechanisms by which dietary constituents suppress tumorogenesis often involve the modulation of signal transduction pathways, leading to altered gene expression (MANSON *et al.*, 2000). A model has been proposed, according to which certain polyphenols, at low concentrations, activate mitogen-activated protein kinase pathways, leading to the activation of the transcription factor Nrf2 and the antioxidant-response element (ARE) with subsequent induction of phase II and other defensive genes (OWUOR and KONG, 2002).

Several reports suggest that olive oil polyphenols can interfere with tumor necrosis factor alpha- (TNFα), estradiol- and epidermal growth factor- (EGF) mediated pathways, whose significance in carcinogenesis is well recognized. TNFα released from monocytes, a key player in inflammation, induces colonic COX-2 expression (JOBIN *et al.,* 1998). The suppression of TNFα release by estradiol via an estrogen receptor β-dependent mechanism (SRIVASTAVA *et al.,* 1999) may account for the inhibitory effects of the hormone on early stages of carcinogenesis (WEYANT *et al.,* 2001b).

Furthermore, recent genetic evidence strongly implicates the EGF receptor family of protein tyrosine kinases in breast cancer development (YU *et al.,* 2001). The EGF receptor activates focal adhesion kinase (FAK) and thus leads to proliferation of pre-malignant mammary cells (ASSOIAN and SCHWARTZ, 2001). Furthermore, EGF protects such cells from apoptosis induced by loss of adhesion to the stroma (SCHULZE *et al.,* 2001). In this respect, it is notable that pinoresinol inhibits the production of TNFα (CHO *et al.,* 2001), while hydroxytyrosol exhibits proapoptotic activity toward human HL60 myeloid leukemia cells and activates peripheral blood mononuclear cells. Interestingly, a combination of p- and m-hydroxylphenyl groups of hydroxytyrosol is needed to promote apoptosis. Hydroxytyrosol-dependent apoptosis is associated with an early release of cytochrome c from mitochondria, which precedes caspase 8 activation, thus ruling out the involvement of cell death receptors in the apoptotic process. However, hydroxytyrosol did not cause cell death in two colorectal cell lines (HT-29 and Caco2). These results suggest that this olive oil polyphenol down-regulates the immunological response, thus providing a possible explanation for the anti-inflammatory and chemopreventive effects of olive oil at the intestinal level (DELLA RAGIONE *et al.,* 2000). BOSKOU *et al.* (2001) reported the inhibition of human breast and prostate cancer cell lines by olive oil phenolic acids. They observed that these substances inhibited nitric oxide synthase (NOS), which suggests the possible involvement of the NO/NOS system in the mode of action of phenolic acids.

Recently, a number of tea polyphenols bearing the galloyl structure (catechins) have been shown to reduce the risk of cancer in a variety of animal tumor and/or proliferative bioassay systems (YANG *et al.*, 2000). Using different experimental systems, it was found that:

(i) the tea polyphenols, (-)-epigallocatechin-3-gallate (EGCG) and theaflavin-3,3'-digallate, inhibit the Ras-MAP kinase signaling pathway in a H-ras-transformed cell line (CHUNG *et al.*, 2001).

(ii) EGCG and (-)-epicatechin-3-gallate (ECG) strongly induced antioxidant response element-mediated luciferase activity, while EGCG showed potent activation of all three MAP kinases (ERK, JNK and p38) and caspase-3 and induced apoptosis (CHEN *et al.*, 2000) in human hepatoma HepG2-C8 cells and squamous carcinoma HeLa cells.

(iii) EGCG decreased the level of ornithine decarboxylase, which is a signal for cellular proliferation, in transformed (NIH-pATMras) but not in normal fibroblasts and preferentially decreased the levels of the oncogenes Ras and Jun in the transformed cells (WANG and BACHRACH, 2002).

(iv) Catechin inhibited FAK (target of EGF receptor as well as integrin signaling) and decreased intestinal tumour formation in APC-/+ mice by 75% (WEYANT *et al.*, 2001 a).

(v) (-)- Epicatechin (EC), (-)-epigallocatechin (EGC) and EGCG drastically inhibited the growth of HCT 116 colorectal carcinoma cells (UESATO *et al.*, 2001).

(vi) EGCG also blocked angiogenesis which led to starvation of the tumor (SINGH *et al.*, 2002) and inhibited Her-2/neu signaling and proliferation, and transformed the phenotype of breast cancer cells (PIANETTI *et al.*, 2002). Both EC and EGC are phenolics with a pyrogallol structure that are found in olive fruits and are probably transferred to the olive oil (BOTIA *et al.*, 2001).

Bioavailability of olive oil phenolics

It has been shown that in humans tyrosol and hydroxytyrosol are dose-dependently absorbed after ingestion and are excreted in the urine as glucuronide conjugates (VISIOLI *et al.*, 2000a; MIRO-CASAS *et al.*, 2001). When olive oil samples containing increasing amounts of an olive oil phenolic extract were administered to human volunteers, a dose-dependent decrease in the urinary excretion of 8-iso-PGF (2alpha), a biomarker of oxidative stress, was observed. In addition, a statistically significant negative correlation was found between homovanillyl alcohol (Hvalc, a major metabolite of hydroxytyrosol, formed through the catechol-O-methyltransferase system) and isoprostane excretion. Thus, Hvalc reflects the *in vivo* activities of hydroxytyrosol (VISIOLI *et al.*, 2000b). Recently, evidence was presented suggesting postprandial absorption of olive oil phenols in humans. In this case, both tyrosol and hydroxytyrosol were found in plasma lipoproteins involved in atherosclerotic processes (BONANOME *et al.*, 2000); urinary excretion of the hydroxytyrosol metabolites, Hvalc and homovanillic acid, were also found (CARUSO *et al.*, 2001). The mechanism of intestinal transport of hydroxytyrosol has also been examined using differentiated Caco-2 cell monolayers as the model system. Evidence has been presented indicating that the transport occurs via a passive diffusion and that the molecule is quantitatively absorbed at the intestinal level (MANNA *et al.*, 2000). Concerning olive oil lignans, no experimental evidence exists regarding their absorption by humans, although their absorption is unlike to be due to their structure. However, it has been recently shown that berries containing relatively high concentrations of plant lignans contribute to plasma and urinary levels of mammalian lignan enterolactone in human subjects (MAZUR *et*

al., 2000). Moreover, *in vitro* metabolism of the olive oil lignan, pinoresinol, by human fecal microflora produces only mammalian lignans (HEINONEN *et al.*, 2001).

Tocopherols

Tocopherols are important non-polar phenolic constituents of virgin olive oil which contribute to the remarkable stability of the oil. The tocopherol content of olive oil is highly dependent on olive variety. The values usually reported for good quality oils vary between 100 and 300 ppm. However, these values are considerably lower than those of tocopherols present in other vegetable oils (WHITE and XING, 1997). The main homologue of vitamin E (-TOH, vitamin E) present in olive oil is α-tocopherol, which makes up 95% of the total tocopherols. The remaining 5% is made up of β+γ tocopherols (BOSKOU, 1996). –TOH is a highly effective antioxidant in the lipid phase of cell membranes, capable of breaking the chain reaction by scavenging a peroxyl radical (BURTON *et al.*, 1982). The addition of α-tocopherol to purified olive oil which had been stripped of antioxidants and pro-oxidants conferred protection against oxidative damage (BLEKAS *et al.*, 1995).

It is commonly believed that vitamin E only exerts a protective role against free radical damage. However, it is also known that tocopherols participate in other biological functions in signal transduction and gene regulation (BOSKOBOINIK *et al.*, 1991). In a recent study, diets with plant oils (corn oil or olive/sunflower oils) rich in α- and γ-tocopherols were compared with respect to their impact on DNA damage in humans. It was found that a diet with a combination of γ-tocopherol and α-tocopherol gave better protection against DNA damage than one with α-tocopherol alone (ELMADFA and PARK, 1999).

Carotenoids

Small quantities of carotenoids are present in olive oil, including lutein (the major constituent), β-carotene, violoxanthin and neoxanthin. Total carotenoids usually range from 1 to 20 ppm, with the β-carotene content ranging from 0.5 to 4 ppm (BOSKOU, 1996). These concentrations are considerably lower than those found in other vegetables and fruits such as carrots, spinach and tomato (ROCK, 1997). The predominant carotenoids found in plasma are β-carotene, lycopene, lutein, β-cryptoxanthin, and α-carotene (ROCK, 1997).

Carotenoids have a typical conjugated polyene structure and represent a major family of efficient ROS quenchers and singlet oxygen traps. The antioxidative function of carotenoids in mammals, however, is still unclear, with clinical trials showing conflicting results (reviewed in PRYOR *et al.*, 2000).

Although the level of carotenoids in olive oil is very low, the possibility of a synergistic antioxidative/antiproliferative action with α-tocopherol or polyphenols at physiological concentrations cannot be excluded (PASTORI *et al.*, 1998; FUHRMAN *et al.*, 2000).

NON-ANTIOXIDANT CHEMOPREVENTIVE COMPONENTS

Squalene

The triterpene squalene is an intermediate in the cholesterol biosynthesis pathway and represents more than 90% of the hydrocarbon fraction of olive oil. Its concentration ranges from 200 to 12,000 mg/kg oil (PSOMIADOU and TSIMIDOU, 1999), depending on the olive cultivar and oil extraction technology; it is dramatically reduced during refining (reviewed in PSOMIADOU and TSIMIDOU, 1999). While squalene does not express radical scavenging activity, its weak anti-

oxidant activity in olive oil may be due to competitive oxidation of the different lipids present which reduces the oxidant rate. For comparison purposes, the average intake of squalene in the United States is 30 mg/day, while in countries where consumption of olive oil is high, such as for example in Mediterranean countries, the intake can reach 200-400 mg/day (reviewed in SMITH, 2000).

It has been suggested that the lower risks of various cancers associated with high olive oil consumption (as compared to other human foods) may be due to the presence of squalene (SMITH, 2000; NEWMARK, 1997; 1999). Experimental studies have shown that squalene can effectively inhibit chemically-induced colon, lung and skin tumorigenesis in rodents. The protective effect was observed when squalene was given before and/or during carcinogenic treatment. A mechanism has been proposed for the tumor-inhibitory activity of squalene based on its known strong inhibitory effect on the catalytic activity of β-hydroxy-β-methylglutaryl-CoA reductase *in vivo* and, hence, reduced availability of farnesyl pyrophosphate for the prenylation of the Ras oncogene. Prenylation is necessary for the relocation of Ras to cell membranes and is required for the signal-transducing function of this oncogene (SMITH, 2000; NEWMARK, 1997; 1999).

β-Sitosterol

Sterols are important nonglyceridic constituents of olive oil. They are related to olive oil quality and are used to check its genuineness. The total sterol content of olive oil ranges from 155 to 265 mg/100g and decreases during storage and processing. The main olive oil sterol is β-sitosterol, which makes up 75-90% of the total sterol fraction (KIRITSAKIS, 1990; BOSKOU, 1996).

β-Sitosterol represents one of the most common phytosterols (plant sterols that are structurally similar to cholesterol), along with campesterol and stigmasterol. The western diet typically contains 80 mg of phytosterols/day, whereas vegetarian and Japanese diets contain 345 and 400 mg/day, respectively (AWAD and FINK, 2000). Epidemiological and experimental studies suggest that dietary phytosterols (the best sources are unrefined plant oils, seeds, nuts and legumes) may offer protection against the most common cancers in western society, such as colon, breast and prostate cancer (AWAD and FINK, 2000; NORMEN *et al.*, 2001; AWAD *et al.*, 2001 a; b). The exact mechanism by which sitosterol protects against cancer is not known. However, several theories have been proposed (reviewed in AWAD and FINK, 2000), based on possible effects of sitosterol and other phytosterols on (i) membrane structure, (ii) membrane fluidity, (iii) membrane-bound enzymes, (iv) signal transduction pathways, (v) apoptosis, (vi) immune function and (vii) the presence of neutral and acidic sterols in the colon. Recent results have indicated that β-sitosterol offers protection against breast cancer metastasis by inhibiting cell invasion of the basement membrane. This action may be mediated by the ability of phytosterol to limit the adhesive interaction between the tumor cell and the basement membrane (AWAD *et al.*, 2001 b).

Table 1 summarizes the putative potential modes of action and cellular targets, in connection with cancer chemoprevention, of various minor olive oil components.

CONCLUDING REMARKS

Many molecular entities found in virgin olive oil appear to be endowed with properties which imply an anticarcinogenic potential. If further research confirms this potential, it would justify considering this dietary oil as a "golden functional food" with real chemopreventive activity against a number of common

Table 1 - Putative potential modes of action and cellular targets of various minor olive oil components in connection with cancer chemoprevention.

Components	Potential	Cellular target
Phenolic compounds (simple phenols: hydroxytrosol, tyrosol; linked phenols: secoiridoids, lignans)	antioxidant non-antioxidant	signal transduction gene expression apoptosis
Tocopherols	antioxidant non-antioxidant	signal transduction gene regulation immunologic response
Carotenoids Squalene	antioxidant non-antioxidant	β-hydroxy-β-methylglutaryl-CoA reductase (inhibition)
β-sitostero	non-antioxidant	membrane properties signal transduction apoptosis immune function

cancers. However, for an appropriate use of a chemopreventive agent it is essential to understand its mechanism of action and its interaction with other chemopreventive and genotoxic agents at all levels, namely the molecular, cellular, tissue and organ levels, as well as in the animal as a whole. Without this knowledge we can only make intuitive decisions in selecting preventive agents.

The process of identifying potential synergistic combinations of chemopreventive constituents of olive oil should be based on a systematic *in vitro* and *in vivo* experimental approach which uses appropriate models of carcinogenesis. If evidence of a chemopreventive effect is found in animal models, then systematic studies in humans are indicated. With regard to the latter, several critical factors must be taken into consideration, including the effective dose (of each constituent separately, or of each component present in a functional mixture) and the duration of exposure. Although animal studies have enhanced our understanding of the potential of some specific olive oil constituents, such as polyphenols, vitamin E, squalene and β-sitosterol, to prevent carcinogenesis, one must be cautious in extrapolating such information to humans, especially if habitual consumption of excessive amounts of specific micronutrients is involved. It is important that any dietary advice given to the public must be based on an adequate understanding of the biological effects of the fat and minor components of olive oil as well as of the bioavailability restrictions of these components.

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PAPER

ANTIOXIDANT ACTIVITY OF THE EXTRACTS OF THE EDIBLE PART OF ARTICHOKE (*CYNARA SCOLYMUS* L.) VAR. SPINOSO SARDO

VALUTAZIONE DELL'ATTIVITÀ ANTIOSSIDANTE DI ESTRATTI DELLA PARTE EDIBILE DI CARCIOFO (*CYNARA SCOLYMUS* L.) VAR. SPINOSO SARDO

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ABSTRACT

The present work was carried out to evaluate the polyphenolic composition of the fresh alcoholic extract of *Cynara scolymus* var. spinoso sardo. Three different methods were used: HPLC/UV, direct spectrophotometric at 330 nm and spectrophotometric with Folin-Ciocalteu reagent (770 nm). The antioxidant properties were evaluated by determining its ability to scavenge the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) free radical and by measuring the induction period of soybean oil in its presence or

RIASSUNTO

Il presente lavoro fu condotto per valutare la composizione polifenolica dell'estratto alcolico fresco di *Cynara scolymus* var. spinoso sardo. Sono stati utilizzati tre diversi metodi: HPLC/UV, spettrofotometrico diretto a 330 nm e spettrofotometrico col reattivo di Folin-Ciocalteu (770 nm). Le proprietà antiossidanti furono valutate determinando la sua capacità di catturare il radicale libero 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) e misurando il periodo di induzione dell'olio di soia in sua presenza o

- Key words: artichoke; $DPPH^{\cdot}$, natural antioxidants; polyphenolic analysis -

in its absence. The activity was compared with that of the same extract after lyophilization, with standard polyphenols either isolated or mixed (in conformity with artichoke composition) and with synthetic antioxidants (BHA and BHT). There was a considerable quantity of polyphenols in the fresh extract of *Cynara scolymus* L. var. spinoso sardo and their antioxidant activity in both of the tests used was comparable with BHA and chlorogenic acid activity.

assenza. L'attività fu confrontata con quella dello stesso estratto dopo liofilizzazione, con standards di polifenoli sia isolati che in miscela (in conformità con la composizione del carciofo) e con antiossidanti sintetici (BHT e BHA). I polifenoli nell'estratto fresco di *Cynara scolymus* L. var. spinoso sardo furono in quantità considerevole e la loro attività antiossidante in entrambi i test utilizzati è risultata essere comparabile con l'attività antiossidante del BHA e dell'acido clorogenico.

INTRODUCTION

The determination of polyphenols in plants is of great interest because of their natural antioxidant activity. They are synthetized by plants as a defence mechanism against microorganisms and strong UV radiation (LE FLOCH *et al.*, 1998). Antioxidants are added to fat-containing food to prevent the formation of off-flavours and toxic compounds resulting from lipid oxidation. Plant extracts are natural alternatives to synthetic antioxidants as they possess similar or even higher antioxidant activity (ARUOMA *et al.*, 1996).

The edible flower of artichoke (*Cynara scolymus L.*) is a typical vegetable in Mediterranean countries. In studies carried out to determine the polyphenolic composition of the artichoke (LATTANZIO and MORONE, 1978; LATTANZIO and VAN SUMERE, 1987) o-diphenolic acids and flavonoids have been found to be the most important polyphenols. It is reputed that this vegetable has a marked antioxidative and health protective potential (GEBHARDT, 1997; CHEN and HO, 1997). In vivo and in vitro studies have shown its hepatoprotective functions (also against toxic compounds) and the inhibition of cholesterol biosynthesis in hepatocytes (ADZET *et al.*, 1987; GEBHARDT, 1998).

Cynara scolymus var. spinoso sardo is the most commonly grown artichoke in Sardinia but it has not been completely characterized. ALAMANNI and COSSU, 2000; COSSU and ALAMANNI, 2001, identified and quantified the main polyphenols present in ethanolic extracts of the different structural parts of the artichoke. They also evaluated the variability in the polyphenolic composition caused by decoction and drying.

Chlorogenic acid (46%), isochlorogenic acids (44.3%), caffeic acid and flavonoids as cynaroside and scolymoside were isolated, identified and quantified in some artichoke samples harvested in Northern Sardinia (ALAMANNI and COSSU, 2000). By HPLC/UV it was determined that the polyphenolic composition was modified due to decoction both qualitatively and quantitatively (cynarine formation), while the drying process gave rise to less marked and only qualitative alterations.

The objective of the present investigation was to evaluate the polyphenolic composition of a fresh alcoholic extract of *Cynara scolymus* var. spinoso sardo, using three different methods: HPLC/UV, direct spectrophotometric and spectrophotometric with Folin Ciocalteau reagent. Its antioxidant properties were then evaluated by determining its ability to scavenge the $DPPH^{\cdot}$ free radical and by

measuring the induction period of soybean oil in its presence or in its absence, according to the HADORN and ZÜRCHER method (1974). This activity was compared with those of the same extract after lyophilization of standard polyphenols either isolated or mixed (in conformity with artichoke composition) and of synthetic antioxidants such as BHA and BHT.

MATERIALS AND METHODS

Chemicals and standards

The following commercial standards were used: chlorogenic acid (3-O-caffeoylquinic acid), caffeic acid, from Sigma-Aldrich Chem. Co. (Milan, Italy); cynaroside (luteolin-7-O-glucoside), from Extrasynthese Co. (Genay, France). Cynarine, isochlorogenic acids and scolymoside (luteolin-7-O-rutinoside) were purified by HPLC and preparative TLC and identified in our laboratory by a standardized TLC method, according to a thin layer chromatographic atlas of drug extracts (WAGNER *et al.*, 1984). The purity of the standards was 95-99%.

The Folin-Ciocalteu reagent was purchased from Carlo Erba (Milan, Italy). 1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$), BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) were purchased from Sigma Chemical Co (Milan, Italy).

The solvents used for chromatography were: ultra pure-water prepared by MilliQ R4 system (Millipore, Milan, Italy), methanol used for gradient elution in HPLC and to prepare standard solutions, 95-97% sulfuric acid from Riedel-deHaen (Milan, Italy). 0.45 µm pore size membrane filters from Millipore were used for filtration of the mobile phase and of the samples. Pure HPLC solvents were used in all cases. Celite 545, 20-45 µm (Fluka Milan, Italy) was used for sample purification. Soybean oil was purchased on the local market.

Calibration curves

Standard calibration curves were made by plotting the area of peaks against different concentrations of phenolic compounds varying from 2.5 to 25.0 mg/L. The linearity of the detector responses for the standards was assessed by means of linear regression analysis regarding the amounts of each standard (mg) introduced in the chromatographic system and the area of the corresponding peak on the chromatogram. The identification of the phenolic compounds was achieved by comparing the retention times and each compound was quantified by comparison with a calibration curve obtained with the corresponding standard.

Sample preparation

The samples consisted of 30 artichokes var. spinoso sardo from Northern Sardinia farms. After removing the leaves, fibrous bracts and stem cortex, the residual edible part was immediately homogenized in a blender.

Extraction of phenolic compounds

An amount of 700 g of fresh (94.0 g dry matter) and homogenized artichokes was treated with 600 mL of methanol. The extracts were obtained after 10 days of maceration in the dark and under N_2 at 18°-20°C. The extraction efficiency of the polyphenols was evaluated at five different times: 2, 4, 6, 8 and 10 days, by HPLC analysis.

The hydroalcoholic extracts containing the polyphenols were purified by column chromatography (Celite, 50 g) and reconstituted with a hydroalcoholic solution (1:1 water/methanol) in order to obtain a final concentration of 2 g fresh artichoke in 1 mL of hydroalcoholic solution.

The extracts were divided into two parts: one part was analysed as it was,

the other part was lyophilized after methanol removal. It was reconstituted with the same volume of hydroalcoholic solution for analysis. All samples were always filtered through a 0.45 µm membrane Millipore chromatographic filter before HPLC analysis.

Determination of polyphenol content

Spectrophotometric analysis

The amount of total extractable artichoke phenols was determined according to the Folin-Ciocalteu method (SINGLETON and ROSSI, 1965), and also by the direct spectrophotometric absorption procedure at 330 nm. The results are expressed in milligrams of chlorogenic acid per 100 g of edible artichoke. The calibration curves were prepared using concentrations of chlorogenic acid ranging from 2.5 to 25.0 mg/L. The total phenol content was determined using a Hitachi U-2001 spectrophotometer (Hitachi Instruments Inc., Milan, Italy) with a 10 mm path length. All absorbance values were corrected in conformity with the dilutions carried out.

HPLC analysis

The following instruments were used: Hewllet-Packard 1050 series quaternary pump, variable-wavelength UV-Vis spectrophotometer detector and autosampler. The peak areas, determined with a 3390 integrator, were used for quantification (Hewllet-Packard Waldbronn, Germany).

The chromatographic separation was performed using an ODS Adsorbosil 5 µm column, 250 mm, 4.6 mm I.D. stationary phase, preceded by a Nucleosil RP-C_{18} 5 µm guard column, (Alltech, Milan, Italy). Ten microlitres of extract samples or calibration standards were directly injected onto the column and eluted with a gradient comprised of methanol (A) and a solution of 0.01N sulphuric acid (B). Zero-time conditions were A-B (30:70). After 15 min, the gradient was adjusted to A-B (50:50), at 20 min to A-B (100:0) until termination of the run at 25 min, the flow rate was 1 mL/min at room temperature. The system was equilibrated for 15 min using the starting conditions before injecting the next sample. Detection was carried out by monitoring the absorbance signals at 340 nm. At the end of each working day, the column was washed with the zero-time solvent mixture. Mobile phases were filtered with a 0.45 µm pore size membrane filter, and degassed before use.

Validation

Validation procedure, parameters and acceptance criteria were based on USP 23 (1995) guidelines and recommendations in the literature. The method was validated regarding accuracy and precision (ALAMANNI and COSSU, 2000), concentration range, linearity, limit of quantification (LOQ) and limit of detection (LOD).

Determination of antioxidant activity

Spectrophotometric method

The antioxidant activity of fresh extracts, lyophilized fresh extracts and standard polyphenols was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in a spectrophotometric test, according to the modified method of VON GADOW *et al.* (1997). One millilitre of a methanolic solution of $DPPH^{\cdot}$ (10^{-1} mM) was mixed with 1.0 mL of a polyphenolic solution of artichoke (10^{-2} mM) in methanol and, after standing for 15 min, the absorbance of the mixture was measured at 517 nm against methanol as the blank. The same concentration was also used to determine the times of inhibition for BHT, BHA, chlorogenic acid, caffeic acid, cynaroside, cynarine, isochlorogenic acids and scolymoside. $DPPH^{\cdot}$ scavenging activity was also measured as a function of the concentration of fresh extracts, lyophilized fresh extracts and mixed standard polyphenols in conform-

ity with the composition of the methanolic extract.

Measurements were carried out using a Hitachi U-2001 UV-vis spectrophotometer with a 10 mm pathlength. The absorbance of the $DPPH^{\cdot}$ radical (control) was measured daily. All measurements were performed in triplicate. The percentage of inhibition of the $DPPH^{\cdot}$ radical by the tested samples was calculated according to the following formula:

$$\% \text{ inhibition} = [(At_0 - At_{15}) / At_0] \times 100$$

where At_0 is the absorbance of the control at t = 0 min and At_{15} is the absorbance of the $DPPH^{\cdot}$-antioxidant solution after 15 min.

Conductometric method

The antioxidant activity was also evaluated by the conductometric method of HADORN and ZURCHER (1974). The principle of this test is to bubble air through heated oil and to monitor continuously the conductivity of the water in which the effluent gas is trapped. The highly volatile organic acids produced and absorbed in the water are used to indicate the induction time measuring the variation in electric conductivity with a YSI mod. 33 conductimeter (Yellow Spring Instruments Co., Inc., Yellow Spring, Ohio).

Oxidations were conducted on soybean oil in the presence and absence (Test) of fresh and lyophilized extracts with a concentration in phenolic compounds of 5 mmol per kilogram of oil. This concentration was also used to determine the induction periods of BHT, BHA, chlorogenic acid as well as of the mixed standard polyphenols.

The air flow-rate and the temperature used were 20 L/h and 110°C.

The antioxidant index was calculated as:

$$AI = It_s / It_o$$

where It_s is the induction period of oil with antioxidant addition and It_o is the induction period of oil alone.

RESULTS AND DISCUSSION

The compositions of the fresh and lyophilized methanolic extracts of the artichoke edible part are reported in Table 1. There were slight differences

Table 1 - Polyphenolic compounds in the extract of the edible parts of *Cynara scolymus* (var. spinoso sardo) fresh and lyophilized, expressed in mg/100g of fresh plant evaluated by HPLC[a].

		R.T. (min)	Fresh extract	Lyophilized extract
1	chlorogenic acid (3-O-caffeoylquinic acid)	6.3	246.9±31.2	174.2±10.6
2	cynarin (1,5- dicaffeoylquinic acid)	7.0	0.8±0.2	1.1±0.1
3	caffeic acid	8.1	1.4±0.1	1.2±0.3
*	isochlorogenic acids (R,R1-O-dicaffeoylquinic acids)	*	188.6±16.3*	188.0±13.3*
4	cynaroside (luteolin-7-O-glucoside)	14.3	33.9±7.9	29.7±4.5
5	scolymoside (luteolin-7-O-rutinoside)	17.5	136.7±28.5	116.8±27.3
	Total polyphenols			
	HPLC method		607.0±68.3	510.9±71.6
	Folin-Ciocalteu method		589.3±58.7	491.6±60.5
	Direct method at 330 nm		572.6±83.4	479.3±49.5

* - expressed as sum of the peak areas;
a - Means ±SD of three determinations.

between the fresh alcoholic extract and the lyophilized alcoholic extract for each of the compounds. The total polyphenols determined using three different methods are also reported. The HPLC method gave results comparable to those obtained by the direct spectrophotometric method and by the Folin-Ciocalteau method. Fig. 1 shows the HPLC profile of the polyphenol standards. Fig. 2 shows the chromatogram of the fresh artichoke alcoholic extract, as an example. The peaks of the alcoholic extracts were identified by comparing the retention time of each peak with that of the corresponding standard. In the chromatogram of the extracts, two peaks were also present, probably corresponding to some minor polyphenols (ADZET and PUIGMACIA, 1985). They have not yet been isolated and purified and are indicated as "n" or not identified.

In Fig. 3 the free radical scavenging activities of the tested polyphenolic standard compounds show a marked $DPPH^{\cdot}$ inhibition by caffeic acid, in comparison with the other polyphenolic and antioxidant (BHA, BHT) standards, at the same concentration of 10^{-1} mmol/L.

Fresh methanolic extract, lyophilized extract and mixed standard phenols antioxidant activities as well as BHA and BHT were measured using the $DPPH^{\cdot}$ test as a function of concentration (Fig. 4), and at the same concentration (10^{-2} mmol/L) (Fig. 5). The total polyphenols contained in the extracts are expressed as mmol/L of chlorogenic acid. In both graphs, there is a marked inhibition activity of both fresh (63.3%) and lyophilized extracts (58.8%) in comparison with the mixed standard polyphenols, BHT and BHA activities.

Fig. 6 shows the induction curves of soybean oil (for the test), of chlorogenic acid, the fresh methanolic extract, lyophi-

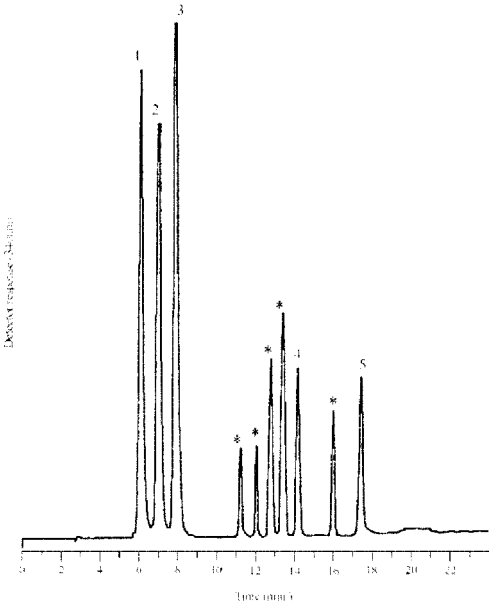

Fig. 1 - Chromatographic profile of polyphenolic standard compounds. Peaks: 1=chlorogenic acid, 2= cynarin, 3= caffeic acid, *= isochlorogenic acids, 4= cynaroside, 5= scolymoside.

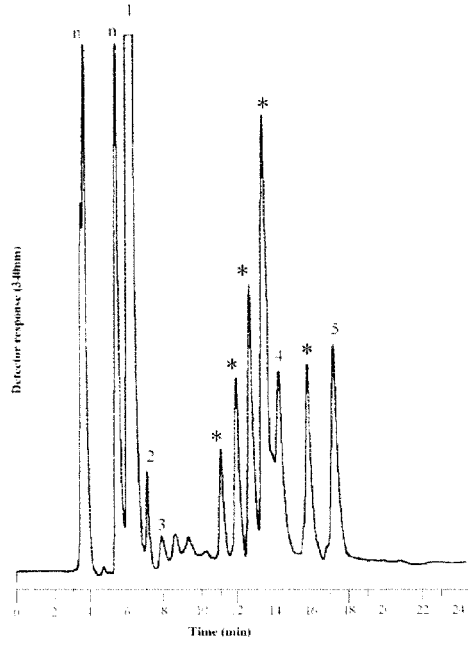

Fig. 2 - Chromatographic profile of a methanolic extract of the edible part of *Cynara scolymus* (var. spinoso sardo) recorded at 340 nm. Numbering is the same as that in Fig. 1. n= peak not identified.

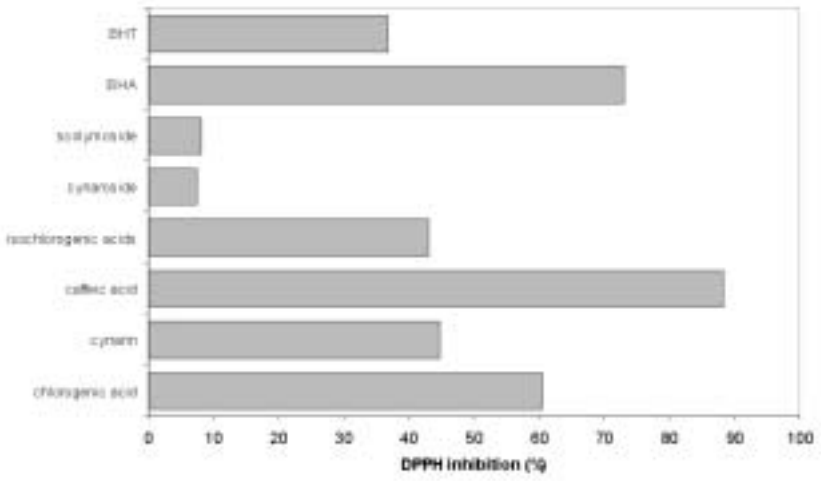

Fig. 3 - Antioxidant activity determined by the DPPH· radical scavenging method of standard compounds representing the main polyphenols at a concentration of 10^{-1} mmol/L.

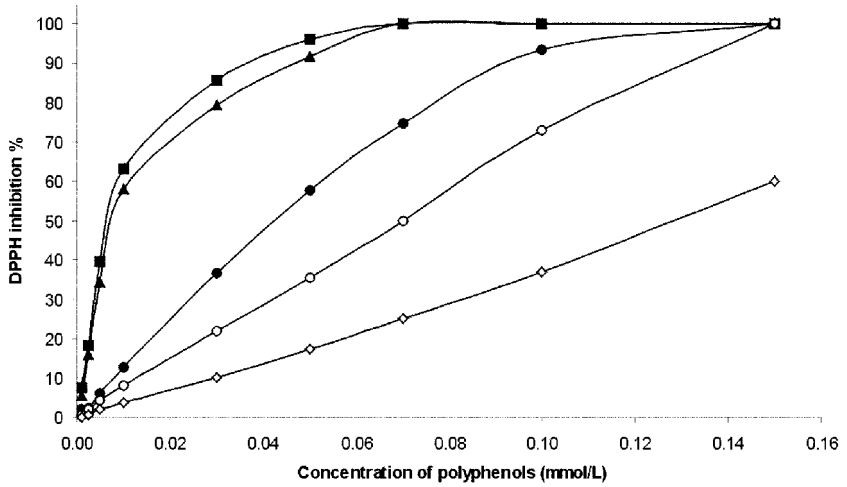

Fig. 4 - DPPH· scavenging activity of the methanolic extracts from artichoke (var. spinoso sardo), fresh (■)*, lyophilized (▲)*, standard mix of polyphenols (●)*, BHA (○) and BHT (◊) as a function of concentration (* expressed as mmol/L of chlorogenic acid).

lized extract, mixed standard polyphenols, BHA and BHT. The induction time and corresponding antioxidant index of each tested compound are reported in Table 2. All the antioxidants were tested at the same molar concentration (5 mmol/kg of oil). The results demonstrate a comparable activity for fresh extract, chlorogenic acid and BHA and a lower activity for the lyophilized extract and BHT.

The concentration of the polyphenols in *Cynara scolymus* L. var. spinoso sardo fresh extract are much more than what has been reported for other arti-

Table 2 - Induction time[a] (It) and Antioxidant Index (AI) of soybean oil (Test), fresh extracts[b] and standards at a concentration of 5 mmol/kg of oil.

	It (min) ± SD	AI
Soybean oil	405±6.3	
Fresh extract	625±12.5	1.54
Fresh lyophilized extract	570±2.2	1.40
Chlorogenic acid	645±3.1	1.59
BHT	540±11.8	1.33
BHA	670±4.5	1.65
Standard mix	609±7.2	1.50

[a] - Means ±SD of five determinations; [b] - expressed as total polyphenols (mmol/kg of chlorogenic acid).

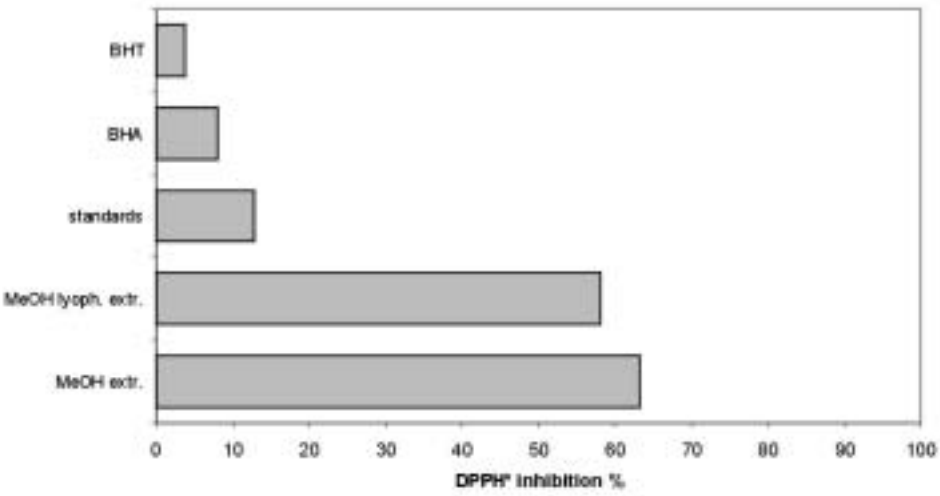

Fig. 5 - DPPH· scavenging activity of a methanolic fresh extract, methanolic lyophilized extract from artichoke (var. spinoso sardo), standard mix of polyphenols, BHT and BHA, at a concentration of 10^{-2} mmol/L.

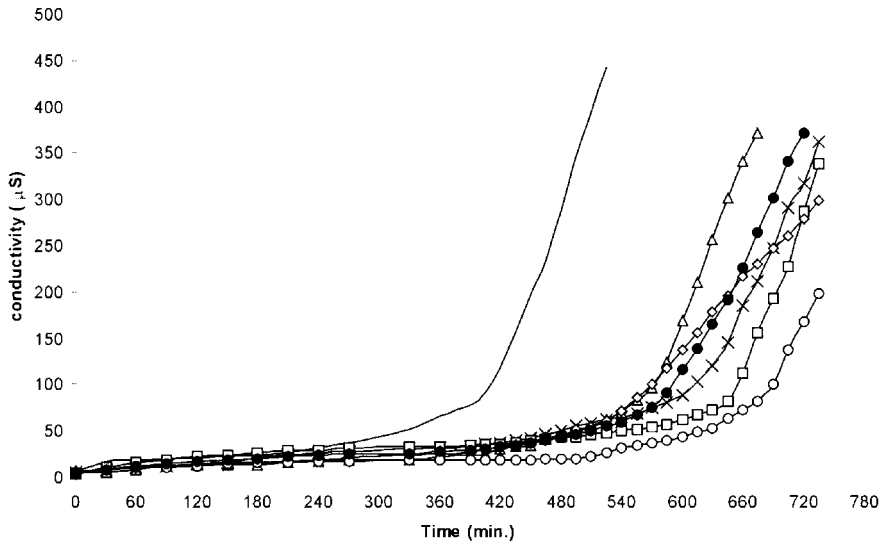

Fig. 6 - Induction curve of soybean oil (test), fresh extracts and standards at a concentration of 5 mmol/kg of oil. Test (—),fresh extract (x), lyophilized fresh extract (△), chlorogenic acid (□), BHT (◊), BHA (○) and standard mix of polyphenols (●).

choke varieties (LATTANZIO and VAN SUMERE, 1987; ADZET and PUIGMACIA, 1985). Their antioxidant activity was comparable with BHA and chlorogenic acid activity in both of the tests used. The results of the conductometric method confirm that the fresh artichoke extract was good against oxidative degradation of lipids, the major limiting factor for the shelf life of foods.

Plant extracts are of increasing interest in the food industry because they slow down the oxidative degradation of lipids. Hence the natural polyphenols of *Cynara scolymus* var. spinoso sardo could be a natural alternative to synthetic antioxidants for food preservation.

In future studies the antioxidant activity of *Cynara scolymus* var. spinoso sardo edible part will be evaluated after cooking, drying and freezing.

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PAPER

FLAVONOIDS IN LEAVES OF BLACK CABBAGE (*BRASSICA OLERACEA* VAR. *ACEPHALA* DC. SUBVAR. *VIRIDIS* CV. *SEROTINA*) GROWN ON DIFFERENT SOILS AND AT DIFFERENT ELEVATIONS

ANALISI DI FLAVONOIDI IN FOGLIE DI CAVOLO NERO (*BRASSICA OLERACEA* VAR. *ACEPHALA* DC. SUBVAR. *VIRIDIS* CV. *SEROTINA)* ALLEVATO IN DIFFERENTI SUOLI E A ALTITUDINI DIFFERENZIATE

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ABSTRACT

Leaves of black cabbage (*Brassica oleracea* var. *acephala* DC. subvar. *viridis* cv. *serotina*) from plants grown on soils developed from two parent materials (sandstone and calcareous marl) and at different elevations were

RIASSUNTO

Foglie di cavolo nero (*Brassica oleracea* var. *acephala* DC. subvar. *viridis* cv. *serotina*), provenienti da piante cresciute su suoli originati da due rocce madri (arenaria e marna calcarea) e a differenti altitudini, sono state campionate

- Key words: calcareous marl soil, HPLC / DAD; HPLC / MS; kaempferol glycosides; quercetin glycosides; sandstone soil -

collected and analysed to evaluate their flavonol content. Eight different flavonols, kaempferol and quercetin derivatives, were identified. HPLC/DAD quantitative analyses showed that both elevation and soil characteristics affect the amount of flavonols in the leaves. The total flavonol content was higher in plants from sandstone soils than in those from calcareous marl soils, and in both cases, it increased with the elevation of the sampling location.

ed analizzate per il loro contenuto in flavonoidi. Sono stati identificati otto flavonoli diversi, derivati del kaempferolo e della quercetina. L'analisi quantitativa HPLC/DAD ha messo in evidenza che, sia l'altitudine sia il tipo di suolo, hanno influenza sulla quantità di flavonoli presenti. In particolare il contenuto in flavonoli è risultato maggiore nelle piante sviluppatesi su arenaria rispetto a quelle su marna calcarea e, in entrambi i casi, incrementava con l'altitudine.

INTRODUCTION

Flavonoids are phenolic compounds which occur widely in many vegetables, fruits and herbs as products of secondary plant metabolism (HARBORNE and WILLIAMS, 2000) and their quantities are known in many species. Flavonoids have been reported to exhibit various biological effects, including antibacterial, antioxidant, antitumor, antiviral, and anti-inflammatory actions, in both animals and humans (WANG, 2000). In fact, regular consumption of fruits and vegetables provides a significant protection against breast, colon and other types of cancer (LUGASI *et al.*, 1999).

In particular, the consumption of carrots and green, leafy vegetables gives a substantial protection against lung cancer, while the consumption of broccoli, cabbage and cauliflower provides protection against colorectal cancer (NASTRUZZI *et al.*, 1996, GRAHAM *et al.*, 1978). Attribution of this protection against cancer is often based on experimental testing of crude extracts and epidemiological data (BEECHER, 1994). In a recent study, performed on a large group of males in the USA, the intake of cruciferous vegetables was inversely associated with bladder cancer risk and this result was statistically significant for broccoli and cabbage. In contrast, the intake of yellow or green leafy vegetables or carotenoid-rich vegetables was not associated with this risk (MICHAUD *et al.*, 1999).

It is evident that flavonoids may be regarded as the most beneficial phytochemicals in food (WANG *et al.*, 1998; HOLLMAN and ARTS, 2000), since their content in fruit, vegetables, nuts and grains is correlated to extensive biological properties which promote human health. The antioxidant activity of some flavonoids is even higher than vitamins C and E (RICE-EVANS *et al.*, 1995) and they may protect animal and plant cells from the negative effects of reactive oxygen species (ROS) which are formed under different stress conditions (HUSAIN and CILLARD, 1987; CHU *et al.*, 2000). In effect, they inhibit oxidation of low-density lipoprotein (LDL), reduce the incidence of coronary heart disease, exhibit anti-inflammatory activity, and act in different ways on various blood components such as platelets, monocytes and smooth muscles (HARBORNE and WILLIAMS, 1992).

Black cabbage (*Brassica oleracea* var. *acephala* DC. subvar. *viridis* cv. *serotina*) has traditionally been cultivated in Tuscany where it is used as food, both raw and cooked, but, differently

from other cabbages, its polyphenol composition has not yet been studied (PRICE *et al.*, 1998; NIELSEN *et al.* 1993, 1998). In the present study the flavonol content in the leaves of black cabbage grown on different soils and at different elevations was determined to ascertain if pedo-ecological factors may affect the qualitative and/or quantitative content of these secondary metabolites.

MATERIALS AND METHODS

Plant material

Outer fully-expanded green leaves of black cabbage were collected from plants growing on soils derived from two parent materials: a sandstone called "Arenaria del Falterona" and a calcareous marl called "Alberese". For each lithology, leaves were sampled from fully mature cabbage growing at three different elevations (Table 1). The soils on sandstone had a sandy loam texture and a sub-acid pH (6.4), while those on calcareous marl had a loam texture and an alkaline pH (7.8). From each site three samples were collected. The 30 cm of topsoil from the sandstone soil had a mean bulk density of 1.34 (standard error 0.10) kg/dm^3, and an organic C content of 11.5 (s.e. 3.2) g/kg; the topsoil of the corresponding layer from the calcareous marl soil had a mean bulk density of 0.89 (s.e. 0.06) kg/dm^3, and an organic C content of 12.8 (s.e. 1.7) g/kg. All the soils had adequate concentrations of the principle available nutrients such as NH_4^+, K^+, Ca^{2+}, Mg^{2+}, NO_3^-, and phosphates.

Extraction, fractionation and analysis of polyphenol compounds

Leaf lamina were frozen in liquid nitrogen and stored at -80°C before proceeding with the analysis. Frozen leaf tissue was then ground with a mortar and pestle under liquid nitrogen. A subsample of 2 g of fresh tissue was extracted with 4x30 mL of 80% *v*/*v* CH_3OH. The solution was then defatted with 3x20 mL of *n*-hexane, evaporated to dryness under reduced pressure at room temperature and then rinsed with a 60:20:20 $CH_3CN/CH_3OH/H_2O$ mixture (pH 2) to a final volume of 5 mL. A sample of 8 µL was analysed by HPLC/DAD and HPLC/MS.

HPLC/DAD analysis

Analyses were conducted using a HP 1100L liquid chromatograph equipped with a DAD detector (both from Agilent Technologies, Palo Alto, California, USA). Polyphenol compounds were separated using a 150x3.0 mm (5 µ) Luna C18 (2) column (Chemtek Analytica, Bologna, Italy) operating at 26°C. The eluents were A: H_2O (at pH 3.2 by HCOOH), B: CH_3CN and C: CH_3OH at a flow rate of 0.6 mL/min following a four-step linear solvent system gradient (53 min) starting from 0% B to 100% C (Table 2).

Table 1 - Sampling locations.

Sampling sites	Parent material	Elevation (m)	Exposure
Puliggiano (Arezzo)	Sandstone	350	North-East
S. Donato in Fronzano (Siena)	Sandstone	400	South
Vallombrosa (Firenze)	Sandstone	1000	West
S. Maria a Grignano (Siena)	Calcareous marl	350	North-West
Malpensata (Siena)	Calcareous marl	500	East-South-East
Sommavilla (Siena)	Calcareous marl	540	South-East

Table 2 - Linear solvent gradient used for analytical HPLC-DAD and HPLC-MS analyses.

H_2O (%)	CH_3CN (%)	Time (min)
100	0.0	0.1
85.0	15.0	20.0
85.0	15.0	25.0
75.0	25.0	35.0
0.0	100.0	43.0
0.0	100.0	53.0

HPLC/MS analysis

The HPLC-DAD apparatus was interfaced with a HP 1100 MSD API-ES (Atmospheric Pressure Ionization-Electrospray, Agilent Technologies, Palo Alto, California, USA) operating in both negative and positive ionization mode with the following operating conditions: gas temperature 350°C, N_2 flow rate 12.0 L/min, nebulizer pressure 30-40 psi, quadrupole temperature 30°C, and capillary voltage 3,500 V.

The fragmentor operated in the range 80-180 V. The interface geometry, with the orthogonal position of the nebulizer with respect to the capillary inlet, permitted the use of the same analytical conditions of the HPLC/DAD analysis. Qualitative analysis was carried out comparing the retention times, UV-Vis spectra and mass spectra with those of commercial standards (Extrasynthèse, Lyon, Nord-Genay, France).

Quantitative analysis

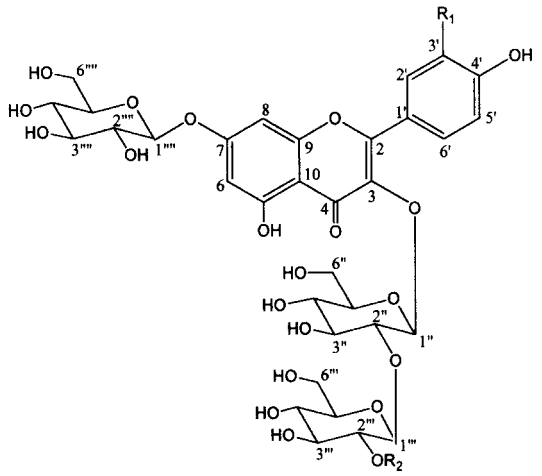
Quantification of individual polyphenols was performed by using a four-point regression curve (r^2>0.998) developed through the use of authentic standards, operating in the range 0-40 µg. In particular, flavonols were calibrated at 350 nm using kaempferol 3-O-glucoside and rutin as reference compounds, respectively. In both cases actual concentrations of both quercetin and kaempferol derivatives were calculated after applying corrections for changes in molecular weight. For example, in the case of quercetin derivatives, knowing the molecular weight of each compound (PM_Q), its actual concentration was obtained applying a multiplication factor of PM_Q/610, where 610 is the molecular weight of rutin.

RESULTS AND DISCUSSION

Qualitative analysis

The flavonol compounds determined in black cabbage leaf extracts, and their relative chemical structures, are listed in Fig. 1. As an example, the chromatographic profile of an extract recorded at 350 nm is presented in Fig. 2. Eight distinct flavonol glycosides with kaempferol and quercetin backbones were found and identified according to NIELSEN (1993) and WILSON (1998). In some cases, a hydroxycinnamic acid moiety (caffeoyl, feruloyl, synapoyl and p-coumaroyl) was linked *via* an ester bridge to a glucose. Among kaempferol and quercetin derivatives, the most abundant were: kaempferol-3-O-sophoroside-7-O-glucoside, kaempferol-3-O-(2-feruloylsophoroside)-7-O-glucoside, kaempferol-3-O-(2-caffeoyl-sophoroside)-7-O-glucoside, and quercetin-3-O-sophoroside-7-O-glucoside, quercetin-3-O-(2-caffeoyl-sophoroside)-7-O-glucoside. NIELSEN *et al.* (1998) reported the occurrence of four kaempferol tetraglucosides in *Brassica oleracea* L. var. *capitata*, however these compounds seem to be absent or present only in trace amounts in the black cabbage leaves tested in the current investigation.

To characterize flavonol glycosides, the HPLC-DAD analysis was coupled to HPLC-MS, operating in negative ion mode with modulated fragmentation patterns. As an example, the mass spectra of kaempferol-3-O-(2-O-caffeoylsophoroside)-7-O-glucoside and quercetin 3-O-sophoroside-7-O-glucoside are reported in Fig. 3a and Fig. 3b, respectively. The



	R_1	R_2
1	OH	H
2	OH	Caffeoyl
3	H	H
4	H	Caffeoyl
5	OH	Feruloyl
6	H	Synapoyl
7	H	Feruloyl
8	H	p-Coumaroyl

R_1 = OH quercetin derivatives
R_2 = H kaempferol derivatives

Fig. 1 - Chemical formulas of flavonol compounds determined in black cabbage leaf extracts.

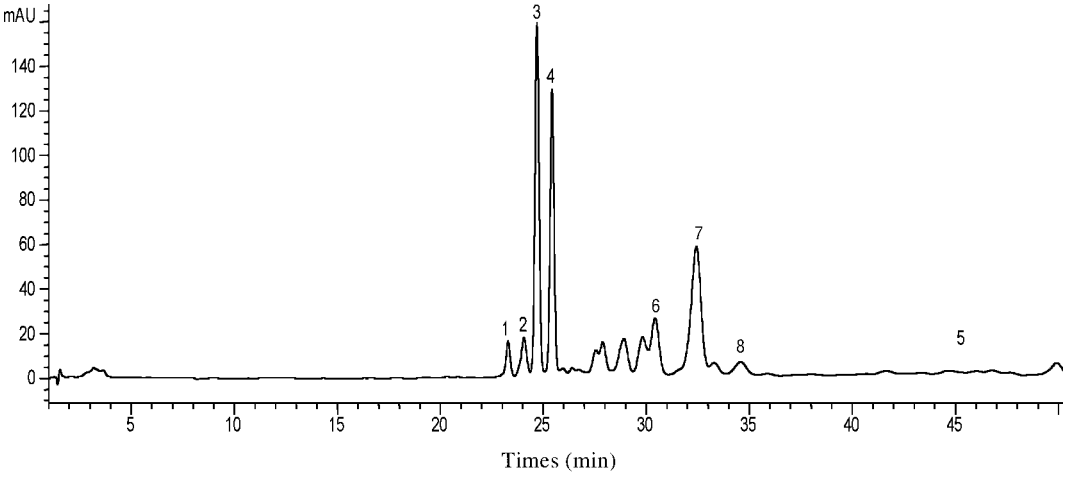


Fig. 2 - Chromatographic profile of black cabbage leaf extracts acquired by HPLC-DAD at 350 nm. *Peaks*: 1. quercetin-3-O-sophoroside-7-O-glucoside; 2. quercetin-3-O-(2-caffeoyl-sophoroside)-7-O-glucoside; 3. kaempferol-3-O-sophoroside-7-O-glucoside; 4. kaempferol-3-O-(2-caffeoyl-sophoroside)-7-O-glucoside; 5. Quercetin feruolylglycoside; 6. kaempferol-3-O-(2-synapoylsophoroside)-7-O-glucoside; 7. kaempferol-3-O-(2-feruloylsophoroside)-7-O-glucoside; 8. kaempferol-3-O-(2-p-cumaroylsophoroside)-7-O-glucoside.

fragmentation pattern of kaempferol-3-O-(2-O-caffeoylsophoroside)-7-O-glucoside shows five signals at 933, 771, 609, 447 and 285 m/z, corresponding to the quasi-molecular ion [M-H]-, to fragments after the loss of three glucosidic moieties $[M-162]^-$, $[M-324]^-$, $[M-486]^-$, and the kaempferol aglycone $[Agl-H]^-$, respective-

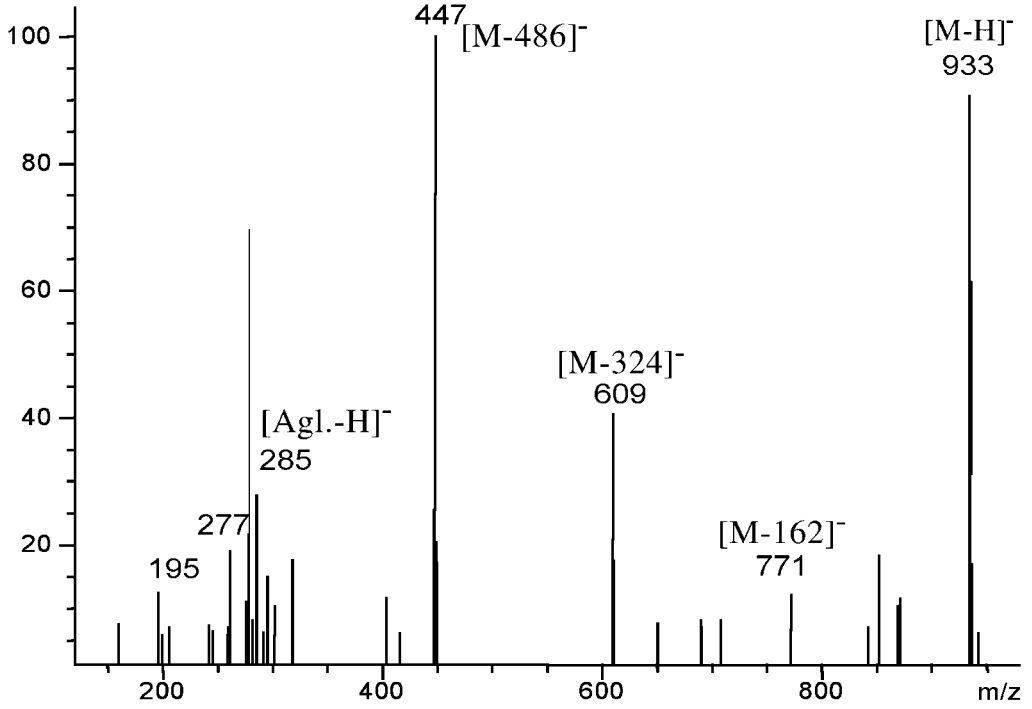


Fig. 3a - Negative-ion mass spectrum of kaempferol-3-O-(2-O-caffeoylsophoroside)-7-O-glucoside acquired by API-electrospray HPLC-MS analysis at 150 negative fragmentor potential.

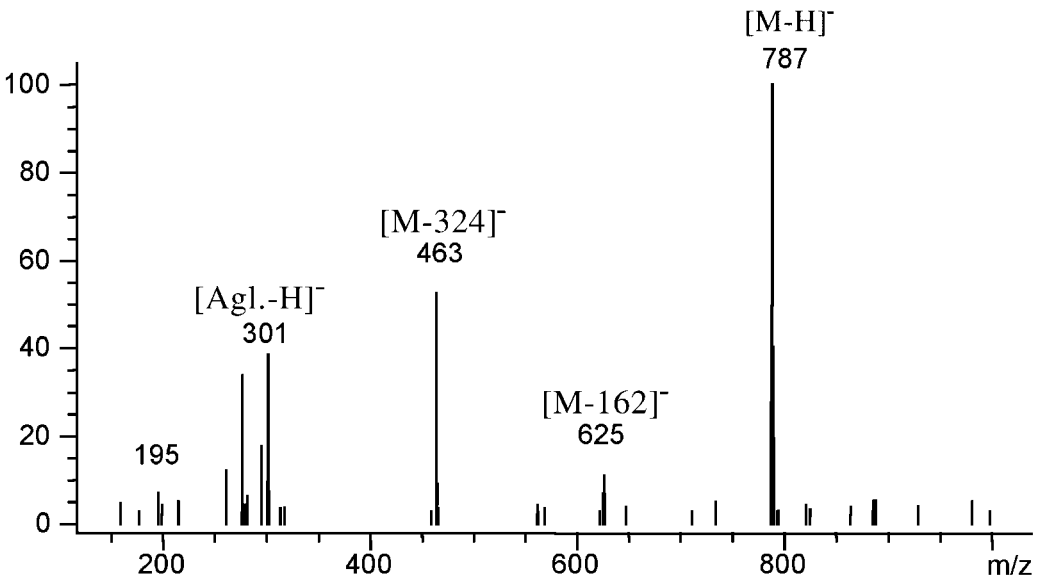


Fig. 3b - Negative-ion mass spectrum of quercetin 3-O-sophoroside 7-O-glucoside acquired by API-electrospray HPLC-MS analysis at 150 negative fragmentor potential.

Table 3 - Kaempferol and quercetin glycosides (mg/g of fresh weight) in the leaves of black cabbage grown on sandstone and calcareous marl at different elevations. Numbers in parentheses are the standard errors.

	Sandstone			Calcareous marl		
	350 m	400 m	1000 m	350 m	500 m	540 m
Kaempferol glycosides	0.608 (0.020)	0.680 (0.020)	1.228 (0.020)	0.413 (0.015)	0.463 (0.015)	0.758 (0.025)
Quercetin glycosides	0.400 (0.010)	0.100 (0.050)	0.495 (0.010)	0.085 (0.050)	0.063 (0.050)	0.120 (0.050)

ly. In the case of quercetin 3-O-sophoroside-7-O-glucoside, four ion fragments were recorded, at 787, 625, 463 and 301 m/z, corresponding to the quasi-molecular ion $[M-H]^-$, the loss of glucosidic moiety $[M-162]^-$, $[M-324]^-$ and quercetin aglycone $[Agl-H]^-$, respectively.

Quantitative analysis

Table 3 shows the amounts of kaempferol and quercetin glycosides found in the leaves of black cabbage grown on sandstone and calcareous marl at different elevations; values are expressed as mg/g of fresh weight. For kaempferol derivatives, each value represents the sum of five glucosides which varied from 0.413 to 1.228 mg/g. In the case of the quercetin derivatives, each value is the sum of three glucosides which varied from 0.063 to 0.495 mg/g. As shown in Fig. 4, the total flavonol content was higher in plants from sandstone soils than in those from calcareous marl soils, however in both cases, it increased with the elevation of the sampling location.

In a recent study carried out on onion bulbs (PATIL *et al.*, 1995), the total querce-

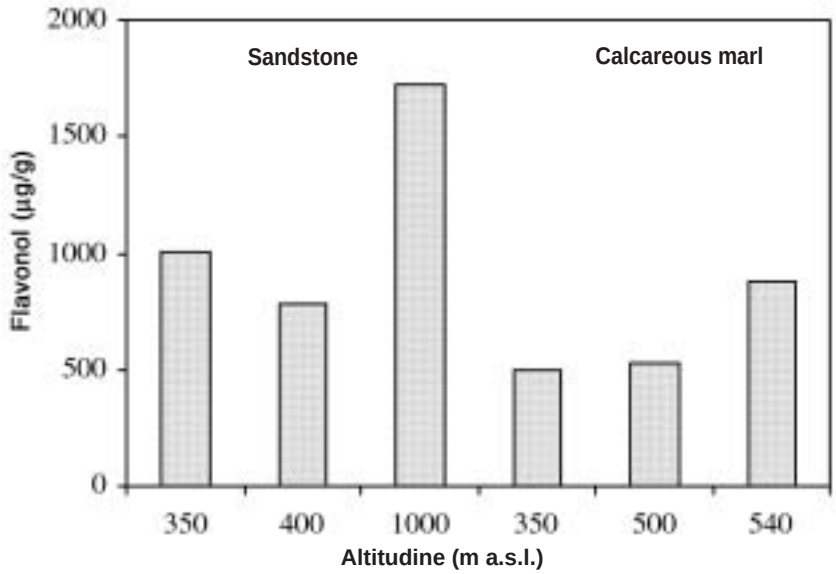


Fig. 4 - HPLC-DAD evaluation of the total flavonol content in hydroalcoholic extracts of black cabbage leaves. Data are expressed as µg/g of fresh weight.

tin concentration varied significantly as a function of location, rather than growing stage and soil type. Thus, the growing site seems to be the major environmental factor in determining quercetin concentration. Similar cultural practices were employed at both locations, but with different planting times; there were no differences in elevation between the sites. However, one location received greater light intensity, with respect to the other and this was held responsible for the greater quercetin content. In the present study the differences in the location and soil type influenced the biosynthesis of flavonols. The two soils had different textures and different amounts of organic matter and available macronutrients, thus some differences are to be expected: in particular, there was a trend for both glycoside flavonol classes to increase with increasing altitude on the different soils. This is in agreement with PATIL (1995) and may be explained by the increase in ultraviolet (UV) radiation with altitude. In fact, the physiological role of flavonoids is to act as a barrier for damaging UV radiation because of their adsorption maxima in the UV region; for this reason, they are often localized in the epidermal cells (LANDRY *et al.*, 1995; COOPER-DRIVER and BHATTACHARYA, 1998). Moreover, the sugar moiety has no, or very little, effect on the UV absorption of these compounds, but acylation with hydroxycinnamic and other phenolic acids may increase the absorption at certain wavelengths. Thus, as can be expected, the penetration of UV-B radiation in plant tissue determines an increased flavonoid glycoside and acylglycoside content in the epidermal layer of the mesophyll (OLSSON *et al.*, 1998: TATTINI *et al.*, 2001).

In Chinese cabbage (*Brassica pekinensis*), UV-B radiation promotes the expression of phenyl-alanine-ammonia-lyase (PAL), which leads to the accumulation of flavonoids in the leaves (HUANG *et al.*, 1999). The present investigation shows that the flavonol content in the leaves of black cabbage is relatively high. In a recent study on several edible vegetables, MIEAN and SUHAILA (2001) found the highest contents of flavonoids in onion leaves (1.497 mg/g quercetin, 0.391 mg/g luteolin and 0.832 mg/g kaempferol) while, for Brassica species, Chinese cabbage was reported to contain a relatively low amount of flavonoids, expressed as apigenin (0.787 mg/g). CHU *et al.* (2000) focused their investigation not only on determination of the flavonoid content, but also on the evaluation of the antioxidant properties of sweet potato and onion extracts with respect to those from cabbage, spinach and potato. Sweet potato leaves and the outer skin of onions possessed antioxidative activities higher than other vegetables, including purple, Chinese and white cabbages. *Lactuca sativa* L. is another important crop species which possesses large amounts of polyphenols, such as caffeic acid derivatives and glycosides of kaempferol and quercetin (between 1.352 and 3.370 mg/g), although in this case the flavonol percentage is relatively low (6 and 30%) (ROMANI *et al.*, 2002). Since in the leaves of black cabbage the flavonol concentration reached values of 1.723 mg/g, this species could be an interesting source of natural antioxidant.

CONCLUSION

The flavonol content of black cabbage is relatively high. The concentration of these molecules seems to depend on environmental conditions, in particular elevation, since their concentration increased with it. The nature of the soil also appears to play a key role in the synthesis of flavonoids, which were more abundant on the sandstone soils than on the calcareous marl.

As both types of soils contained a sufficient amount of available nutrients (macroelements), it is assumed that other pedological factors or availability of micronutrients could be responsible for

the differences in flavonoid production by black cabbage. Production and the use of this vegetable as a food should be promoted in order to preserve a typical biodiversity and to better exploit a species that contains beneficial phytochemicals.

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PAPER

DETERMINATION OF THE HYDROPHILIC AND LIPOPHILIC ANTIOXIDANT ACTIVITY OF WHITE- AND RED WINES DURING THE WINE-MAKING PROCESS

LA DETERMINAZIONE DELL'ATTIVITÀ ANTIOSSIDANTE IDROFILA E LIPOFILA NEI VINI BIANCO E ROSSO DURANTE LA VINIFICAZIONE

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ABSTRACT

The ABTS/H_2O_2/HRP decoloration method was used to evaluate the antioxidant activity of one white and two red wines. During the wine-making process, the red wines showed higher antioxidant activity than the white wine. The correlation between total phenols, anthocyanin content, colour index and antioxidant activity was also examined. These parameters, normally determined in the vinification process, were not necessarily correlated with the antioxidant properties of the wines. Corre-

RIASSUNTO

Il metodo di decolorazione ABTS/H_2O_2/HRP è stato utilizzato per valutare l'attività antiossidante di un vini bianco e di due vini rossi. Durante il processo di vinificazione, i vini rossi hanno mostrato una capacità antiossidante superiore a quella del vino bianco. È stata anche valutata la correlazione tra i polifenoli totali, il contenuto in antociani, il colore e la capacità antiossidante. Tutti i parametri analitici normalmente valutati nel corso della vinificazione non sono apparsi automa-

- Key words: ABTS, anthocyanins, antioxidant activity, phenols, vinification, wine -

lations between the parameters were strongly dependent on the grape variety. The determination of hydrophilic antioxidant activity (HAA) and lipophilic antioxidant activity (LAA) in the three wines showed that nearly 25% of the total antioxidant activity (TAA) was due to LAA.

ticamente correlati con la capacità antiossidante, mentre è emersa una forte correlazione tra l'attività antiossidante e la varietà delle uve. La determinazione dell'attività antiossidante idrofila (HAA) e di quella lipofila (LAA) nei tre vini ha evidenziato che circa il 25% dell'attività antiossidante totale (TAA) è legata a quella lipofila (LAA).

INTRODUCTION

Wine, along with other moderately alcoholic beverages, has been associated with reduced risk of cardiovascular diseases and other illnesses related to oxidative stress. A number of epidemiological studies have demonstrated that a moderate consumption of alcohol is associated with reduced mortality and coronary heart disease (St LEGER *et al.*, 1979; RENAUD and DE LOGERIL, 1992; GOLDBER *et al.*, 1995; FUHRMAN *et al.*, 1995; RIMM *et al.*, 1996). Wines contain a complex mixture of compounds which have great significance in reducing health problems caused by reactive oxygen and nitrogen species (FRANKEL *et al.*, 1995; CAMPOS and LISSI, 1996; SATO *et al.*, 1996; SIMONETTI *et al.*, 1997; PELLEGRINI *et al.*, 2000). This property is related to the antioxidant potential (or activity) of plant-derived foodstuffs.

Several methods are used to measure antioxidant activity. The most commonly used are those that use chromogen radical compounds to simulate reactive oxygen and nitrogen species. CANO *et al.* (1998) developed a method based on the ABTS radical ($ABTS^{+\cdot}$) generated by horseradish peroxidase (HRP) to evaluate the antioxidant activity of pure compounds and plant-derived samples. The method is easy, accurate and rapid to use and has numerous advantages since there are no unwanted side-reactions, high temperatures are not required to generate ABTS radicals and the antioxidant activity can be studied over a wide range of pH values. This method is capable of determining both hydrophilic (in buffered media) and lipophilic (in organic media) antioxidant properties (CANO *et al.*, 2000). The method has been used to determine the antioxidant activity in a range of foodstuffs (ARNAO *et al.*, 1998; 1999; 2001a;b). The $ABTS^{+\cdot}$ chromogen used in this method has been compared with another widely used radical chromogen, $DPPH^{\cdot}$; it has been concluded that, in the determination of the antioxidant potential of citrus and wine samples, the $DPPH^{\cdot}$ method could significantly under-estimate TEAC (Trolox Equivalent Antioxidant Capacity) by up to 36% compared to $ABTS^{+\cdot}$ (ARNAO, 2001). The method has also been used to study the total antioxidant activity of different vegetable soups, obtaining relevant data on the relative contribution of hydrophilic (ascorbic acid and phenols) and lipophilic (carotenoids) components to their total antioxidant activity (ARNAO *et al.*, 2001a).

In this study the $ABTS/H_2O_2/HRP$ method was used to study the antioxidant properties of wines in the various stages of the vinification process. The contribution of the hydrophilic and lipophilic antioxidant activity to the total antioxidant activity was determined. The correlation between total phenols, anthocyanin content, colour index and antioxidant activity was also examined.

MATERIAL AND METHODS

Reagents and standards

2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and gallic acid were purchased from Sigma Chem.Co (Madrid, Spain). H_2O_2 (30%, v/v) was obtained from Aldrich Chem.Co. (Madrid, Spain). The concentration of ABTS and hydrogen peroxide was determined by measuring their absorbance using ε_{340nm}=36 mM^{-1} cm^{-1} for ABTS (CHILDS and BARDSLEY, 1975) and ε_{240nm}= 43.6 M^{-1} cm^{-1} for H_2O_2 (BIELSKI and ALLEN, 1977). Type 4B horseradish peroxidase (HRP-C) was obtained from Biozyme (Blaenavon, UK) and checked using ε_{403nm}= 100 mM^{-1} cm^{-1}.

Spectrophotometric measurements were recorded with a UV-VIS Perkin-Elmer Lambda-2S spectrophotometer. The temperature was controlled at 25±0.1°C using a Haake D1G circulating bath with a heater/cooler.

Plant material

The different musts used in this study were obtained from the grape varieties (*Vitis vinifera* L.): "Airen" (white), "Cabernet-Sauvignon" (red) and "Cencibel", also called "Tempranillo" (red). All samples were provided by the "Sociedad Cooperativa Cristo de la Vega" winery (Socuéllamos, Ciudad Real, Castilla-La Mancha, Spain). The must samples were obtained from the winery and rapidly transported to the laboratory at -20°C, to stop the fermentation process.

In this winery, white wine "Airen" is produced by a cool-controlled fermentation process at 18°C. In this case, samples were taken at the following selected points during white-vinification: juice after grape pressing (wA), prior to filtration (wB), at the start (wC), in the middle (wD) and at the end (wE) of controlled fermentation and from the finished wine (wF). In the red-grape varieties, wines are produced according to a traditional fermentation process. The selected sampling points during red vinification were: initial must (rA), start of alcoholic fermentation (rB), remontage (pump-overs) (rC), racking (rD) and finished wine (rE).

Generally, musts and wines were used directly to determine hydrophilic antioxidant activity (direct HAA), total phenols, anthocyanin content and colour index. Homogeneous vinification samples were centrifuged at 1,075 g in a refrigerated (4°C) Sorvall RC-5B Plus to eliminate solid residues.

For lipophilic antioxidant activity (LAA), wine (5 mL) and ethyl acetate (2 mL) were mixed in a decanting funnel. The total amount was washed with 50 mM sodium phosphate buffer (2 mL, pH 7.5). This procedure guarantees that no hydrophilic compounds are retained in the organic phase. This procedure also brings the constituents of the aqueous phase to pH 7.5, the pH value used in determining the hydrophilic antioxidant activity. When the phases were separated, HAA and LAA were determined immediately in the aqueous and organic phase, respectively.

Hydrophilic and lipophilic antioxidant activity

These were measured using the ABTS/HRP decoloration methods (CANO *et al.*, 1998; 2000). The methods are based on the capacity of a sample to scavenge the ABTS radical cation ($ABTS^{+\cdot}$) compared to a standard antioxidant (ascorbic acid or Trolox) as measured on a dose-response curve. For hydrophilic antioxidant activity (HAA), the reaction mixture contained 2 mM ABTS, 30 µM H_2O_2 and 0.25 µM HRP in 50 mM Na-phosphate buffer (pH 7.5) in a total volume of 1 mL. The assay temperature was 25°C. The reaction was monitored at 730 nm until

a stable absorbance was obtained. Then, 10 µL of the aqueous sample was added to the reaction medium and the decrease in absorbance, which is proportional to the $ABTS^{+\cdot}$ quenched, was determined after 5 min.

For lipophilic antioxidant activity (LAA) the reaction mixture contained 1 mM ABTS, 30 µM H_2O_2 and 6 µM HRP in acidified ethanol (pure ethanol with phosphoric acid, 0.7% w/v), in a total volume of 1 mL. In this case, 10 µL of the organic phase was added to the reaction medium and the decrease in absorbance at 730 nm was determined after 5 min. The total time needed to carry out each assay was approximately 7 min, including ABTS radical generation by peroxidase, the addition of antioxidant and acquisition of the final absorbance value. The decrease in absorbance was determined from the difference between the A_{730} values before and 5 min after sample addition. Antioxidant activity was calculated as moles of $ABTS^{+\cdot}$ quenched by 1 mol of Trolox. In both cases, the antioxidant activity is expressed as Trolox equivalents per 100 mL of must/wine (mg · 100 mL^{-1}).

Total phenols, anthocyanins and colour index

Total phenols were determined using the Folin Ciocalteau reagent using gallic acid as standard. Thus, the data are expressed as gallic acid equivalents (GAE, mg·100 mL^{-1}). Anthocyanins were determined spectrophotometrically at 516 nm using malvidin-3-monoglucoside as standard (ε_{516nm} = 4.48 $mM^{-1}cm^{-1}$). Colour Index (C.I.) is conventionally used as a parameter to determine the colour intensity of must/wines. The parameter was measured using the equation:

$$C.I. = (A_{420nm} + A_{520nm} + A_{620nm}) \cdot 1/b;$$

where b is the cuvette light pathlength.

Statistical analysis

Results were statistically analysed using the SPSS program, applying the LSD multiple range test to establish significant differences between the parameters.

RESULTS AND CONCLUSIONS

The antioxidant activity of wines can be measured by determining the scavenging activity with chromogen free radicals. Generally, there is a direct correlation between the total phenol content of a wine and its antioxidant activity (KANNER *et al.*, 1994; VINSON and HONTZ, 1995; PELLEGRINI *et al.*, 2000). To study changes in the antioxidant activity, phenol content and other parameters during the wine-making process in three wines, these parameters were determined at six critical points of the vinification process for the white wine and at five for the red wines (see Materials and Methods section). The antioxidant activity was determined using the established assay that uses the ABTS radical cation as chromogen. The presence of an antioxidant leads to the disappearance of this species.

The antioxidant activity was determined directly using must and wine, without separating the components (HAA). Fig. 1a shows the evolution of hydrophilic antioxidant activity (HAA) in the white wine "Airen". The initial must (stage wA) had an HAA of 26.0 mg·100 mL^{-1} (as Trolox equivalents) and, after a slow increase during fermentation (stages wC-wE), the final white wine had an HAA value of 25.7 mg·100 mL^{-1}. In the case of red wines (Figs. 1b and 1c), the HAA values of the initial must and final wines differred greatly. In the case of red wines, the second-stage (rB, start of fermentation) provoked the incorporation of many antioxidants into the musts; thus in "Cencibel" the HAA increased

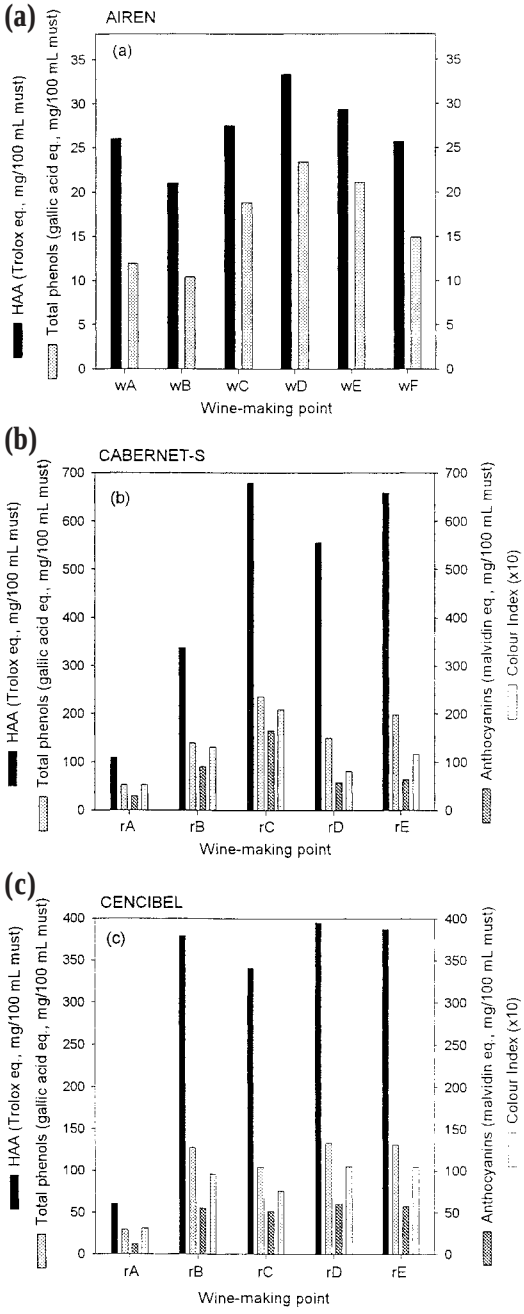

Fig. 1 - Hydrophilic antioxidant activity (HAA), total phenols, anthocyanins and color index at different phases of the wine-making process for the grape varieties: (a) Airen; (b) Cabernet-Sauvignon and (c) Cencibel. The nomenclature for the wine-making points appears in the Materials and Methods section.

from 60 mg·100 mL^{-1} (stage rA) to 387 mg·100 mL^{-1} (stage rB). A similar increase occurred for "Cabernet", going from 109 (stage rA) to 658 mg·100 mL^{-1} (stage rC).

Quantitatively, the final wines had important differences: white wine "Airen" had less HAA than red wines. The red wines had significant differences; "Cabernet" wine had a final HAA of 658 mg·100 mL^{-1} and "Cencibel" wine had 387 mg·100 mL^{-1}. These HAA values of the final wines correspond with those reported by other authors when the most important grape variety-dependent variations in antioxidant activity are considered (CAMPOS and LISSI, 1996; RICE-EVANS *et al.*, 1997; PELLEGRINI *et al.*, 2000; ARNAO, 2001).

Figs. 1a, 1b and 1c also show the change in total phenols during vinification. In all three cases, total phenols varied in a similar-way to HAA and in all cases the total phenol values were lower than HAA, indicating that a possible direct correlation exists between the parameters. Table 1 shows the correlation parameters between HAA and total phenols for the three wines. In the case of the red wines, there was a good correlation between HAA and total phenol content (r^2>0.9), but for white wine the correlation index was lower. Figs. 1b and 1c also show the anthocyanin content and colour index (C.I.) during red wine production. Apparently, a direct relationship exists between these parameters and antioxidant activity in both wines. Table 1 shows good correlations between HAA, total phenols, anthocyanin content and C.I. during the vinification of the "Cencibel" variety. Contrary to "Cabernet", in which a good correlation existed only between HAA and total phenols, the correlations between HAA and anthocyanins and C.I. was very poor, even though there was a good correlation between anthocyanins and C.I. for this variety. The parameters normally determined in the vinification process, such

Table 1 - Linear correlation between the different parameters estimated during the wine-making process of the three grape varieties.

	Parameters correlated	Linear Regression Coefficient (r^2) (n=5, P<0.05)
White must/wine "Airen"	HAA vs Total phenols	0.856
Red must/wine "Cabernet"	HAA vs Total phenols	0.905
	HAA vs Anthocyanin content	0.341
	HAA vs Colour Index	0.440
	Total phenols vs Anthocyanin content	0.612
	Total phenols vs Colour Index	0.717
	Anthocyanin content vs Colour Index	0.962
Red must/wine "Cencibel"	HAA vs Total phenols	0.985
	HAA vs Anthocyanin content	0.996
	HAA vs Colour Index	0.943
	Total phenols vs Anthocyanin content	0.979
	Total phenols vs Colour Index	0.984
	Anthocyanin content vs Colour Index	0.938

as total phenols and C.I. are not necessarily correlated with the antioxidant properties of wines. Correlations between the parameters are strongly dependent on the grape variety.

In order to establish the partial contributions of hydrophilic and lipophilic antioxidants to the total antioxidant activity of wines, these components were extracted separating the aqueous and organic phases of the final wine. Simultaneously, the hydrophilic antioxidant activity (HAA) and the lipophilic antioxidant activity (LAA) were determined in the wine made from the three grape varieties. Table 2 shows the values of HAA and LAA of the three wines, expressed as Trolox equivalents. Comparing these values with the values of direct hydrophilic antioxidant activity (without phase-separation: direct HAA), in all cases the Total Antioxidant Activity (TAA= HAA + LAA) was higher than the direct HAA of the wines. This means that in these samples there are hydrophilic and lipophilic compounds that

Table 2 - Lipophilic (LAA) and hydrophilic (HAA) antioxidant activity of wines and their contribution to the total antioxidant activity (TAA).

	White wine "Airen"	Red wine "Cabernet"	Red wine "Cencibel"
HAA[1]	20.0±1.0[3]	546±18	329±10
	(76.6)[4]	(81.3)	(76.6)
LAA	6.1±0.3	125±6	100±5
	(23.4)	(18.3)	(23.4)
TAA	26.1±1.3	672±24	429±15
Direct HAA[2]	25.7±1.4	658±23	387±11

[1]HAA, LAA and TAA are expressed as trolox equivalents (mg/100 mL).
[2]Direct HAA was estimated directly from wine, without phase-separation.
[3]Mean (n=5) ± standard error.
[4]In parentheses, percentage contribution of HAA and LAA to TAA.

express their antioxidant activity only in the adequate medium. It was possible to sum HAA and LAA because in both determinations the same radical scavenger was used, expressing the antioxidant activity in the same units (trolox equivalents). Generally, summing of different antioxidant activities has been problematic because antioxidant activities estimated with different tests (with distinct reactive principles) has not permitted the summing of the antioxidant activities expressed in each one. In this case, due to the excellent properties of the ABTS/H_2O_2/HRP system it was possible to separately determine the antioxidant activity due to hydrophilic compounds (HAA) and those due to lipophilic compounds (LAA), both contributing to the Total Antioxidant Activity (TAA) of the samples.

Thus, although quantitatively there were great differences between the three wines, percentage-wise, all had an LAA close to 25% of TAA, the remainder (75%) being HAA (Table 2). In the case of the white wine "Airen", the addition of sulphite could have contributed moderately to the HAA (HUA LONG *et al.*, 2000). In all cases, the high percentages of HAA can be explained by synergistic effects (the easily oxidized phenols are regenerated by less active phenols) (KANNER *et al.*, 1994), and/or by unidentified compounds with specific antioxidant activities in the wine.

In conclusion, an experimental approach has been proposed to quantify the hydrophilic and lipophilic antioxidant activity in wine. The application to the wine-making process provides interesting data about the evolution of the antioxidant potential during vinification. The antioxidant activity of red wines was fifteen- (Cencibel) and twenty-five-times (Cabernet-S) that of the white wine (Airen). In the three wines assayed nearly 25% of the total antioxidant activity was due to lipophilic antioxidant activity.

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PAPER

EVALUATION OF A HEDONIC SCALING METHOD FOR MEASURING THE ACCEPTABILITY OF SCHOOL LUNCHES BY CHILDREN

VALUTAZIONE DI UN METODO EDONISTICO PER LA DETERMINAZIONE DELL'ACCETTABILITÀ DEI PASTI FORNITI AI BAMBINI A SCUOLA

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ABSTRACT

Few studies concerning product testing by children have been published, and practically no such studies have been carried out in Italy. The aim of this work was to set up an evaluation card and a procedure to test grading of meals supplied to school refectories by the Municipality of Milan. Children from four second grade classes were selected from two different primary schools, which received meals from the same meal service. The aims of the study were explained to the children involved (88),

RIASSUNTO

Le pubblicazioni relative a test di prodotto condotti su bambini sono poche e, in Italia, praticamente inesistenti. Scopo di questo lavoro è stato la messa a punto di una scheda di valutazione e di una procedura per testare la gradibilità dei pasti forniti nelle mense delle scuole del Comune di Milano. Sono state scelte quattro seconde classi elementari appartenenti a due scuole diverse e servite dallo stesso centro cottura. I bambini coinvolti (88), tutti di età compresa tra i 7 e gli 8 anni, dopo essere

- Key words: acceptability, children, scaling, school lunch -

7 and 8-year-olds who were trained in the use of scaling. They then provided acceptability scores for a series of first courses, second courses, vegetables and fruits. While it was not difficult to obtain judgements on grading of meals, some problems were encountered in setting up a model that was useful for both a real time evaluation of meals and any changes in meal formulations. Results were processed by Analysis of Variance, and comparable grading assessments were obtained from the four grades. No significant differences were found in most formulations between the two schools.

stati istruiti sulle finalità del lavoro e sull'utilizzo della scala di valutazione, hanno fornito un punteggio di accettabilità per una serie di primi piatti, di secondi piatti, di contorni e di frutta. Infatti, non è difficile ottenere dei giudizi di gradibilità ma impostare un modello che sia utile per la valutazione in tempo reale dei pasti e che si possa utilizzare per eventuali modifiche delle formulazioni. I risultati, dopo elaborazione con l'Analisi della Varianza, sono confrontabili in termini di gradibilità, per le quattro classi considerate ed inoltre, si può concludere che, per la maggior parte delle formulazioni, non esistono differenze significative tra le due scuole.

INTRODUCTION

Few studies concerning product testing by children have been published, and practically no such studies have been carried out in Italy. Hedonic rating of school meals by children is affected by various factors. Children may smell and taste products differently than adults do, but the differences in reported smells and tastes may also be caused by their lack of skill in communicating perceptions (MOSKOWITZ, 1985; JAMES *et al.*, 1997). Testing with children poses an entirely new set of problems, combining new food preferences with procedural difficulties due to problems with behaviour and comprehension. Odour discrimination and preference are apparent in children as young as 3 years old, and the hedonic quality of an odour may influence how food is accepted (GUINARD, 2001). Children's preferences or food acceptance are also mediated by familiarity with and the sweetness of the food, as well as social learning (SPAETH *et al.*, 1992).

Because children are the consumers of school meals, their responses need to be studied. Difficulty in measuring children's responses may be caused by variability among individuals and the effects of age differences on verbal skills, comprehension, and attention span (SCHRAIDT, 1991). In addition, the responses of some children are difficult to study because they have trouble expressing their feelings and explaining their behaviour, have difficulty remembering and describing objects or previous events, and do not comprehend questions that require abstract thinking (SPAETH *et al.*, 1992). Hence, under the current circumstances, grading of school meals does not depend on a conscious analysis and independent reflection by children. Rather, it results from some factors that make quality assessments of individual meal formulations difficult. It is not difficult to obtain judgements on the grading of meals, but it is difficult to set up a model that is useful for both a real time evaluation of meals and any changes in meal formulations. In addition, little is known about the sensory percep-

tion of food by children (LEON *et al.*, 1999). The lack of studies involving children can be partially explained by the limited availability of methodologies to measure food preference in children. These methods have to be simple in order to be perfectly understandable by young children and, at the same time, they must be robust enough to reliably measure food preferences (KROLL, 1990; CHEN and RESURRECCION, 1996; LEON, 1998; LEON *et al.*, 1999; GUINARD, 2001). Sensory evaluation with children typically requires a specifically designed introduction to the test methodology and more extensive training. Young children are subject to limitations in cognition, including the concepts of quality, time/frequency/periodicity, and serialization/progression; abstraction vs concreteness; and cause/effect relationships (KIMMEL *et al.*, 1994). Knowledge of children's food preferences, the factors influencing them, and their perceptions about the healthiness of foods is needed if school caterers and those involved in nutrition education are to work together to help children choose a nutritionally balanced meal (NOBLE *et al.*, 2000; BAXTER *et al.*, 2000).

The aim of this study was to set up, for the first time in Italy, an evaluation card and a reference procedure to test grading of meals supplied to school refectories by the Municipality of Milan using 7-8-year-old children as the consumers.

MATERIALS AND METHODS

Sample selection

The formulations (i.e. first course, second course, vegetables, fruit) of the meals that were evaluated are reported in Table 1.

Subjects

Two schools, where meals were supplied by the same food service, were selected: a primary school, designated R,

Table 1 - List of the formulations tested.

Formulations	First course	Second course	Vegetables	Fruit
A	Tortellini soup	Tuna-fish	Tomatoes	Apple
B	Pasta with garlic and oil	Turkey with tomato sauce	Roast potatoes	Pear
C	Risotto with saffron	Roulades of beef and tomato	French beans	Apple
D	Pasta with tomato sauce	Roast chicken	Tomatoes	Grapes
E	Pasta with tomato sauce	Raw ham	Boiled potatoes	Apple
F	Minestrone	Hamburger	Boiled potatoes	Pear
G	Pasta with tomato sauce	Chicken escalope	Red salad	Grapes
H	Pasta with butter	Spreadable cheese	Carrots	Apple
I	Pasta with tomato sauce	Cooked ham	Carrots	Apple
L	Ravioli with spinach and ricotta cheese filling	Roast turkey	Boiled potatoes	Pear
M	Vegetable puree	Fish sticks	Green salad	Yogurt
N	Risotto with Parmesan cheese	Meatballs with sauce	Potato puree	Orange
O	Pasta with Bolognese sauce	Potato omelette	Tomatoes	Orange
P	Pasta with pesto	Fontal cheese	Roast potatoes	Pear
Q	Pasta rainbow	Pork loin with lemon sauce	Roast potatoes	Orange
R	Soup	Breasts of chicken	Tomatoes	Banana

where meals were cooked and served directly, and a primary school, designated L, not far from the food service, where meals were served after hot transport (15 min). Four classes were selected as reference models in the schools: two second grade levels (R_1 and R_2) consisting of 23 children each (percentage of girls = 43%), from school R and two second grade levels (L_1 and L_2) consisting of 20 (percentage of girls = 40%) and 22 children (percentage of girls = 36%), respectively, from school L. All the children had lunch between 12:30 and 13:00. During this preliminary study, the second grade pupils were selected to determine whether filling out of the cards would cause problems to children who had just learned how to write.

Training procedure

After a preliminary exploratory session carried out by the respective teachers in the classroom, all the children (88) involved in the study were asked to participate in a lesson on the meaning of sensory analysis and the role they would play as the assessors representing all the children (about 70,000) who have their lunch daily at municipal schools. During this 90 min lesson, suitably selected teaching material (i.e. transparencies and slides) was used to thoroughly explain to the children the task they would be performing. The main aim of this training session was to make the children fully aware of the role they would undertake by providing them information about determining factors for an objective evaluation of food quality, methods for expressing a reliable judgement and aspects of food hygiene and nutrition (i.e. the importance of carbohydrates, fat and vitamins and, hence, the importance of eating fruit and vege-

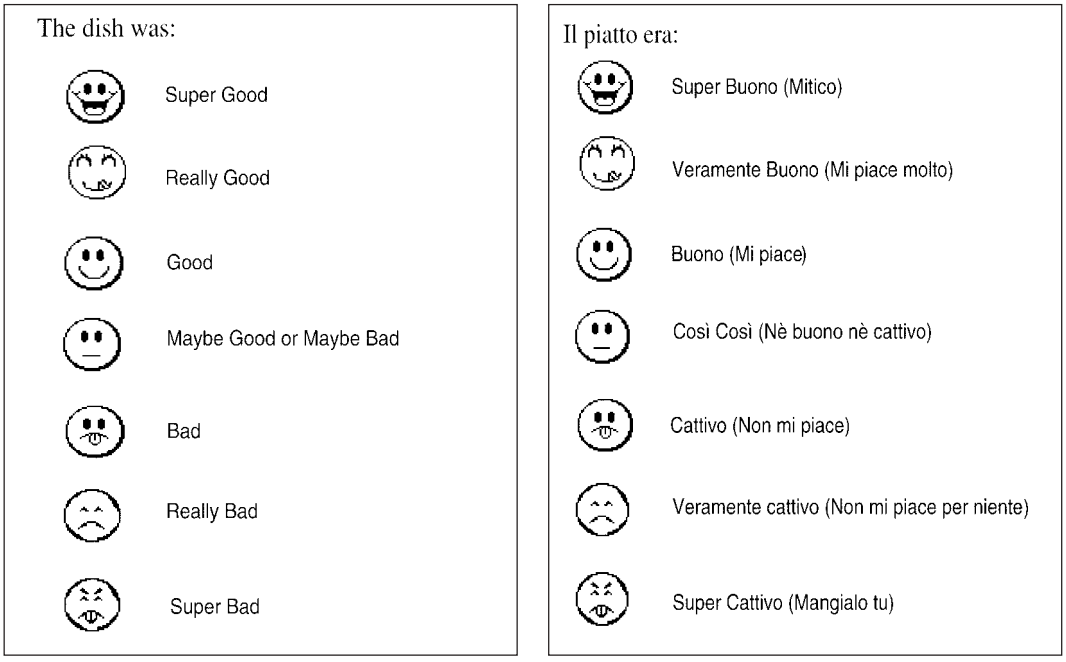

Fig. 1 - An example of the evaluation card used to assess the degree of liking of individual meal formulations.

tables every day). In order to determine whether all the children had understood the instructions, a test trial was carried out on how to fill out the cards.

Methodology

After a thorough investigation on the different ballot types used in other studies (KROLL, 1990; SPAETH *et al.*, 1992; KIMMEL *et al.*, 1994; GUINARD, 2001), a 7-point facial hedonic scale from super good (7) to super bad (1) was chosen to identify grading of the four meal components. An identification card was also provided, as reported in Fig. 1. Children were requested to fill out their identification cards with the date, name, surname, class and school, to write the name of the dish they had just eaten and to mark an X on the face reflecting their evaluation.

The use of facial hedonic scales is popular in determining preferences. These may be simple smiley face scales or depict a popular cartoon character and are primarily intended for use with children and those with limited reading or comprehension skills (RESURRECTION, 1998).

Tests started in May and ended in June with a total of 16 sessions over four successive weeks. On fixed test days, before leaving for lunch, children changed their classroom into a "Testing Laboratory" by rearranging the desks into given intervals, and hanging a "Testing Laboratory" sign poster. This helped the children pay more attention to the lunch they were eating so they were better able to remember their sensations when filling out the evaluation cards. The tasks were performed in a suitable environment, which was less noisy than the school cafeteria, and the children could be checked more easily. The children filled out their evaluation cards approximately 5 min after finishing their lunch. The procedure required 5-10 min, which was then recovered during playtime prior to beginning of teaching activities in the afternoon.

Data analysis

The means of the acceptability from the grading were subjected to the Student's t test to determine whether there were significant differences between formulations for the two schools (R_1 and R_2 for school R and L_1 and L_2 for school L, respectively). Data for acceptability were submitted to one-way Analysis of Variance (Anova) by applying the Least Significance Difference (LSD, $p<0.05$) test to determine whether there were significant differences between formulations. The sources of variations taken into account were four meal components (i.e. first course, second course, vegetables and fruit). The significance of these effects was tested with F tests and LSD by Statgraphics® Plus (version 5) software.

RESULTS AND DISCUSSION

The aim of this work was to study the preferences of 7-8-year-old children for individual dishes of formulations supplied at school. A quantitative method, similar to the Focus Group (YOUNKIN, 1989) or Repertory Grid Method (BAXTER *et al.*, 1998; BAXTER *et al.*, 1999) was applied to evaluate preferences. This choice was also dependent on the fact that qualitative methods (GUINARD, 2001) may be used with older children.

Initially, tests were evaluated to determine whether there were any differences in preferences between the two different classes from each individual school. Table 2 shows the means for acceptability for 16 samples (meal formulations) for grades R_1 and R_2.

From the results in Table 2 it can be seen that there were no differences in any dish between children from R_1 and R_2 ($t_{\text{first course}}$ = n.s.; $t_{\text{second course}}$ = n.s.; $t_{\text{vegetables}}$ = n.s.; t_{fruit} = n.s, $p<0.05$). Among the

first courses, pasta with butter (sample H) was the least liked, while pasta with bolognese sauce (sample O) was the most liked dish; among the second courses, potato omelette (sample O) was one of the least liked, while roast chicken (sample D) was one of the most liked; among the vegetables, carrots (sample H) were the least liked and roast potatoes (sample B) were the most liked. In general the children liked fruit although pear (sample P) received the lowest score and orange (sample O) received the highest score.

Table 3 shows the means of acceptability of 16 samples (meal formulations) of grades L_1 and L_2.

There were no significant differences in any dish between children from grades L_1 and L_2 ($t_{first\ course}$ = n.s.; $t_{second\ course}$ = n.s.; $t_{vegetables}$ = n.s.; t_{fruit} = n.s, $p<0.05$). The grading of the children from school L was very similar to that of children from school R, although the former had a slightly higher score. Among the first courses, vegetable puree (sample M) was the least liked and pasta with bolognese sauce (sample O) was the most liked dish; among the second courses, potato omelette (sample O) was the least liked and roast chicken (sample D) the most liked dish; among the vegetables red salad (sample G) was the least liked and roast potatoes (sample B) were one of the most liked dishes; no significant differences in fruit preferences were found.

As a result, a single mean for school R (i.e. a mean of values from grades R_1 and R_2) and one for school L (i.e. a mean of values from L_1 and L_2) were taken into account to compare the means of acceptability between the two schools (Figs. 2-4). These figures show the means of the scores for different formulations from both schools. A line was drawn at point 4, which marked the "maybe good" or "maybe bad" score (mean data point on the evaluation scale).

Table 2 - Means and calculated value for t ($p<0.05$) for grades R_1 and R_2. Average scaling for grades and 16 meal formulations; within columns, values marked with different letters are significantly different ($p<0.05$).

Primary grades	First course t= 0.06 n.s.	Second course t= 0.26 n.s.	Vegetables t= 0.34 n.s.	Fruit t= 0.71 n.s.
R1	4.42	4.68	4.13	5.09
R2	4.40	4.57	4.26	4.78
Formulations	**First course F= 3.52*****	**Second course F= 6.89*****	**Vegetables F= 7.43*****	**Fruit F= 1.81***
A	4.25 bcd	5.42 de	4.67 efg	5.00 abcde
B	4.14 abc	4.12 bc	5.97 h	4.50 abc
C	4.08 abc	4.36 bc	3.22 ab	5.11 bcde
D	4.14 abc	6.23 e	4.25 cdef	5.17 bcde
E	3.39 ab	3.81 b	3.92 bcde	5.08 abcde
F	3.97 abc	5.31 d	3.28 ab	5.33 cde
G	4.56 cdefg	5.45 de	3.33 abc	5.17 bcde
H	3.34 a	4.03 bc	2.83 a	4.81 abcde
I	4.34 cde	5.33 d	3.70 abcd	5.08 abcde
L	5.12 defg	4.39 bc	4.11 bcdef	4.36 ab
M	4.47 cdef	4.78 cd	3.42 abc	4.53 abcd
N	5.31 fg	5.28 d	5.36 gh	5.33 cde
O	5.39 g	2.92 a	4.47 defg	5.58 e
P	4.31 cd	3.89 b	5.64 h	4.19 a
Q	4.56 cdefg	4.14 bc	5.06 fgh	4.31 ab
R	5.23 efg	4.61 bcd	3.83 bcde	5.42 de

Table 3 - Means and calculated value for t ($p<0.05$) for grades L_1 and L_2. Average scaling for grades and 16 meal formulations; within columns, values marked with different letters are significantly different ($p<0.05$).

Primary grades	First course t= 0.67 n.s.	Second course t= 0.39 n.s.	Vegetables t= 0.98 n.s.	Fruit t= 0.95 n.s.
L1	5.17	5.24	5.09	5.69
L2	4.87	5.06	4.66	5.24
Formulations	**First course F= 3.00*** **	**Second course F= 6.55*** **	**Vegetables F= 8.19*** **	**Fruit F= 0.92 n.s.**
A	5.67 cd	6.08 fg	5.19 efg	5.36
B	4.53 ab	4.83 bcd	6.08 h	5.08
C	4.53 ab	4.17 ab	3.81 b	5.94
D	5.17 bcd	6.50 g	5.08 defg	5.50
E	5.14 bcd	5.17 de	4.56 bcde	5.36
F	4.50 ab	5.69 efg	3.92 b	5.42
G	5.28 bcd	6.11 fg	2.78 a	5.81
H	5.00 bc	5.08 cde	4.19 bc	5.36
I	5.67 cd	5.39 def	5.19 efg	5.50
L	5.06 bc	5.53 def	4.25 bcd	4.89
M	3.87 a	5.22 de	4.97 cdef	5.61
N	4.94 bc	5.19 de	5.94 gh	5.17
O	6.00 d	3.72 a	5.25 efgh	5.53
P	5.64 cd	5.31 def	5.83 fgh	5.56
Q	4.64 ab	4.28 abc	5.42 efgh	5.58
R	4.69 ab	4.11 ab	5.53 fgh	5.83

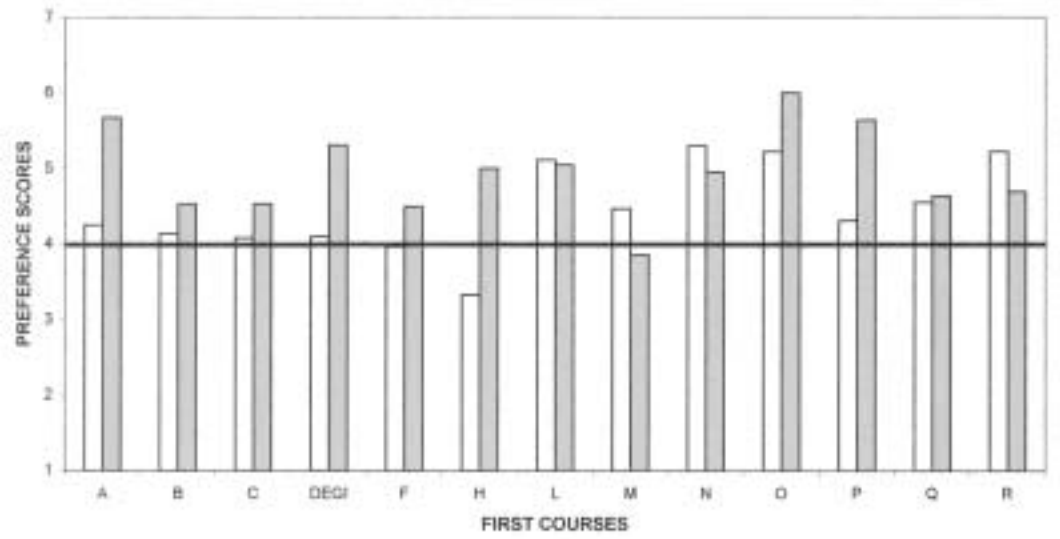

Fig. 2 - Histograms of grading of the first courses with respect to schools R (□) and L (▨). The solid horizontal line indicates "maybe good" or "maybe bad".

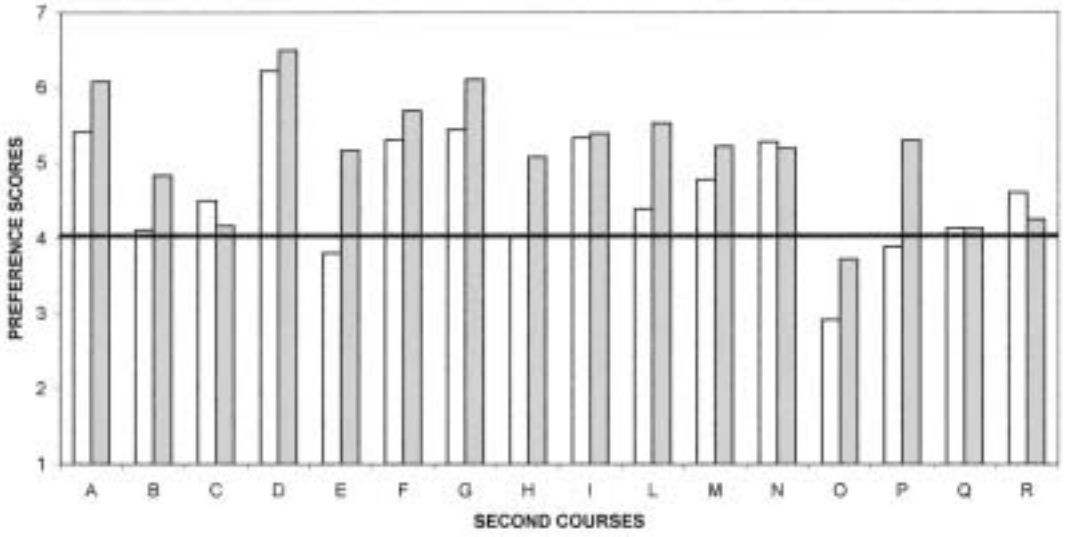

Fig. 3 - Histograms of grading of the second courses with respect to schools R (□) and L (▨). The solid horizontal line indicates "maybe good" or "maybe bad".

From data in Fig. 2 it can be observed that for most first courses, the preference scores were higher than 4. This means most of the formulations were appreciated, particularly, ravioli with spinach and ricotta cheese-filling (sample L), risotto with parmesan cheese (sample N) and pasta with bolognese sauce (sample O) for both schools. In order to facilitate a comparison between formulations D, E, G and I, corresponding to pasta with tomato sauce eaten on four different days, a mean

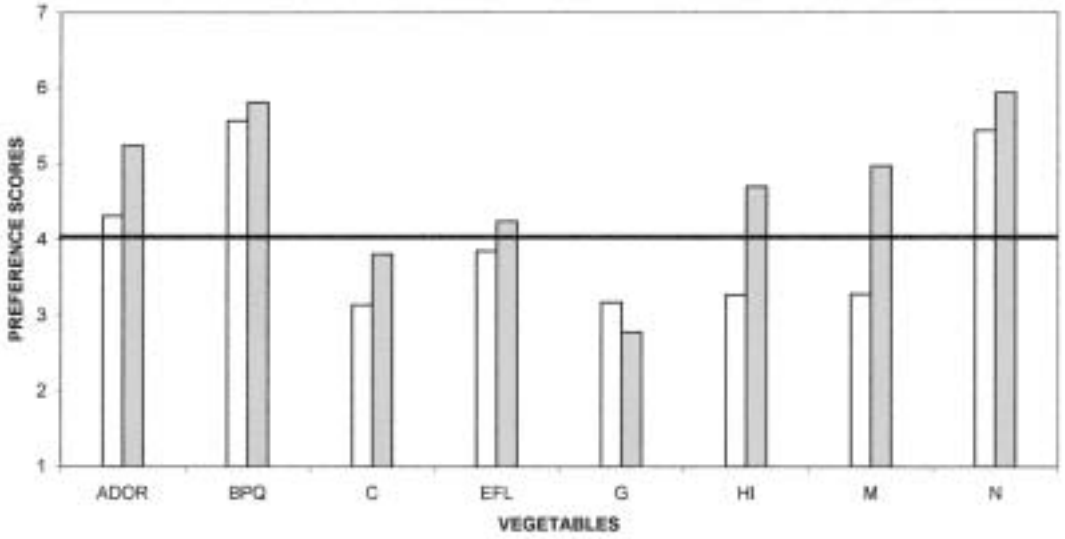

Fig. 4 - Histograms of grading of the vegetables with respect to schools R (□) and L (▨). The solid horizontal line indicates "maybe good" or "maybe bad".

was first calculated, and then all of the formulations were evaluated together. By applying the Student's t test, no significant differences were found in the evaluation of first courses between the two schools (t_A = 1.11 n.s., t_B = 0.35 n.s., t_C = 0.39 n.s., t_{DEGI} = 2.01*, t_F = 0.47 n.s., t_H = 1.47 n.s., t_L = 0.04 n.s., t_M = 0.54 n.s., t_N = 0.27 n.s., t_O = 0.56 n.s., t_P = 1.05 n.s., t_Q = 0.07 n.s., t_R = 0.53 n.s., $p<0.05$), except for pasta with tomato sauce.

From the data in Fig. 3, it can be seen that for the second courses, homogeneous preferences were obtained at the two schools (t_A = 0.47 n.s., t_B = 0.63 n.s., t_C = 0.17 n.s., t_D = 0.18 n.s., t_E = 1.16 n.s., t_F = 0.28 n.s., t_G = 0.47 n.s., t_H = 0.88 n.s., t_I = 0.04 n.s., t_L = 0.90 n.s., t_M = 0.34 n.s., t_N = 0.06 n.s., t_O = 0.87 n.s., t_P = 1.18 n.s., t_Q = 0.00 n.s., t_R = 0.31 n.s., $p<0.05$).

The second courses were appreciated, except for potato omelette (sample O) and pork loin with lemon sauce (sample Q) for both schools.

From the data in Fig. 4 it can be seen that for vegetables very homogeneous preferences were also obtained for the two schools (t_{ADOR} = 1.53 n.s., t_{BPQ} = 0.28 n.s., t_C = 0.67 n.s., t_{EFL} = 0.63 n.s., t_G = 0.46 n.s., t_{HI} = 1.88 n.s., t_M = 1.52 n.s., t_N = 0.35 n.s., $p<0.05$). In this case, most formulations were tasted several times. Hence, for repeated evaluations, a mean was calculated to facilitate comparisons. For both schools french beans (sample C), boiled potatoes (sample EFL) and red salad (sample G) were particularly disliked.

Finally, data on fruit were not included because no significant differences in the children's preferences were found.

CONCLUSIONS

Research with children poses some of the most difficult problems in sensory analysis and product testing. Although it is not easy to evaluate children's reactions to a product, a consistent model to test children's preference for school lunches has been proposed in this preliminary study. From the results it can be inferred that 7-8-year-old children were able to use evaluation cards and express a judgement on different components for meals supplied to school refectories. The present method provides an easy means for determining the eating habits of Italian children and their preferences.

With reference to the formulations tested, among the first courses ravioli with spinach and ricotta cheese-filling, risotto with parmesan cheese, pasta with bolognese sauce were most liked; among vegetables, potato puree and roast potatoes were most liked; among the second courses potato omelette and pork loin with lemon sauce were the most disliked. No significant differences were found in the 16 formulations tested between school R and school L.

This study was not intended to provide an absolute assessment. Rather, it was intended to provide a foundation for further studies about children's preferences for school lunches in Italy to evaluate whether there are any differences based on age, sex and meal combination. The school is still a privileged place where the health and psychological conditions of children can be valued. School lunches, when used properly, may represent an important positive step of education.

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PAPER

DEEP-FRYING OF CHICKEN MEAT AND CHICKEN-BASED PRODUCTS. CHANGES IN THE PROXIMATE AND FATTY ACID COMPOSITIONS

MODIFICHE NEL CONTENUTO DEI COMPONENTI DI BASE E NEGLI ACIDI GRASSI NEL CORSO DELLA FRITTURA DI CARNE DI POLLO E PRODOTTI A BASE DI POLLO

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ABSTRACT

The aim of this study was to determine the proximate and fatty acid composition of chicken meat (C), chicken burgers (B) and chicken-sausages (S) and the changes that they underwent after being fried in olive oil (CO, BO, and SO, respectively) or in sunflower oil (CS, BS, and SS, respectively). The changes in composition were related to temperature changes in the oil and in the food. Chicken-based products contained significantly less water and protein and more fat and ash than the

RIASSUNTO

Scopo di questo studio è la valutazione dei componenti di base e degli acidi grassi di carne di pollo (C) burger di pollo (B) e salsicce di pollo (S) e delle loro modificazioni a seguito della frittura in olio di oliva (CO), (BO) e (SO) od in olio di girasole (CS), (BS) e (SS). I cambiamenti di composizione osservati sono correlati alle variazioni di temperatura dell'olio e degli alimenti. I prodotti a base di pollo contengono significativamente meno acqua e proteine e più grasso e ceneri delle carni di pollo. I carboidrati sono pratica-

- Key words: chicken, chicken-based products, chicken burger, fat, fatty acids, pan-frying, proximate composition, sausage -

chicken meat. Carbohydrates were almost null in C, ~9% in B, and ~2% in S. True retentions of protein and ash decreased in CO but increased in BO and BS samples. Frying increased the fat content in C and B samples but it decreased in S. Fat increase was higher in C and B samples when frying with sunflower oil. These fat-changes seem related to the water loss that changed the temperature of the oil and to the structure of the food product. Fatty acid percentage of the raw products (B and S) did not differ significantly from that of C. Frying with olive oil increased the percentage of oleic acid in CO very significantly and frying with sunflower oil that of linoleic acid in CS. These increases were higher in B than in S samples. Labeling of chicken-based-products should include the fat content and fatty acid composition. Final content in fat and fatty acids in chicken and in fried chicken-based products depends on (i) the fat content of the raw product, (ii) the presence of an edible coating which limits the penetration of fat during frying, and (iii) the composition of the oil used.

mente assenti in (C), ca. 9% in (H) e ca. 2% in (S). La ritenzione delle proteine e delle ceneri diminuisce in (CO) ma aumenta in (BO) e (BS). La frittura aumenta il contenuto in grassi in (C) ed (B) ma ne diminuisce il contenuto in (S). L'aumento in grassi è maggiore in (C) ed (B) quando fritti con olio di girasole. Questi cambiamenti nel contenuto in grassi sembrano correlati alla perdita di acqua che fa variare la temperatura dell'olio ed alla struttura del prodotto. Il contenuto percentuale in acidi grassi dei prodotti crudi (B ed S) non differiscono in modo significativo da quello di (C). La frittura con olio di oliva aumenta la percentuale di acido oleico in (CO) in modo molto significativo, mentre la frittura con olio di girasole aumenta il contenuto in acido linoleico in (CS). Questi aumenti sono più alti in (B) che in (S). Si consiglia che l'etichetta dei prodotti a base di pollo indichi il contenuto in acidi grassi ed in grasso totale. Il contenuto finale in grassi ed acidi grassi nel pollo fritto e nei prodotti fritti dipende da: a) il contenuto in grassi del prodotto crudo, b) la presenza di un rivestimento edibile che limiti la penetrazione del grasso durante la frittura e c) la composizione dell'olio utilizzato.

INTRODUCTION

Frying is a culinary method widely used in food preparation. It is a complex process, since many factors are at work. Some of them depend on the process itself and others on the food and the type of fat used (VARELA, 1988; CUESTA and SÁNCHEZ-MUNIZ, 2001).

The use of the domestic fryer and/or pan-frying has spread to all parts of the world because this method of food preparation is carried out in a short time and gives appealing characteristics to the food (ROMERO *et al.*, 2001; VARELA and RUIZ-ROSO, 2000). Despite potato chips being the leading fried product, a large number of other foods (e.g. chicken and chicken-based products) are prepared by frying.

During frying, the fat or oil acts as a heat transfer medium and becomes an important ingredient of the fried food because water loss as well as penetration of oil into the food takes place (SÁNCHEZ-MUNIZ *et al.*, 1992; CASTRILLÓN *et al.*, 1997; VARELA and RUIZ-ROSO, 2000). Modifications (losses and gains) in the nutritional value of the final products have been described (FILLION and HENRY, 1998; BOGNÁR, 1998). Moreover, undesirable changes might occur during frying including thermal oxidation (BASTIDA and SÁNCHEZ-MUNIZ, 2001; ROMERO *et al.*, 2000 a) and losses

of some nutrients such as essential fatty acids (ROMERO *et al.*, 2000 b; SÁNCHEZ-MUNIZ *et al.*, 1994), minerals, and vitamins (FILLION and HENRY, 1998). Nevertheless, according to BOGNÁR (1998) the losses of some vitamins when frying are not greater than those produced by stewing or by other cooking methods. VARELA and RUIZ-ROSO (2000) showed that the fat content of different foods (e.g. hake and minced meat) was lower after frying than after stewing.

Consumers are becoming more concerned about eating healthy diets with a limited amount of dietary fat and cholesterol and increased amounts of vitamins and minerals. Because of that, chicken has become one of the most preferred foods. Out of 115 foodstuffs which provide 95% of the total energy consumed in Spain, chicken occupies the fifth place with an average consumption of 58 g per person per day (INE, 1995).

This high level of consumption is partly due to its pleasing texture and partly to its high nutritional value which is why it is recommended for specific population groups including children, convalescents and elderly people (GRANDE-COVIÁN and VARELA-MOSQUERA, 1991; FARRÉ ROVIRA and FRASQUET PONS, 2001).

The energy and nutrient composition of chicken meat (breast and leg) have been reported in many Food Composition Tables (MATAIX *et al.*, 1995; MOREIRAS *et al.*, 1998; SOUCI *et al*, 1994; HOLLAND *et al.*, 1992) and in many review papers (GARCÍA de FERNANDO and SANZ-PÉREZ, 1986; PÉREZ-LLAMAS *et al.*, 1997). These data show chicken to be a meat with a low energy content due to its low fat content (3-5%), while maintaining a high protein content (20%) and a very low quantity of carbohydrates in comparison with other types of meat. On the other hand, it is fairly rich in vitamins and minerals (MATAIX *et al.*, 1995; MOREIRAS *et al.*, 1998; SOUCI *et al.*, 1994; HOLLAND *et al.*, 1992).

Information on the nutrient content of meat-based products (sausages and hamburgers) is available in the bibliography (MOREIRAS, *et al.*, 1998; SOUCI *et al.*, 1994; HOLLAND *et al.*, 1992). However, frequently they do not specify from which type of meat the products were made. Since meat is the main component of these products, the final composition not only depends on the type and nutritional quality of the meat used as raw material but also on the variety, quality, proportion of the ingredients added in order to obtain the final product (e.g. carbohydrates, salt, and fat) and the technology used. It must, also be taken into account that the main changes in both the content and composition of food occur during industrial and culinary processing (GALL *et al.*, 1983; MAI *et al.*, 1978; GARCÍA-ARIAS, 1989). Physicochemical changes produced in oils have been subject to intensive investigations in the last decades; however, few nutritional studies have focused on the nutritional properties of fried foods and even fewer on those of fried chicken-based products.

In order to contribute new reliable data about fried foods (e.g. chicken-based products) not yet included in food composition tables, this study was conducted: (i) to determine how deep-fat frying In a pan with olive oil or sunflower oil affects the proximate and fatty acid composition of both chicken meat and chicken burgers and sausages; (ii) to compare fatty acid changes induced by frying of the foodstuffs with the fatty acid composition of the frying oil; (iii) to relate those changes with those of food and oil temperatures.

MATERIALS AND METHODS

Material and reagents

Olive oil with an acidity value of 0.4 and refined sunflower oil were purchased at a local store. The choice of

such fats was based on their fat composition (olive oil being very high in monounsaturated fatty acids (MUFA) and sunflower oil being high in polyunsaturated fatty acid (PUFA)). Chicken meat and chicken-based products (chicken burgers and sausages) were supplied by the largest local food company in León, Spain (Huevos León). Boron trifluoride-methanol complex (20% solution in methanol), hexane, chloroform, methanol, sodium chloride, and petroleum ether were purchased from Merck AG (Darmstadt, Germany). Chromatographic standards were obtained from Sigma (St. Louis, MO). Chicken breasts and legs (50:50) were deboned and the resulting meat was minced to obtain about 1 kg of a homogeneous sample (C). Raw sausages (1 kg) and raw burgers (1 kg) were separately minced to obtain S and B samples, respectively. Six aliquots from each pooled chicken (C), chicken sausages (S), and chicken burgers (B) were analyzed raw.

The geometrical size of the chicken meat and chicken-based products ready to fry was for chicken burgers: raw-weight 103.05±8.20 g; thickness 0.72±0.17 cm; olive oil fried-weight 81.66±11.12 g; sunflower oil fried-weight 88.96±6.55 g; and for sausages: raw-weight 43.79±18.34 g; length 15.11±1.09 cm; thickness 1.45±0.06 cm; olive oil fried-weight 37.59±15.52 g; sunflower oil fried-weight 38.22±16.23 g.

Methods

About 1 kg of pooled chicken and 1 kg of each chicken-based product were fried in olive oil (CO, SO, and BO, respectively) and in sunflower oil (CS, SS, and BS, respectively). Frying was performed on an electric stove at 180°C in domestic stainless steel-teflon coated pans with a 2L-capacity. Culinary fats were used once with the food/oil ratio being 250 g/500 mL. The frying lasted 6 min for C samples, 4 min for S, and 4 min for B. Oil and food-core temperatures were checked using a quartz electronic thermometer (HUGER, Oregon Scientific Trade Mark, Villingen-Schewenningen Germany) during frying. Once fried, samples were drained for about 2 min, homogenized, and the water content was determined immediately. The rest of the samples were freeze-dried and kept at -20°C under nitrogen atmosphere until analyses. Determinations were performed on six aliquots of C-, S-, and B-fried samples.

Moisture was determined by drying the samples at 100°C to constant weight (AOAC, 1993). Protein was determined by the Kjeldahl procedure (AOAC, 1993).

Total fat was determined by extracting samples (0.5 g) with petroleum ether (BP 40°-60°C, Soxtec System 1040 Tecator, Sweden).

Ash was determined by the AOAC method 310.12; (1993), heating dried samples in a muffle furnace at 500°C.

Carbohydrates were determined by subtracting the sum of moisture, protein, fat and ash percentages from 100%.

Fatty acid composition. Under nitrogen atmosphere, food-fat was extracted with chloroform-methanol according to the method of BLIGH and DYER (1959), saponified for 30 min at 60°C with 0.5 N sodium hydroxide, and then methylated for 60 min with boron-trifluoride at 90°C following a modification of the method of METCALFE *et al.* (1966) as stated by SANCHEZ-MUNIZ *et al.* (1992). The fatty acid methyl esters were analyzed by gas chromatography in a Hewlett Packard 6890 chromatograph equipped with a capillary column Omegawax 250 (30 m x 0.25 mm x 0.25 m) (Supelco, Barcelona, Spain). The oven was temperature programmed from 150° to 200° at 10°C/min, and then programmed from 200° to 240° at 1°C/min until completion of the analyses.

Split 50:1 injection was used and the injection port and detector were maintained at 200° and 300°C, respectively. Helium was the carrier gas. The fatty acids were identified by comparing their relative and absolute retention times with commercial standards (Lipid Standard Fatty Acids Methyl Ester Mixture 189-19. Sigma) analyzed under the same experimental conditions. The different amounts of fatty acids were calculated on the basis of percentage in the fat and the proportion of fat in the food, using the conversion factors suggested by HOLLAND *et al.* (1992): 0.956 for the oils, 0.945 for raw chicken, burgers, and sausages, and of those previously obtained in our laboratory (0.950 for fried chicken, fried burgers and fried sausages).

True nutrient retention (%) of moisture, protein, fat, ash, and major fatty acids was calculated according to BOGNAR (1998) by applying the following equations:

$$\text{Weight yield} = (V/U)*100=d;$$
$$\text{Nutrient retention (\%)} = (Z/X)* d$$

Where:
V = weight of food after cooking in g; U = weight of food before cooking in g; Z = content of nutrient per 100 g of cooked food; X = content of nutrient per 100 g of raw food.

The data were analyzed using one-way analysis of variance (ANOVA) followed by the NEWMAN-KEULS test. Significance was established at the $p<0.05$ level.

RESULTS AND DISCUSSION

Temperature changes

Figs. 1 to 3 show the temperature changes in the oil and in the core of the three food samples. During the first min oil temperature decreased at a different rate in the three samples with B samples having the greatest decrease (about 30°C). However, after 3 min, oil temperature changes were similar in oils used for frying S and B samples. Sunflower oil in the pan tended to have lower temperatures than olive oil. Moreover, during frying, the temperature of the oil remained below 150º C for almost the entire process. These results are different from those found in frying sardines (SÁNCHEZ-MUNIZ *et al*, 1992) in which the olive oil bath temperature was lower than the sunflower oil. These differing results could be related to the different frying methods used (domestic fryer *vs*. pan), the amount of food fried (250 g *vs*. 500 g), and the food/oil ratio used (250 g/500 mL oil *vs*. 500 g/3 L).

The decrease in the oil temperature should first be related to the initial water content and the water lost by the samples (CUESTA and SÁNCHEZ-MUNIZ, 2001; VARELA, 1988). According to the temperature profiles of both oils, changes in the water content of C samples were quite similar (Fig. 1b), but the water loss seemed higher in B and S samples when frying with sunflower oil (Figs. 2b and 3b).

The food-core temperature increased during frying. However, the end temperature was the lowest in C (70°-85°C), medium in B (81°-92°C) and highest in S (100°C) (Figs. 1a–3a). It is interesting to observe that the core temperature did not surpasses 100°C during frying. The reason why the temperature inside the food did not go above 100°C is due to water evaporation. According to CUESTA and SÁNCHEZ-MUNIZ (2001) and VARELA (1988), the fat does not begin to penetrate the food until a substantial amount of the water it contains has evaporated. Because the hot fat acts on a large part of the food for a very short time, it can be thought that the nutritional damage should be small.

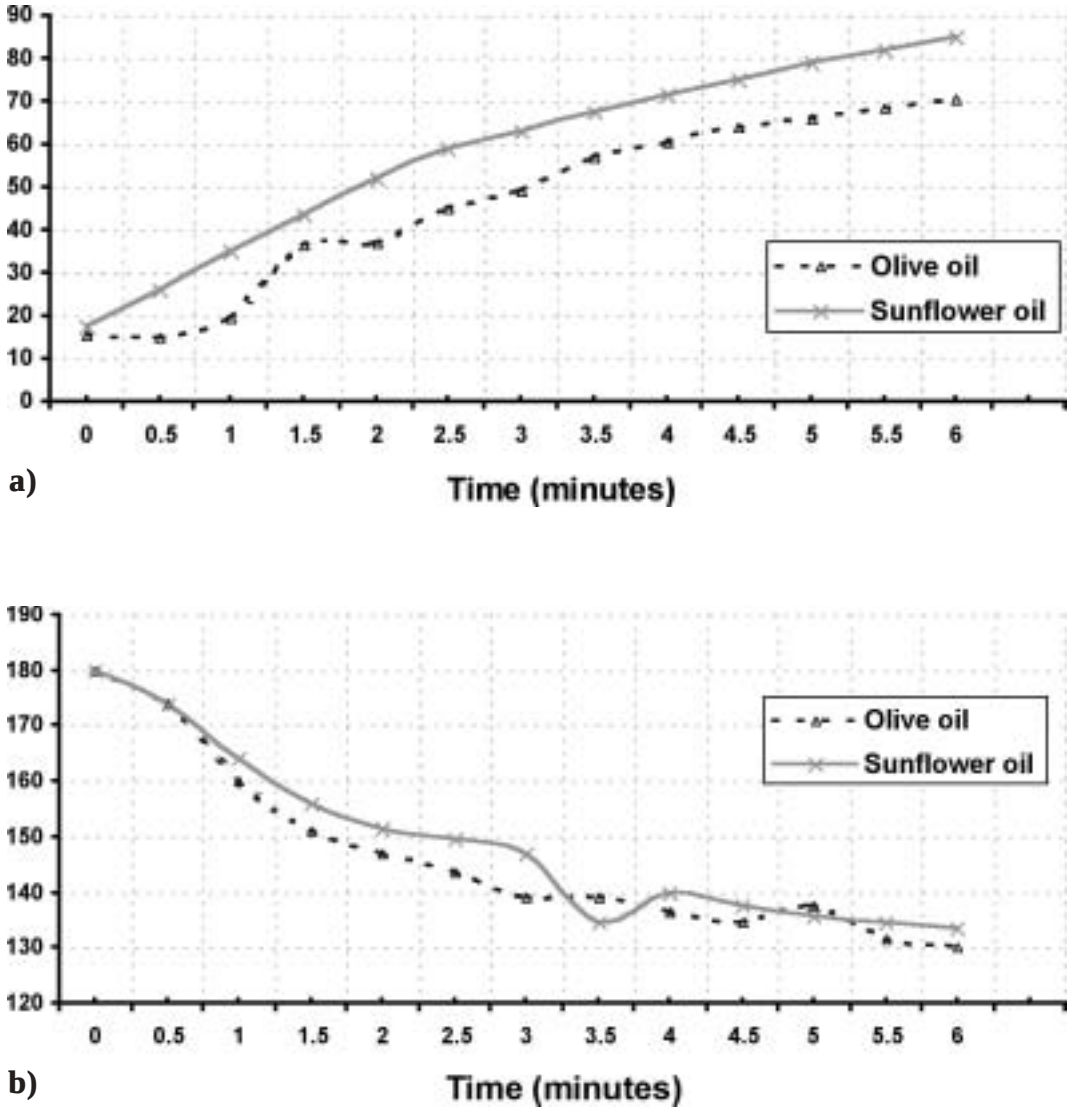

Fig. 1 - Time-temperature changes during pan-frying of chicken.
a - Food-core temperature change. b - Frying oil temperature change.

Proximate composition changes

Table 1 shows the proximate composition of the raw samples: C, B and S. Moisture and protein were higher, while fat and ash were lower in C samples. These differences should be related to the addition of fat to the original chicken meat to obtain B and S samples. Differences were also relevant when data were expressed on a dry matter basis. These results are in agreement with

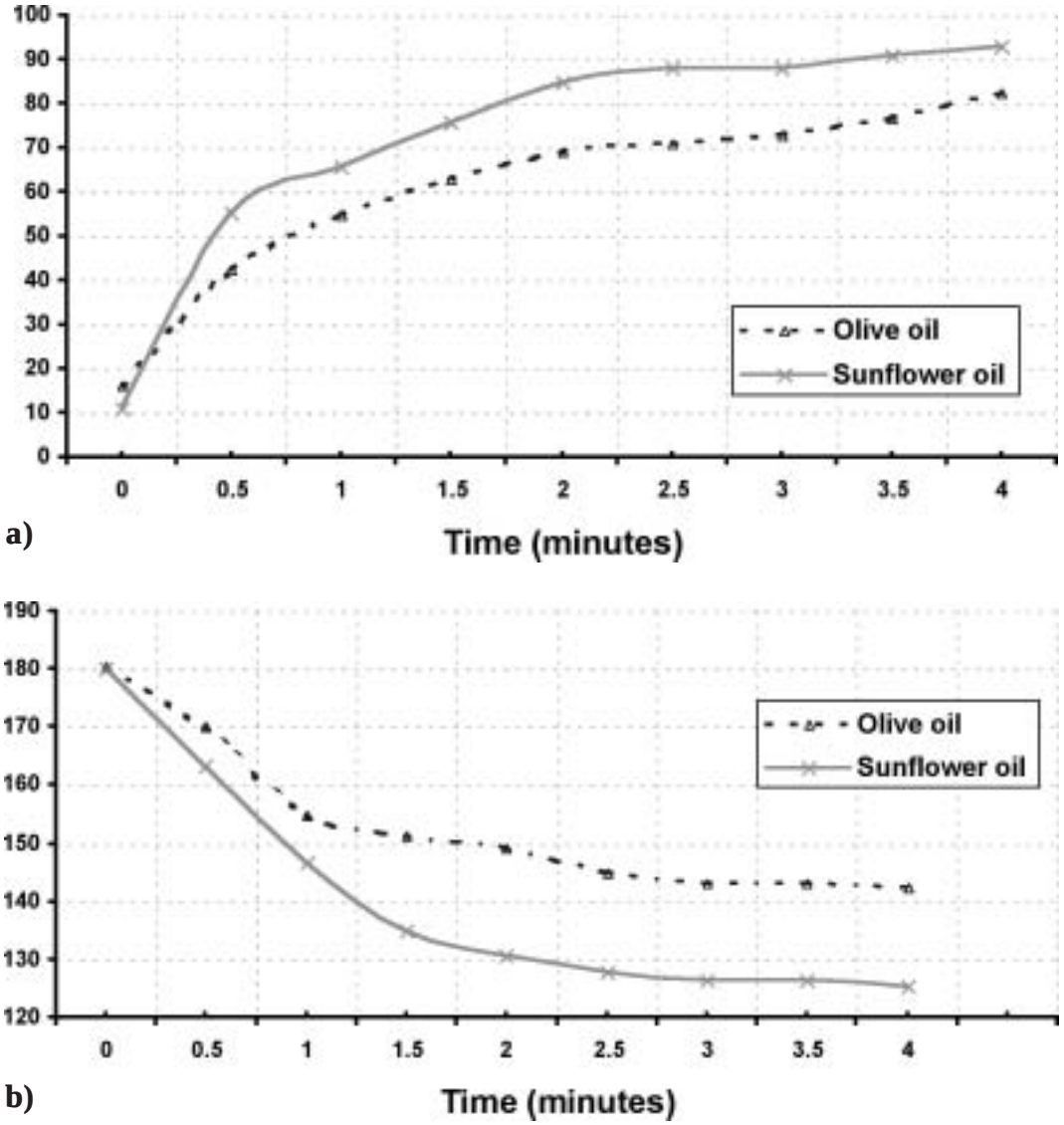

Fig. 2 - Time-temperature change during pan-frying of chicken burgers.
a - Food-core temperature change. b - Frying oil temperature change.

those found in the Food Composition Tables (MOREIRAS *et al.*, 1998; SOUCI *et al.*, 1994), where it can be observed that meat-based products contain a higher amount of fat than the base meat. Enrichment with fat increases the palatability (CUESTA and SÁNCHEZ-MUNIZ, 2001) and makes the resulting product less expensive. The higher ash content in B and S *vs*. C was due to the salt added during the preparation of the chicken-based products. In fact the amount

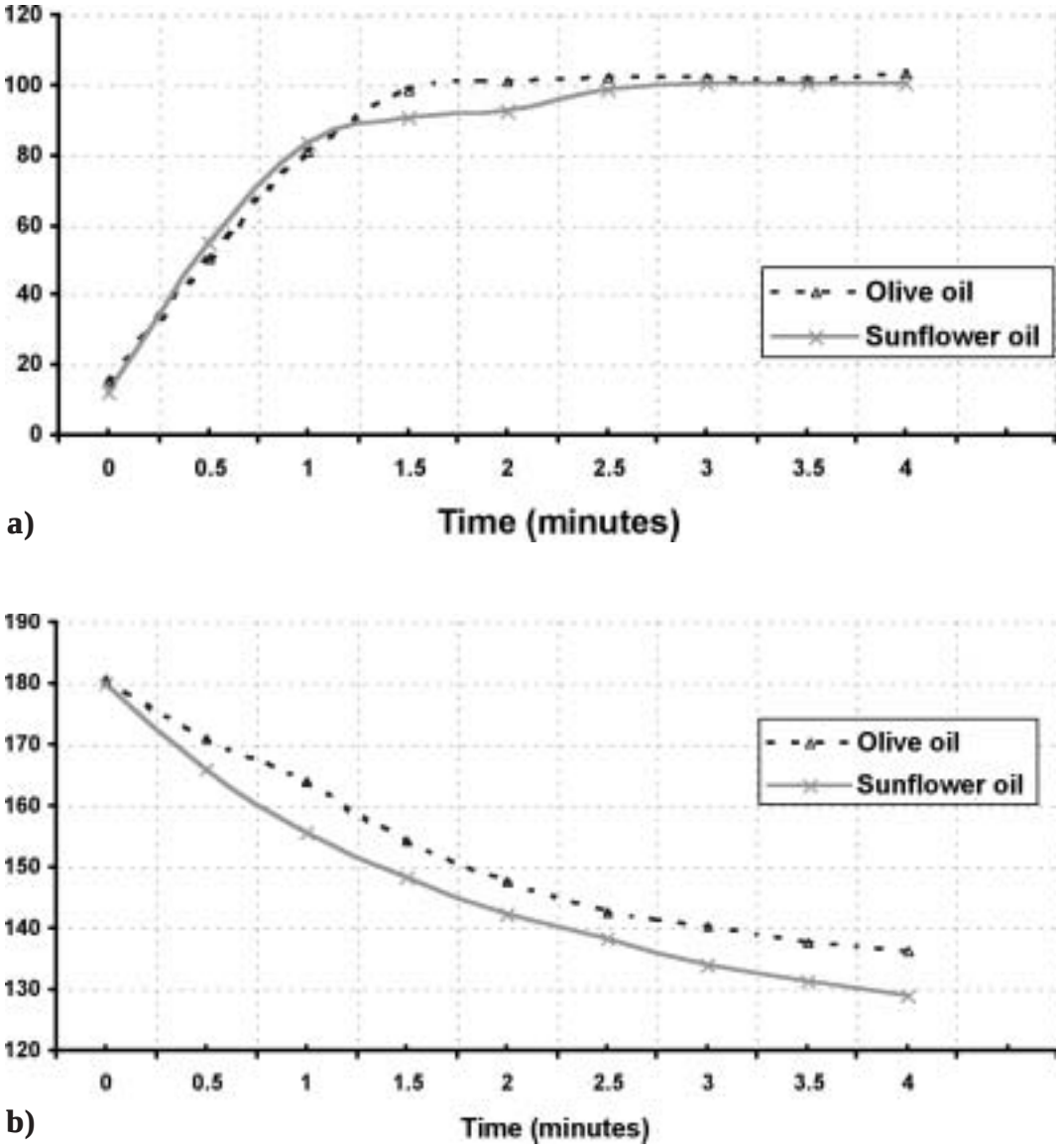

Fig. 3 - Time-temperature change during pan-frying of chicken sausages.
a - Food-core temperature change. b - Frying oil temperature change.

of Na+ (mg/100 g edible food) was significantly higher ($p<0.001$) in B (501.8±61.7) and in S (589.3±20.9) *vs.* C (87.3±20.7).

Table 1 shows the changes in water, fat, protein, ash, and carbohydrates of the various fried samples. When C and B samples were fried (in olive or sunflower oils), the water content fell significantly, which caused the amounts of protein, fat and ash to increase significantly. Similar results were found by

Table 1 - Proximate composition (g/100 g edible portion) of raw chicken (C), chicken burgers (B) and chicken sausages (S) and after frying them in olive oil (CO, BO, and SO, respectively) and in sunflower oil (CS, BS and SS, respectively).

	Moisture	Protein	Fat	Ash	Carbohydrates*	Protein	Fat	Ash	Carbohydrates*
	Wet matter basis					Dry matter basis			
C	74.09±0.97 ^{aA}	21.6 8±0.19 ^{aA}	3.11±0.10 ^{aA}	1.12±0.10 ^{aA}	0.00	83.69±0.75 ^{aA}	11.99±0.37 ^{aA}	4.32±0.37 ^{aA}	0.00
CO	68.14±0.60 ^{bA}	25.26±0.14 ^{bA}	4.45±0.22 ^{bA}	1.1 6±0.16 ^{aA}	0.99	79.28±0.45 ^{bA}	13.96±0.69 ^{bA}	3.65±0.52 ^{bA}	3.11
CS	66.08±0.56 ^{cA}	26.75±0.24 ^{cA}	5.05±0.05 ^{cA}	1.35±0.04 ^{bA}	0.77	78.87±0.72 ^{bA}	14.88±0.16 ^{cA}	3.99±0.11 ^{bA}	2.26
B	62.22±2.38 ^{aC}	13.56±1.64 ^{aB}	12.96±1.14 ^{aC}	2.11±0.26 ^{aC}	9.19	35.88±4.33 ^{aB}	34.30±3.03 ^{aC}	5.60±0.69 ^{aC}	4.28
BO	54.50±1.11 ^{bC}	19.84±0.72 ^{bC}	17.02±0.62 ^{bC}	4.02±0.35 ^{bB}	4.62	43.60±1.59 ^{bB}	37.40±1.37 ^{abB}	8.83±0.90 ^{bB}	10.17
BS	57.59±0.55 ^{cC}	18.48±0.42 ^{cB}	16.74±0.53 ^{bC}	3.72±0.42 ^{cC}	3.47	43.57±0.99 ^{bB}	39.47±1.25 ^{bB}	8.77±0.99 ^{bC}	9.19
S	63.98±0.56 ^{aB}	14.59±1.22 ^{aB}	16.97±0.24^{aB}	2.72±0.30 ^{aB}	1.74	40.50±3.40 ^{aB}	47.11±0.66 ^{aB}	7.56±0.85 ^{abB}	4.83
SO	59.04±0.95 ^{bB}	18.10±0.76 ^{bB}	15.92±0.48 ^{bB}	3.54±0.25 ^{bB}	3.40	44.20±1.86 ^{bB}	38.87±1.18 ^{bB}	8.65±0.61 ^{aB}	8.28
SS	58.49±0.40 ^{bB}	17.22±1.00 ^{bB}	16.13±0.29 ^{bB}	2.83±0.36 ^{aB}	5.33	41.48±2.41 ^{bB}	38.85±0.71 ^{bB}	6.82±0.88 ^{bB}	12.95

Each value is the mean±SD of six determinations. *Calculated by difference. Values in the same column for the same food (raw or fried) bearing different small letters are significantly different at p<0.05. Values in the same column for raw or for samples fried in the same oil bearing different capital letters are significantly different at p<0.05.

CASTRILLÓN *et al.* (1997) in frying sardines. However, a different behavior was observed in S samples because the fat content did not change. Differences in moisture between CO and CS were significant with chicken water losses being lower when fried in olive oil than in sunflower oil. However, the temperature of both oils changed in a similar manner (Fig. 1b).

When data were expressed on a dry matter basis (Table 1), protein and ash fell significantly in C samples. This may be due to fat enrichment but leaching and dripping could also have occurred. Similar results were obtained for protein and ash in CO but not in CS samples, by using the BOGNÁR (1998) nutrient retention formula. BOGNÁR (1998) observed that the decrease in retention of protein after roasting of meat and fish occurred mainly due to leaching and dripping. MOREIRAS-VARELA *et al.* (1988) indicated that the variations in the mineral composition of fish due to frying are rather small. Lipid enrichment was greater in CS (Tables 1 and 2) – which is in agreement with the higher core-temperature of this sample with respect to the CO sample (Fig. 1a).

Frying burgers resulted in an increase in fat, protein and ash and a decrease in the water content (Table 1). The amount of fat on wet and dry matter basis (Table 1) and nutrient retention (Table 2) was similar in BO and BS, a fact that cannot be related to the higher core-temperature of BS (Fig. 2a), although it tended to absorb more fat. Moreover, the loss of water in burgers was higher when fried in olive oil than in sunflower oil and it was not related to changes in the oil temperature which was higher in olive oil.

The loss of water and the increase in protein were similar in SO and SS (Tables 1 and 2). However, surprisingly, the fat content (on dry matter basis, Table 1; or in terms of nutrient retention, Table 2) markedly decreased because of frying. This suggests that when a sam-

Table 2 - Weight yield and nutrient retention after frying samples in olive oil (CO, BO, and SO, respectively) and sunflower oil (CS, BS and SS, respectively).

	Weight yield (%)	% Nutrient retention		
		Protein	Fat	Ash
CO	79.49	92.6±0.52 ^{aA}	113.67±5.64 ^{aA}	82.68±11.65 ^{aA}
CS	81.90	101.06±0.92 ^{bA}	132.85±1.38 ^{bA}	98.57±2.80 ^{aA}
BO	79.24	115.92±4.21 ^{aB}	104.04±3.82 ^{aA}	118.66±8.01 ^{aB}
BS	86.33	117.63±2.67 ^{aB}	111.49±3.55 ^{aB}	152.26±17.16 ^{bB}
SO	85.60	106.23±4.46 ^{aB}	80.31±2.41 ^{aB}	111.53±7.97 ^{aB}
SS	88.42	104.32±6.10 ^{aA}	84.01±1.54 ^{aC}	92.00±11.94 ^{aA}
ANOVA		***	***	***

Values in the same column for the same food bearing different small letters are significantly different at $p<0.05$. Values in the same column for different foods that have been fried in the same oil bearing different capital letters are significantly different at $p<0.05$.

ple has a high fat content, such as sausages, frying does not increase its fat content. MAI *et al.* (1978), in a study on fresh water fish with a different fat content, found that the higher the fat content in the fish, the fewer were the lipid changes during frying. In contrast, fish that have a low fat content absorb more fat. CASTRILLÓN *et al.* (1997) found that during frying there was a decrease in the fat content of sardine fillets which was only shown when expressing the data on a dry matter basis. It is also interesting to note that the ash content was higher in the SO samples than in the SS samples, suggesting that with a similar water loss the mineral retention differed.

Fatty acid composition

Table 3 shows the fatty acid composition of raw and fried chicken and chicken-based products. The fatty acid composition slightly differed among the C, B and S samples; however, B samples contained slightly more SFA and more PUFA, and less MUFA than C and S samples. The amount of arachidonic, eicosapentaenoic and docosahexaenoic acids was rather low in all the samples. Moreover, n-6 and n-3 PUFA tended to decrease with frying. Previous studies (SANCHEZ-MUNIZ *et al.*, 1992) have shown that these long chain fatty acids decreased during repeated frying. However, the PUFA + MUFA / SFA (P+M/S) ratio was not different in the three raw samples. This means that the fat used in the S samples had a similar fatty acid profile as the C samples with only myristic, palmitic, stearic, palmitoleic, oleic and eicosatrienoic acids being significantly different. However, B samples differed in all the fatty acids except for lauric, pentadecanoic, 18:1 n-7 and arachidonic acids with respect to the C samples.

Adipose tissue from different body parts differs in fatty acid composition (PINNEY *et al.*, 1994), with abdominal adipose tissue having a significantly higher percentage of SFA and lower MUFA than subcutaneous adipose tissue. Thus, it can be suggested that fat from a peripheral location was used to make the B samples. Nevertheless, according to the data (Table 1) carbohydrates were also added to the B samples. Flour contains 1-3 g fat / 100 g edible portion (MOREIRAS *et al.*, 1998) with a higher percentage of PUFA than MUFA and SFA, partially explaining the higher percentage in B samples than in C and S samples.

Table 3 - Fatty acid composition (g/ 100 g total fat) of raw chicken (C), sausages (S) and burgers (B) fried in olive oil (CO, BO, and SO, respectively) and in sunflower oil (CS, BS, and SS, respectively).

Fatty Acid	C	CO	CS	B	BO	BS	S	SO	SS	Olive oil	Sunflower oil
C12:0	0.11±0.02^{aA}	-	0.27±0.05^{bA}	0.11±0.01^{aA}	-	0.33±0.01^{bA}	0.07±0.00^{abA}	0.16±0.01^{aB}	0.05±0.01^{bB}	-	-
C14:0	0.92±0.02^{aA}	0.84±0.13^{aA}	0.75±0.01^{bA}	1.08±0.01^{aB}	0.84±0.03^{bA}	0.84±0.03^{bB}	1.03±0.03^{aB}	1.03±0.03^{aB}	1.03±0.09^{aC}	-	-
C15:0	0.13±0.01^{A}	-	-	0.12±0.02^{aA}	-	0.42±0.04^{b}	0.14±0.01^{A}	-	-	-	-
C16:0	25.85±0.06^{aA}	20.82±0.18^{bA}	18.34±0.06^{cA}	24.46±0.08^{aB}	23.92±0.07^{aB}	22.50±0.08^{aB}	25.04±0.14^{aC}	24.88±0.04^{aC}	24.15±0.08^{aB}	13.29±0.15	6.61±0.01
C17:0	-	-	-	0.27±0.01^{a}	0.20±0.01^{b}	0.11±0.01^{c}	-	-	-	-	-
C18:0	7.52±0.05^{aA}	6.02±0.05^{bA}	5.79±0.08^{cA}	8.87±0.03^{aB}	7.48±0.06^{bB}	8.85±0.20^{aB}	8.35±0.14^{aC}	7.79±0.08^{bC}	8.03±0.08^{bC}	2.48±0.03	3.81±0.03
C20:0	-	-	-	-	-	-	-	-	-	0.43±0.02	0.31±0.01
C22:0	-	-	0.01±0.00^{A}	-	0.01±0.00^{a}	0.01±0.00^{aA}	-	-	-	0.10±0.01	0.30±0.02
SFA total	34.51±0.05^{aA}	27.68±0.26^{bA}	25.14±0.08^{cA}	34.91±0.09^{aB}	32.45±0.16^{bB}	33.06±0.18^{cB}	34.64±0.04^{aA}	33.86±0.15^{bC}	33.27±0.09^{cB}	16.30±0.18	11.03±0.01
C16:1	5.53±0.05^{aA}	3.06±0.01^{bA}	3.04±0.03^{bA}	4.20±0.04^{aB}	3.41±0.02^{bB}	2.34±0.06^{cB}	4.25±0.04^{aB}	4.03±0.03^{bC}	4.19±0.07^{aC}	1.07±0.02	tr
C18:1	39.89±0.06^{aA}	47.65±0.23^{bA}	33.83±0.23^{cA}	38.27±0.17^{aB}	46.04±0.09^{bB}	39.56±0.19^{cB}	40.74±0.13^{aC}	43.01±0.15^{bC}	39.70±0.13^{cB}	67.32±0.34	28.91±0.23
C18:1 n-7	2.48±0.06^{aA}	2.17±0.03^{aA}	1.45±0.09^{bA}	2.64±0.01^{aA}	2.28±0.10^{bA}	2.04±0.11^{bB}	2.54±0.09^{aA}	2.39±0.02^{aB}	2.34±0.08^{aC}	2.05±0.03	0.62±0.01
C20:1	-	-	-	-	-	-	-	-	-	0.31±0.02	0.22±0.03
C22:1	-	-	-	0.06±0.01^{a}	0.05±0.01^{a}	0.05±0.02^{a}	-	-	-	-	-
MUFA total	47.90±0.10^{aA}	52.89±0.27^{bA}	38.33±0.31^{cA}	45.17±0.14^{aB}	51.78±0.13^{bB}	43.99±0.21^{cC}	47.53±0.01^{aA}	49.43±0.15^{bC}	46.24±0.08^{cB}	70.75±0.32	29.75±0.23
C18:2	10.07±0.31^{aA}	14.01±0.01^{bA}	29.28±0.08^{cA}	11.84±0.19^{aB}	10.05±0.15^{bB}	17.40±0.07^{cB}	10.22±0.03^{aA}	10.79±0.03^{bC}	14.58±0.14^{cC}	9.38±0.14	56.25±0.20
C18:3	t r	-	-	0.74±0.02	tr	-	tr	tr	tr	0.65±0.01	0.15±0.03
C20:3	-	-	-	0.03±0.02^{aA}	0.02±0.01^{aA}	0.02±0.01^{aA}	0.02±0.00^{aA}	0.02±0.01^{aA}	0.02±0.01^{aA}	-	-
C20:4	0.34±0.02^{aA}	0.18±0.02^{bA}	0.16±0.02^{bA}	0.37±0.03^{aA}	0.21±0.02^{bA}	0.21±0.03^{bA}	0.35±0.03^{aA}	0.28±0.02^{aB}	0.29±0.04^{aB}	-	-
C20:5	0.28±0.02^{aA}	0.14±0.03^{bA}	0.14±0.02^{bA}	0.56±0.05^{aB}	0.32±0.02^{bB}	0.29±0.02^{bB}	0.28±0.01^{aA}	0.25±0.02^{aC}	0.24±0.01^{aC}	-	-
C22 :5	-	-	-	0.15±0.02^{a}	0.08±0.01^{b}	0.07±0.02^{b}	-	-	-	-	-
C22 :6	0.20±0.02^{aA}	0.10±0.01^{bA}	0.09±0.02^{bA}	0.38±0.02^{aB}	0.20±0.02^{bB}	0.20±0.01^{bB}	0.20±0.03^{aA}	0.15±0.01^{aC}	0.16±0.01^{aC}	-	-
PUFA total	10.89±0.07^{aA}	14.43±0.01^{bA}	29.40±0.08^{cA}	14.07±0.21^{aB}	10.88±0.15^{bB}	18.19±0.07^{cC}	11.07±0.03^{aA}	11.49±0.03^{bC}	15.29±0.14^{cB}	10.03±0.14	56.40±0.20
(P+M)/S ratio	1.70±0.01^{aA}	2.43±0.03^{bA}	2.69±0.02^{bA}	1.70±0.01^{aA}	1.93±0.01^{bB}	1.88±0.01^{cB}	1.69±0.01^{aA}	1.80±0.01^{bC}	1.85±0.01^{cB}	4.95±0.06	7.81±0.01

Each value is the mean±SD of six determinations; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. Values in the same row for the same food (raw or fried) bearing different small letters are significantly different at $p<0.05$. Values in the same row for raw food or for samples fried in the same oil bearing different capital letters are significantly different at $p<0.05$. - indicates "not detected"; tr = trace.

Frying involves an exchange of fatty acids between the fat in the food and the cooking oil used. This causes significant changes in the fatty acid composition of the chicken and chicken-based products, which were related to the fatty acid composition of the oils and to the fat and fatty acid composition of the raw products (Table 3). The SFA content decreased and MUFA increased in the C samples when frying in olive oil, while SFA and MUFA decreased, and PUFA increased when frying C samples in sunflower oil. Similar results were found when frying sardines in olive oil or sunflower oil (CANDELA *et al.*, 1997; CASTRILLÓN *et al.*, 1997; SÁNCHEZ-MUNIZ *et al.*, 1992). Likewise, CANDELA *et al.* (1998) showed that the percentages of SFA and MUFA decreased and that of PUFA increased in chicken breast when fried in sunflower oil.

According to MOREIRAS *et al.* (1998) the P + M / S ratio is used to indicate the fat quality of a food. Foods with a high P + M / S ratio have a more adequate fatty acid profile with respect to cardiovascular and other degenerative diseases (MOREIRAS *et al.*, 1998). Thus frying in both oils improves this ratio.

Changes in the S and B samples were less relevant due to the lesser fat enrichment of the food. These results suggest that when the fat content of the food is high, changes in the fatty acid composition are less than when a lean food is fried. However, B became more enriched in the major fatty acids of the frying oil than S. Thus, there was more oleic acid in BO than in SO, while linoleic acid was higher in BS than in SS. These differences are most likely related to the structure of the chicken-based products because the amount of fat in the B and S samples before frying was quite similar. Because the B samples did not have an edible coating, water could exit more easily and the fat from the fryer and the food could exchange. Moreover, the water loss from S was less than that of B, thus the water-fat exchanged more quickly. The rapid increase in the food-core temperature in the S samples supports this hypothesis. These results agree with those of CANDELA *et al.* (1997); CASTRILLÓN *et al.* (1997) and SÁNCHEZ-MUNIZ *et al.* (1992).

Fatty acid changes observed in C, B and S samples after frying were quantitatively much greater than those observed in the respective oil baths (data not shown). This is related to the bath/food fat ratio and to the number of fryings. According to ROMERO *et al.* (2000b) the amount of fatty acids belonging to the food increases in the oil bath after repeated fryings.

Table 4 reports the fatty acid content (g/100 g edible food) of raw and fried samples. The most relevant change was produced in lauric, and oleic acids in CO, and in lauric and linoleic acids in CS. A 56.9% increase in oleic acid occurred in BO with respect to B, while an almost non-noticeable change took place in SO with respect to S. However, linoleic acid increased 88.4% in BS and 35.3% in SS with respect to B and S, respectively. The presence of an edible coating in S but not in B should explain these differences. Retention of the three major fatty acid is shown in Table 5. As expected, because of the higher fat content of the sunflower oil-fried samples (Table 2), the true retention of linoleic acid was higher in all samples fried in sunflower oil than in those fried in olive oil, however oleic acid retention was higher in CO and BO samples (Table 5). Thus, the data suggest a distinct frying behavior of triglycerides containing oleic acid with respect to those containing linoleic acid. SÁNCHEZ-MUNIZ *et al.* (1992) suggested that during frying changes in fatty acids occur because of the existence of fatty acid gradients from the frying media to the food and from the food to the fryer, which in turn dilute or increase the fatty acid content of both the culinary fat and the food. However, not all of the fat-

Table 4 - Fatty acid composition (mg/100 g edible portion) of raw chicken (C), chicken sausages (S), and chicken burgers (B) fried in olive oil (CO, BO, and SO, respectively) and in sunflower oil (CS, BS, and SS, respectively).

Fatty Acid	C	CO	CS	B	BO	BS	S	SO	SS
C12:0	3.42±0.71^{aA}	-	13.63±2.60^{bA}	14.26±0.71^{aC}	-	55.24±1.45^{bC}	11.88±0.17^{aB}	25.47±2.47^{bB}	8.06±1.44^{bB}
C14:0	28.92±0.50^{aA}	37.38±1.36^{bA}	37.87±0.43^{bA}	142.56±1.12^{aB}	144.67±4.43^{bB}	142.29±4.40^{abB}	176.49±4.95^{aC}	165.57±4.68^{bC}	166.14±2.93^{bC}
C15:0	4.04±0.01^{A}	-	-	15.55±0.02^{aB}	-	70.31±1.17^{b}	23.76±0.08^{C}	-	-
C16:0	810.47±1.82^{aA}	930.49±2.06^{bA}	929.70±3.14^{bA}	3220.06±10.48^{aB}	4106.93±11.50^{bB}	3799.98±12.55^{cB}	4286.62±24.61^{aC}	3989.55±6.24^{bC}	3922.82±12.48^{cC}
C17:0	-	-	-	36.29±1.95^{a}	34.04±0.84^{a}	18.41±0.60^{b}	-	-	-
C18:0	235.74±1.49^{aA}	269.22±2.97^{bA}	293.40±1.49^{cA}	1167.70±3.57^{aB}	1285.01±10.30^{bB}	1495.88±33.58^{cB}	1428.89±23.87^{aC}	1249.72±13.36^{bB}	1304.92±12.35^{cC}
C22:0	-	-	0.23±0.03^{A}	-	0.76±0.09^{a}	1.67±0.68B^{b}	-	-	-
C16:1	173.54±1.49^{aA}	136.61±2.46^{bA}	154.02±1.69^{cA}	553.39±4.93^{aB}	585.49±2.64^{bB}	395.06±9.48^{cB}	728.01±7.36^{aC}	646.35±4.31^{bC}	680.69±11.73^{cC}
C18:1	1250.84±1.80^{aA}	2129.32±10.95^{bA}	1715.48±11.88^{cA}	5038.85±21.73^{aB}	7905.79±14.52^{bB}	6627.37±31.49^{cB}	6972.97±21.86^{aC}	6894.95±23.64^{bC}	6448.88±20.78^{cC}
C18:1 n-7	77.75±2.04^{aA}	97.01±3.54^{bA}	73.73±4.81^{aA}	347.33±1.44^{aB}	391.46±16.67^{bB}	344.84±18.67^{aB}	434.43±15.78 ^{aC}	383.67±3.73^{bB}	380.67±12.84^{bC}
C22:1	-	-		7.45±1.24^{a}	8.51±1.39^{a}	7.95±2.86^{a}	-	-	-
C18:2	315.66±9.69^{aA}	626.11±2.48^{bA}	1484.70±4.01^{cA}	1559.09±24.94^{aB}	1725.83±23.14^{bB}	2937.87±11.47^{cB}	1749.61±4.81^{aC}	1730.50±4.93^{aB}	2367±22.68^{bC}
C18:3	tr	-	-	97.20±2.28	tr	-	tr	tr	tr
C20:3	-	-	-	3.89±0.21^{aA}	3.40±1.39^{aA}	3.77±1.60^{aA}	2.97±0.19^{aB}	3.18±1.29^{aA}	3.63±1.54^{aA}
C20:4	10.57±0.51^{aA}	8.01±0.73^{bA}	8.08±0.82^{bA}	47.63±4.28^{aB}	35.74±2.78^{bB}	34.73±5.53^{bB}	58.97±5.61^{aB}	44.58±2.60^{bB}	46.37±6.63^{bB}
C20:5	8.71±0.51^{aA}	6.45±1.52^{aA}	7.07±0.82^{aA}	72.58±6.35^{aB}	54.46±2.78^{bB}	49.38±3.20^{bB}	47.09±0.85^{aC}	39.80±2.60^{bC}	39.11±2.03^{bC}
C22:5	-	-	-	19.44±2.12^{a}	13.19±0.85^{b}	11.72±2.73^{b}	-	-	-
C22:6	6.06±0.59^{aA}	4.34±0.22^{bA}	4.54±0.83^{bA}	49.90±2.48^{aB}	34.04±2.78^{bB}	33.06±0.84^{bB}	33.52±4.46^{aC}	23.88±1.30^{bC}	25.40±1.54^{bC}

Each value is the mean±SD of six determinations; Values in the same row for the same food (raw or fried) bearing different small letters are significantly different $p<0.05$. Values in the same row for raw food or for samples fried in the same oil bearing different capital letters are significantly different at $p<0.05$. - indicates "not detected"; tr = trace.

Table 5 - Weight yield and major fatty acid retention in chicken (C), burgers (B) and sausages (S) after frying them in olive oil (CO, BO, and SO respectively) and in sunflower oil (CS, BS and SS, respectively).

	Weight yield (%)	% Nutrient retention		
		C16:0	C18:1	C18:2
CO	79.49	135.32±1.55^{aA}	157.67±0.74^{aA}	91.26±1.81^{aA}
CS	81.90	93.92±0.30^{bA}	111.52±0.73^{bA}	386.08±0.99^{bA}
BO	79.24	82.14±0.22^{aB}	126.47±0.22^{aB}	86.22±1.24^{aB}
BS	86.33	113.68±0.36^{bB}	112.63±0.50^{bA}	146.70±0.59^{bB}
SO	85.60	79.80±0.12^{aB}	82.73±0.27^{aC}	86.49±0.24^{aB}
SS	88.42	78.48±0.24^{bC}	83.85±0.26^{aB}	118.35±1.07^{bC}
ANOVA		***	***	***

Values in the same column for the same food bearing different small letters are significantly different at $p<0.05$. Values in the same column for different foods that have been fried in the same oil bearing different capital letters are significantly different at $p<0.05$.

ty acid exchanges occur in the same proportion.

In conclusion, although the fatty acid composition of the chicken-based products (B and S) did not differ markedly from that of the original chicken used to make them, they appear to be very much enriched in fat. Thus, in the information included on the label of these products, the term "chicken" could be misleading, since consumers relate the term chicken with a lean food. These results call for the need to include the proximate composition as well as the fatty acid profile on the label of these chicken-based products. Frying increased the fat content in the burgers but not in the sausages – a fact that seems related to the water loss and the presence or absence of an edible coating. Moreover, after frying, the major fatty acids of the frying oils increased in the samples, which was more relevant in burgers than in the sausages.

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PAPER

ARTIFICIAL REARING AND INTRAMUSCULAR FATTY ACID COMPOSITION OF UNWEANED LAMBS

ALLATTAMENTO ARTIFICIALE E COMPOSIZIONE ACIDICA DEL GRASSO INTRAMUSCOLARE IN AGNELLI LATTANTI

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ABSTRACT

The effect of artificial rearing on the fat quality (fatty acid composition and cholesterol content) of lambs raised on a farm converting from conventional to organic production was evaluated. Twenty Sarda male lambs were divided into two groups: artificially reared animals (AR), separated from their dams 2 days after birth and fed on reconstituted milk replacer and ewe-reared lambs (ER), left with their mothers to suckle until slaughter. Animals from both groups were slaughtered at 45 d

RIASSUNTO

È stato valutato l'effetto dell'allattamento artificiale sulla qualità del grasso intramuscolare (composizione acidica e contenuto di colesterolo) di agnelli allevati presso un'azienda in fase di conversione dal sistema convenzionale a quello biologico. La prova ha riguardato 20 soggetti maschi di razza Sarda, suddivisi in due gruppi sperimentali. Dieci agnelli (gruppo AR), separati dalle madri 2 giorni dopo la nascita, hanno ricevuto latte ricostituito, mentre altri 10 soggetti (gruppo ER) sono

- Key words: artificial rearing, cholesterol, fatty acids, lamb, organic farming -

of age. Fatty acid composition and cholesterol content were analysed on leg samples. Results showed that milk feeding is a major factor affecting the nutritional characteristics of lamb since the fatty acid profile of the meat reflected that of the corresponding milk source. Although the P/S ratio was lower (P<0.01) in meat from ER lambs, they had a better ω-6/ω-3 ratio, closer to the value recommended by current nutritional guidelines. Rearing system had no significant effect on *trans*-fatty acid and cholesterol contents.

stati lasciati con le madri per l'intero periodo sperimentale. Tutti gli animali sono stati macellati all'età di 45d. La qualità del grasso intramuscolare è stata valutata su un campione omogeneo dei muscoli del coscio. I risultati indicano che l'alimentazione lattea è un fattore in grado di influenzare marcatamente le caratteristiche nutrizionali della carne di agnello in quanto il profilo acidico riflette quello del corrispondente tipo di latte (materno e artificiale). Sebbene sia stato osservato un rapporto polinsaturi/saturi (P/S) più alto negli agnelli AR (P<0,01), le caratteristiche dietetico-nutrizionali, in relazione al rapporto ω6/ω3, sono risultate migliori per la carne prodotta dal gruppo ER. Non è stato evidenziato alcun effetto di rilievo del tipo di allattamento sul contenuto di acidi grassi di tipo *trans* e sul contenuto di colesterolo.

INTRODUCTION

Many different factors may affect the purchase decision-making process of consumers. Perceived healthiness of food is not the only factor, but it is one of the most important ones and, in the case of meat, it essentially depends on fat content and fatty acid composition. In western countries about 35-40% of the total calories in the diet comes from fat, nearly half of which is of meat origin (JIMENEZ-COLMENERO *et al.*, 2001). In particular, high levels of saturated fat may increase low-density lipoprotein cholesterol and plasma cholesterol levels, whereas increased dietary polyunsaturated fatty acids may have beneficial effects on human health. In addition, high levels of ω6/ω3 may increase the risk of atherosclerosis (SIMOPOULOS, 2002).

Different factors such as weight (KEMP *et al.*, 1981), breed (FISHER *et al.*, 2000), age at slaughter (CIFUNI *et al.*, 2000), gender (SOLOMON *et al.*, 1992) and feeding (VELASCO *et al.*, 2001) have been reported to influence fatty acid composition in lamb. Although diet does not change the fat composition of ruminants as much as of monogastric animals, there is evidence that in young lambs the fatty acid profile closely reflects the dietary fat pattern because the rumen is only partially functional and therefore extensive hydrogenation by micro-organisms does not occur (VELASCO *et al.*, 2001).

Organic livestock farming is a potentially important future development, involving ecological, sociological and economic aspects, animal welfare and product safety. Elevated standards for animal well-being are fundamental in the rearing of organic livestock and have a positive effect on the consumers' image of meat quality.

Artificial rearing programmes, in which the lambs are separated from the ewe at a very early age, have been developed to increase sheep-milk availability

for cheese production. Since this practice may adversely affect the welfare of the lambs, due to reduced ability to cope with emotional and nutritional stress (SEVI *et al.*, 1999; NAPOLITANO *et al.*, 2001), artificial rearing is not allowed under current EU organic livestock guidelines (E.U., 1999).

Food safety and healthiness are among the most important factors that contribute to the increased demand for organic products. In fact, a growing segment of consumers perceives organic products as being healthier and safer. In addition, the relationship between animal welfare and meat quality is well known (GREGORY, 1998).

The aim of this study was to assess the effect of the rearing system on the fat quality (fatty acid composition and cholesterol content) of lambs raised on a farm converting from conventional to organic production.

MATERIALS AND METHODS

Twenty Sarda single born male lambs, with an average birth weight of 4.2 kg, were divided into two equal groups, with respect to live weight. Artificially reared (AR) lambs were separated from their dams 2 days after birth and housed in a collective straw-bedded pen. Three times a day, the lambs were fed an artificial milk replacer (25% crude protein, 23% fat, 0.5% crude fibre, 7% ash; Schils Holland, The Netherlands), from buckets with 50 mm long latex teats, at 37°C. The make-up of the milk replacer is shown in Table 1. Ewe-reared (ER) lambs, used as control, were allowed to suckle and stay with their mothers throughout the experiment. The chemical composition of the ewe milk was: 5.15% crude protein, 6.11% fat, 4.9% lactose. From 7 days of age until slaughter all lambs had free access to commercial concentrate (16.5% crude protein, 3.8% fat, 10% crude fibre, 8.5% ash; Martini, Italy) and alfalfa hay.

Table 1 - Make-up of the milk replacer (%).

Skim milk	57
Fat	23
Refined coconut oil (62)	
Refined animal fat (38)	
Whey	17
Wheat flour	2
Vitamins and Minerals	1

All lambs were weighed weekly from birth to slaughter (45d). At slaughter, the AR and ER lambs weighed 12.86±0.80 kg and 9.71±1.00 kg, respectively. Standardised procedures were used to produce and dissect the carcasses (FISHER and DE BOER, 1994). Average carcass yields were 60.16±0.02% and 59.98±0.02% in AR and ER groups, respectively. Carcasses were graded 2 to 3 for fatness and "O" for muscular conformation (E.C., 1992).

The leg muscles were blended together in a small food processor. The lipids were extracted from the leg muscle sample according to a modification of the method of FOLCH *et al.* (1957) as described by MICHAELSEN *et al.* (1991). Briefly, a 5 g homogenised meat sample was blended with chloroform/methanol (2:1, v/v) twice, filtered, placed in separator funnels and mixed with saline solution (0.88% KCl). After separation in two phases, the methanol aqueous fraction was discarded, whereas the lipid chloroform fraction was washed with distilled water/methanol (1:1, v/v). After a further filtration and evaporation by means of a rotary evaporator, lipid extracts were transferred to test tubes for gas chromatographic analysis. Duplicates of 10 mL chloroform extracts, corresponding to 100 mg of lipid, were methylated by adding 1 mL of hexane and 0.05 mL of 2N methanolic KOH (IUPAC, 1987). Fatty acid methyl esters (FAME) were analysed on a Varian chromatograph (model Star 3400

CX). Separations were performed using a CP-88 capillary column (100 m x 0.25 mm i.d. x 0.25 μm). Operating conditions were: helium flow rate of 0.7 mL/min, FID detector at 260°C, split-splitless injector at 220°C with an injection rate of 120 mL/min, injection volume of 1 μL. The temperature programme of the column was: 4 min at 140°C followed by an increase to 220°C at 4°C/min. The retention time and area of each peak were computed using the Varian Star 3.4.1. software. Individual fatty acids were identified by retention time with reference to fatty acid standard mixtures (FAME, Sigma). Fatty acids are expressed as percent of total methylated fatty acids.

To determine the milk fatty acids, lipids were extracted using the method of BLIGH and DYER (1959) and methylated by adding 1 mL of heptane and 0.05 mL of 2N methanolic KOH (I.U.P.A.C., 1987). Gas chromatographic analysis was performed as described for the leg muscles, although the temperature programme for the column differed: 4 min at 40°C and a subsequent increase to 220° at 4°C/min. Identification of fatty acids was performed as described for the meat.

Cholesterol content (mg/100 g of meat) was determined by directly saponifying the samples, extracting the unsaponifiable compounds with cyclohexane, followed by an enzymatic assay (Kit n. 139050, Boehringer Mannheim Gmbh) according to the method of ULBERTH and REICH (1992).

Data were subjected to analysis of variance with rearing system (ewe and artificial rearing) as factor (SAS, 1990). Only one sample of reconstituted milk was available for the determination of the fatty acid profile, whereas the fatty acid content of the ewe milk was measured on a mix of three samples obtained at 10, 20 and 30 days of lactation. Therefore, no statistical analysis was performed for milk fatty acid composition.

RESULTS AND DISCUSSION

As shown in Table 2, meat from ewe-reared lambs was characterised by a higher content of saturated fatty acids ($P<0.01$) and lower percentages of monounsaturated ($P<0.01$) and polyunsaturated ($P<0.05$) fatty acids, compared with artificially-reared lambs. For both groups these results were probably due to the digestive physiology of the unweaned lambs. In the absence of a functional rumen, the fatty acid profile of the diet and the fat deposited are closely related (STOKES and WALKER, 1970; FUENTE *et al.*, 1998; BERIAIN *et al.*, 2000). Therefore, the higher level of unsaturated fatty acids in artificially reared lambs matched the higher percentage of unsaturated fatty acids of the milk substitute (Table 3) that did not undergo ruminal biohydrogenation. In agreement with BERIAIN *et al.* (2000), the saturated caprinic (C10:0; $P<0.01$) and myristic (C14:0; $P<0.001$) fatty acid contents were much higher in the meat obtained from suckled lambs because of the higher levels of these compounds in the ewe milk (GARCIA and COLL, 1976). The palmitic acid (C16:0; $P<0.01$) levels were higher, whereas the behenic acid (C22:0; $P<0.01$) percentage was lower in muscles from the ER group. The percentage of palmitoleic acid (C16:1; $P<0.20$) tended to be higher in the ewe-reared lambs. Conversely, meat from milk-substitute fed lambs was characterised by higher percentages of oleic acid (C18:1; $P<0.01$) and linoleic acid (C18:2ω6; $P<0.01$), thus matching the composition of the diet. The percentages of eicosapentaenoic (C20:5ω3; $P<0.01$) and docosahexaenoic (C22:6ω3; $P<0.20$) acids were higher in the ewe-fed lambs.

A considerable amount of data has been accumulated which supports the view about the health benefits of the long-chain ω-3 polyunsaturated fatty acids (WILLIAMS, 2000). The P/S ratio in

Table 2 - Effect of rearing system on intramuscular composition (%) of leg meat (means±s.e.).

	Rearing system		Significance
	Ewe (n= 10)	Artificial (n= 10)	
Intramuscular fat (mg/100g of meat)	3.25 ± 0.44	2.95 ± 0.44	NS
C10:0	0.21 ± 0.03	0.08 ± 0.03	P<0.01
C12:0	1.00 ± 0.16	1.50 ± 0.05	P<0.05
C14:0	7.00 ± 0.31	5.45 ± 0.31	P<0.001
C14:1	0.23 ± 0.01	0.23± 0.01	NS
C14:1 *trans 9*	0.18 ± 0.02	0.07 ± 0.02	P<0.01
C15:0	0.60 ± 0.07	0.22 ± 0.02	P<0.01
C15:0 *anteiso*	0.21 ± 0.03	0.06 ± 0.03	P<0.01
C16:0 *iso*	0.23 ± 0.03	0.07 ± 0.03	P<0.01
C16:0	25.00 ± 0.84	20.91 ± 0.84	P<0.01
C16:1ω9	0.40 ± 0.06	0.24 ± 0.06	NS
C16:1 *trans*	0.35 ± 0.06	0.39 ± 0.06	NS
C17:0 *anteiso*	2.62 ± 0.15	2.84 ± 0.15	NS
C17:0 *iso*	1.11 ± 0.09	0.57 ± 0.09	P<0.001
C17:1	0.25 ± 0.08	0.12 ± 0.08	NS
C18:0 *iso*	0.65 ± 0.03	0.42 ± 0.03	P<0.001
C18:0	11.37 ± 0.42	11.33 ± 0.42	NS
C18: 1 *trans (isomers)*	0.80 ± 0.15	1.09 ± 0.15	NS
C18:1ω9	31.74 ± 0.88	36.10 ± 0.88	P<0.01
C18:1ω7	0.40 ± 0.09	0.22 ± 0.09	NS
C18:2 t9-t12	0.19 ± 0.01	0.08 ± 0.01	P<0.001
C18:2 c9-t12	0.17 ± 0.01	0.10 ± 0.02	P<0.01
C18:2 t9-c12	0.14 ± 0.02	0.07 ± 0.02	P<0.01
C18:2ω6	7.63 ± 0.50	10.51 ± 0.50	P<0.01
C18:3ω6	0.69 ± 0.06	0.53 ± 0.06	NS
C18:3ω3	0.56 ± 0.03	0.51 ± 0.03	NS
C20:0	0.17 ± 0.01	0.16 ± 0.02	NS
C20:1ω9	0.21 ± 0.04	0.27 ± 0.04	NS
C22:0	0.16 ± 0.02	0.31 ± 0.02	P<0.01
C20:2ω6	0.46 ± 0.04	0.49 ± 0.04	NS
C20:3ω6	0.29 ± 0.02	0.35 ± 0.02	P<0.05
C20:4ω6	3.16 ± 0.25	3.32 ± 0.25	NS
C20:5ω3	0.40 ± 0.04	0.21 ± 0.04	P<0.01
C22:4ω6	0.24 ± 0.01	0.29 ± 0.01	P<0.05
C22:5ω3	0.91 ± 0.08	0.57 ± 0.08	P<0.05
C22:6ω3	0.35 ± 0.03	0.28 ± 0.03	NS
Saturated	50.34 ± 1.12	43.93 ± 1.12	P<0.01
Mono-unsaturated	34.47 ± 0.94	38.74 ± 0.94	P<0.01
Polyunsaturated	15.19 ± 0.60	17.33 ± 0.60	P<0.05
P/S	0.30 ± 0.02	0.39 ± 0.02	P<0.01
Σω6/Σω3	6.03 ± 0.67	9.87 ± 0.67	P<0.01
Trans	1.52 ± 0.17	1.63 ± 0.17	NS
Cholesterol (mg/100g of meat)	96.92 ± 4.32	88.25 ± 4.32	NS

Table 3 - Fatty acid composition of ewe-milk and milk-substitute (%).

	Milk source	
	Ewe	Substitute
C4:0	2.29	1.47
C6:0	2.37	0.19
C8:0	2.70	1.65
C10:0	8.79	1.41
C10:1 *cis 9*	0.28	ND
C12:0	4.85	9.67
C14:0 *iso*	0.16	ND
C14:0	11.82	6.53
C14:1 *trans 9*	0.43	0.12
C14:1 *cis 9*	0.09	0.19
C15:0	1.35	0.28
C15:0 *anteiso*	0.56	0.12
C16:0 *iso*	0.39	0.10
C16:0	27.83	21.88
C16:1ω9	0.28	0.43
C16:1 *trans*	0.58	0.43
C17:0 *anteiso*	1.14	1.75
C17:0 *iso*	1.12	0.60
C17:1 *cis 10*	0.06	0.15
C18:0 *iso*	0.33	0.33
C18:0	9.38	13.56
C18: 1 *trans (isomers)*	0.52	1.94
C18:1ω9	17.67	25.08
C18:1ω7	0.09	1.38
C18:2 t9-t12	0.13	0.30
C18:2 c9-t12	0.07	0.12
C18:2 t9-c12	0.12	0.20
C18:2ω6	2.26	4.71
C18:3ω6	0.69	0.74
C18:3ω3	0.32	0.49
C20:0	0.38	0.70
C20:1ω9	0.07	0.40
C21:0	0.13	ND
C20:2ω6	0.20	0.32
C20:3ω6	0.04	0.20
C20:4ω6	0.22	0.70
C20:5ω3	0.05	0.91
C22:4ω6	0.06	0.91
C22:5ω3	0.03	0.14
C22:6ω3	0.12	0.13
Saturated	75.61	60.23
Mono-unsaturated	20.07	30.14
Polyunsaturated	4.32	9.66
Σω6/Σω3	6.73	4.39
Trans	1.65	2.79

the meat from artificially fed lambs was higher ($P<0.01$) than in the ewe-suckled subjects, although both values were lower than 0.45, the recommended dietary value for the human diet (DEPARTMENT OF HEALTH, 1994).

An important parameter, in terms of human nutrition and health, is the ω6/ω3 ratio (SIMOPOULOS, 2002). In the present study this ratio was lower in meat samples from ewe-reared lambs (6.03 vs. 9.87, $P<0.01$). Although both values were well above the recommended ratio of 4 (DEPARTMENT OF HEALTH, 1994), a range of 5 to 10 (COMMISSION OF EUROPEAN COMMUNITIES, 1993) has been recommended. The ω-6/ω-3 ratio is the only variable that did not follow the trend shown by the corresponding milk source (Table 2). This result was probably due to an earlier intake of concentrate, by artificially reared lambs (data not shown), which led to higher concentrations of ω-6 PUFA (MARMER *et al.*, 1984; MITCHELL *et al.*, 1991; ENSER *et al.*, 1998), that could have escaped rumen biohydrogenation. Omega-6 fatty acids can increase blood pressure, inflammation, platelet aggregation, thrombosis, vasospasm and allergic reactions (MILES and CALDER, 1998); omega-3 series have the opposite effects. These two families of essential fatty acids compete for enzymes involved in their desaturation, thus the excessive consumption of foods rich in ω-6 fatty acids may compromise the conversion of alpha-linolenic (C18:3ω3) to eicosapentaenoic (C20:5ω3) acid, with adverse health affects (OKUYAMA *et al.*, 1997). Current research suggests that the levels of essential fatty acids and the balance between them may play a critical role not only in growth and development, but also in the prevention and treatment of chronic diseases including coronary artery disease, hypertension, type II diabetes, arthritis and other immune/inflammatory disorders and cancer (CHOW, 1993).

In agreement with previous studies on Comisana lambs (NAPOLITANO *et al.*, 2001) and on Colmerana x Rubia lambs (FUENTE *et al.*, 1998), the ewe-reared lambs showed higher amounts of branched fatty acids C15:0 anteiso ($P<0.01$), C16:0 iso ($P<0.01$), C17:0 iso ($P<0.001$) and C18:0 iso ($P<0.001$). It is well-established that the branched-chain fatty acids, mainly of the *iso* and anteiso series, detectable in tissue and milk lipids of ruminants, are components of rumen bacterial derivation (HARFOOT *et al.*, 1988). Since young pre-ruminant animals use fat without much digestive modification, as in non-ruminant mammals, it is reasonable to assume that the methyl-branched fatty acids detected in the meat of lambs from both groups of lambs may, to some extent, have originated in the milk (Table 3).

No effect of rearing was detectable on *trans* fatty acid content, although NAPOLITANO *et al.* (2001) found higher levels of these fatty acids in meat from ewe-reared Comisana lambs. Despite the increasing evidence about the potentially adverse effects of high intakes of *trans* fatty acids with an increased risk of coronary heart diseases (WILLIAMS, 2000), the issue of *trans* fatty acids and human health is still debatable. In addition, possible differences in *trans* fatty acids derived from ruminal metabolism, compared with those formed by industrial hydrogenation, may exist (WILLIAMS, 2000).

Analysis of variance showed no significant effects of rearing system on cholesterol content in the meat.

CONCLUSIONS

Meat may be considered to be a functional food because it contains numerous compounds thought to be functional for health purposes. Results showed that milk feeding is a major factor affecting the nutritional characteristics of lamb since the fatty acid profile of the meat reflected the corresponding milk source. The use of a milk substitute may improve some nutritional characteristics of the meat, such as increasing the P/S ratio, but the high concentration of linoleic acid increases the ω-6/ω-3 ratio which should not exceed the maximum recommended value of 4. Therefore, in conjunction with current EU legislation on organic farming, ewe rearing is recommended in order to obtain healthy, nutritious lamb meat.

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PAPER

EVALUATION OF THE COLOR OF SPANISH SAFFRON USING TRISTIMULUS COLORIMETRY

VALUTAZIONE DEL COLORE DELLO ZAFFERANO SPAGNOLO CON TRISTIMOLO COLORIMETRI

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ABSTRACT

The color of saffron in filament and in powdered form was determined on 245 samples by a colorimetric reflection method. The chromatic parameters (L*, a*, b*, C* and H* coordinates) of saffron filaments were lower than those of powdered saffron. Grinding increased the yellow component and luminosity of the powder. Significant differences were observed between the chromatic parameters of saffron from Castilla-La Mancha and Aragón, the former being redder and darker. The L*, a* and b*

RIASSUNTO

Scopo del presente lavoro è la determinazione del colore di 245 campioni di due tipi di zafferano, in filamenti e in polvere, con il metodo di riflessione del colore. I parametri cromatici studiati sono le coordinate L*, a*, b*, C* e H*, che sono state più basse nello zafferano filamentoso rispetto a quello in polvere. La macinazione fa aumentare la componente gialla e la luminosità dello zafferano in polvere. I risultati mostrano che esistono delle differenze significative tra i parametri cromatici

- Key words: chromatic coordinates, color, coloring power, saffron -

coordinates showed the relationship between the color of the sample and the quantity of style. A linear correlation between the chromatic parameters and coloring power ($R^2 > 0.90$) was obtained for some agricultural areas. Discriminant analysis correctly classified 91% of the Castilla-La Mancha samples and 71% of those from Aragón.

dello zafferano di Castilla-La Mancha e della regione di Aragón, nel senso che il primo presenta un colore più rosso e più scuro. I parametri L*, a* e b* mostrano una correlazione tra il colore del campione e la quantità del trattamento. Emerge anche una correlazione lineare tra i parametri cromatici e il potere colorante ($R^2 > 0,9$) in ogni area di coltivazione. Inoltre, l'analisi discriminante ha permesso di classificare correttamente il 91% dei campioni della regione di Castilla-La Mancha e il 71% di quelli della regione di Aragón.

INTRODUCTION

Saffron is obtained from the dried stigmas of the flowers of *Crocus sativus* L. and is sold as whole filaments or in powdered form. The stigmas of *Crocus sativus* L. consist of three red filaments joined at their base by the style, which is white. It is the most expensive spice on the market and is basically used in foodstuffs for its pleasing yellow color, characteristic taste and exotic aroma. It is used in the food industry as a colorant and in the cosmetic and pharmaceutical industries for other properties it possesses.

Color is the principal quality characteristic of saffron and it is this property that leads to its rejection or acceptance by the consumer. The ISO 3632 (1993) method, which is used worldwide, measures the "coloring power" of saffron, that is, its capacity to impart color, which is considered one of the most influential parameters in deciding saffron quality (ALONSO *et al.*, 1997). The compounds responsible for the characteristic color of saffron are carotenoids, which are derived from crocetin (PFANDER and WITTWER, 1975a, 1975b; TARANTILIS *et al.*, 1995). These compounds, known as "crocins", unlike other carotenoids, are soluble in water (ALONSO *et al.*, 2001).

However, the real color of a foodstuff, as perceived by the consumer, is that resulting from the radiation reflected by the surface of the food, and for this reason is best measured by a colorimetric reflection method. Tristimulus colorimetry, which provides the trichromatic coordinates defined by the CIE (1976) system, is a rapid and easily applied method which is used to measure the external color of many foods but has not been used on saffron. It has been successfully used to measure the visual color of green vegetables (GNANASEKHARN *et al.*, 1992), grapes (CARREÑO *et al.*, 1995), carrots (Sulaeman *et al.*, 2001), rainbow trout flesh (BJERKENG *et al.*, 1997), watermelon (Pardo *et al.*, 1997), tomato fruit and paprika pepper (GÓMEZ *et al.*, 1998) and fruit juices (LEE and CASTLE, 2001). In most of these studies varieties have been able to de differentiated by the color measured by reflection.

The production of saffron spice differs from country to country, as the harvesting, stigma separation and dehydratation processes are different (NEGBI,1999). Spain is one of the principal producers and among the saffron-pro-

ducing countries, is the country generally recognized as producing the highest quality (OBERDIECK, 1991; Raina *et al.*, 1996). The highest quality saffron is known as "Mancha saffron" since it comes from the area known as "La Mancha" in the Castilla-La Mancha region. However, within Spain, saffron differs from one agricultural area to another, so it is of interest to know the range of any color variation between the areas of production.

Because of its high price, saffron is frequently adulterated with other substances (ALONSO *et al.*, 1998a). However, before the color of a saffron sample is determined, it is necessary to assure that it has not been adulterated, and its place of origin and processing methods are known.

In this study a colorimetric reflection method was used for the first time on saffron samples of known origin, which had not been adulterated and which had been processed by known methods. The results obtained were compared to the standard method, which measures the coloring power of saffron. It is hoped that such a reflection method might serve as a complementary method to the coloring power one since it provides an immediate assessment of color and could be included in commonly use quality control procedures. Several colorimetric categories are proposed to classify saffron quality and they are compared with the standard ISO method.

MATERIALS AND METHODS

A total of 245 saffron samples were analyzed, 236 of which were from the most important saffron-producing regions of Spain. Non-adulteration of the samples was assured by obtaining them directly from the producers. One sample was taken for every 10 ha from each area (personal communication from the respective regional Agricultural Ministries of Castilla-La Mancha and Aragón). Table 1 shows the number of samples, their origin and their coloring power. With the exception of samples from the Aragón region (all from Teruel province, the only saffron-producing province in Aragón), the Spanish samples came from four provinces in the Castilla-La Mancha region: Albacete, Ciudad-Real, Cuenca and Toledo, each province being divided into its principal saffron-growing areas. The predominance of samples from Castilla-La Mancha was due to its being the largest saffron producing region in Spain.

Saffron from India, Iran and Italy, all important saffron-producing countries, was used for comparison with Spanish saffron. The non-Spanish samples were obtained from specialized companies and only those with certificates of origin were used. Since non-adulteration could not be ensured, the number of non-Spanish samples was very low and hence they were not included in the statistical analysis.

Saffron was acquired as the dry stigmas in filament form since this was the form in which it was obtained from the saffron producers. Color measurements were also made on powdered saffron, because it is also sold in this form. Reflected color was measured using a Minolta C.R. 300 (Minolta Camera Co. Osaka, Japan) with a CR-A33a cone and a calibrated white plate (Minolta 14333085) with y=95.0; x=0.3154; z=0.3319. The measurement was made letting the projection cone fall over the sample placed on a Petri dish. The quantity and thickness of the sample were such that the background and surroundings did not affect the measurement. D65 illuminant and an angle vision of 2º were used. Previous studies (ALONSO *et al.*, 1997) had shown that particle size, humidity and illumination influenced the results obtained using this technique, so care was taken that all these parameters were kept similar for all the sam-

ples. Measurement of saffron filaments was done directly on the samples. For saffron powder, the stigmas were ground and passed through a 0.5 mm mesh. Moisture was eliminated by heating 1 g of sample in an oven at 40°C with dry (blue) silica gel until constant weight was reached. The moisture percentage was calculated using the following ratio: (initial mass-constant mass/initial mass ratio)x 100. The ground samples were stored in hermetically closed containers in darkness at -20°C until analysis of coloring power.

Three reflection measurements were made and the value reported is the mean of the three measurements made automatically by the colorimeter, giving the chromatic coordinate values, L*, a* and b*. The L* value (brightness) indicates the capacity of a color to reflect white light and varies from 0 (for black) to 100 (for white). The a* value indicates the red-green component and the b* value indicates the yellow-blue component, with positive values, in this case, corresponding to yellowness. The colorimeter also provides the psychophysic coordinates C*, chroma, and H*,hue angle, which are expressed as a function of a* and b*.

The method described by ALONSO *et al.* (1990) was used to determine the coloring power of the samples. For this 20 mg of powdered sample was extracted with 200 mL water for 24 h at room temperature and in darkness. The absorption spectrum of the aqueous extract in a 1 cm thick cell between 200 and 750 nm was determined using water as reference. A Perkin-Elmer Lambda 3B spectrophotometer (Norwalk, CT, USA) was used. The coloring power was calculated as $E_{440}^{1\%}$.This value is in accordance with ISO 3632 (1993) (unpublished data). The coloring power of an aqueous extraction of saffron is obtained by measuring the absorbance at 440 nm (CORRADI and MICHELLI, 1979a 1979b). According to the standard coloring power ISO 3632 (1993) method, there are four quality grades: I, coloring power ≥ 190; II, coloring power ≥ 150; III, coloring power ≥ 110 and IV, coloring power ≥ 80. The colorimetric distance parameter (D) was also calculated in terms of: $D= [(L^*-L_o^*)^2 +(a^*-a_o^*)^2 + (b^*-b_o^*)^2]^{1/2}$ in relation to / an ideal color of a powdered sample according to ALONSO *et al.* (1990).

The SPSS work program, version 8 for Windows (NORUSIS, 1997) was used for statistical analysis. Univariate analysis of variance (ANOVA) with subsequent comparisons (Duncan's multiple range test; α=0.05) and discriminant analysis were used to investigate the differences between saffron as powder and as filament. Comparison was made between districts and provinces. Samples from abroad were not used in the statistical treatment due to the low number of samples.

RESULTS AND DISCUSSION

Table 1 shows the mean colouring power of the samples and the corresponding quality category according to ISO 3632 (1993). Overall, the coloring power ranged from 97.33, in a sample from Rio Jiloca, to 273.29 in a sample from Albacete. The samples from Castilla-La Mancha generally had a greater coloring power than the samples from Aragón. Within Castilla-La Mancha, the saffron from Albacete had the highest mean value.

Tables 2 and 3 show the chromatic coordinate values obtained for saffron as powdered and filament form, respectively. The coefficient of variation of the colorimetric coordinates (0.02-0.18) was lower than that for coloring power (0.15-0.22), indicating the higher homogeneity for each district and province obtained by measuring the samples with the CIE-Lab method. The values of the chromatic parameters measured on the filaments were lower than the corresponding values measured on powdered saffron, with

Table 1 - Number of saffron samples, mean coloring power and quality category according to ISO 3632 in terms of coloring power and according to the CIELab methodology proposed in this paper.

Area	Number of samples	Coloring power mean / cv	ISO Quality category	CIELab quality category
SPAIN				
La Mancha (I)	18	188.62 / 0.17bc	II	A
Sierra	7	203.39 / 0.13bc	I	A
Centro	18	194.94 / 0.23bc	I	A
La Manchuela	47	223.31 / 0.16c	I	A
Hellín	21	187.17 / 0.22bc	II	A
Province of Albacete	**111**	**205.25 / 0.20v**	**I**	A
Alcázar de San Juan	2	212.39 / 0.06bc	I	C
La Solana	8	179.03 / 0.15bc	II	B
Province of Ciudad Real	**10**	**185.00 / 0.15v**	**II**	**B**
Landete	5	187.00 / 0.12bc	II	A
Cuenca	12	184.33 / 0.12bc	II	A
La Mancha (II)	17	189.53 / 0.19bc	II	A
Province of Cuenca	34	187.26 / 0.16v	II	A
Toledo	29	184.15 / 0.22bc	II	B
Province of Toledo	**29**	**184.15 / 0.22v**	**II**	**B**
Total Castilla-La Mancha	***184***	***196.98 / 0.2j***	I	
Río Jiloca	46	186.81 / 0.18bc	II	C
Muniesa	6	164.29 / 0.21ab	II	B
Province of Teruel	**52**	**184.22 / 0.19v**	II	**C**
Total Aragón	***52***	***184.22 / 0.19ij***	II	
OTHER COUNTRIES				
India	2	167.29 / 0.15 ab, uv, ij	II	B
Iran	5	210.84 / 0.02 bc, v, j	I	A
Italy	2	130.39 / 0.01 a, u, i	III	A

The mean was calculated taking into account the number of samples collected in each place. The mean with different letters in a column shows differences at a level of significance of 5% according to Duncan' s test. Letters a,b,c,d and e are used for comparison between districts and other countries; u,v,x and y for comparison between provinces and other countries, and letters i,j and k for comparison between Castilla-La Mancha, Aragón and other countries.

Table 2 - Mean chromatic parameters of saffron powder.

Area	L*		a*		b*		C*		H*	
SPANISH SAFFRON										
La Mancha I	36.91	b-e	36.99	d	45.25	c	58.35	de	50.92	a-c
Sierra	36.18	a-e	36.56	d	43.01	a-c	56.47	b-e	49.66	a
Centro	35.35	a-d	34.38	b-d	41.18	a-c	53.69	a-d	50.07	ab
La Manchuela	35.25	b-d	34.81	b-d	40.92	a-c	53.72	a-d	49.76	a
Hellín	33.40	a	33.32	b-d	39.77	a-c	51.88	a-c	50.14	b
Province of Albacete	35.25	uvx	34.92	x	41.60	u	54.29	u	50.06	u
Alcázar de San Juan	37.22	c-e	31.51	ab	39.44	ab	50.48	ab	51.28	a-c
La Solana	37.09	c-e	36.86	d	44.14	a-c	57.32	b-e	50.43	ab
Province of Ciudad Real	37.12	vxy	35.79	x	43.20	u	55.95	u	50.06	uv
Landete	36.04	a-e	37.23	d	43.74	bc	57.52	cde	49.52	a
Cuenca	35.95	a-e	33.95	b-d	42.53	a-c	54.36	a-d	51.36	a-c
La Mancha II	36.38	a-e	33.84	b-d	42.45	a-c	54.36	a-d	51.46	a-c
Province of Cuenca	36.18	uvxy	34.38	vx	42.67	u	54.82	u	51.14	uv
Toledo	38.79	e	31.76	a-c	41.80	a-c	52.53	a-d	52.90	b-d
Province of Toledo	38.79	y	31.76	uvx	41.80	u	52.53	u	52.90	uvx
TOTAL Castilla-La Mancha	**36.49**	**ij**	**34.37**	**j**	**41.92**	**i**	**54.20**	**i**	**50.73**	**i**
Río Jiloca	38.40	de	31.04	ab	41.20	a-c	51.62	a-c	53.12	b-d
Muniesa	33.74	b-e	29.20	a	37.89	a	49.16	a	53.62	de
Province of Teruel	38.20	xy	30.83	uv	40.82	u	51.34	6u	53.18	vx
TOTAL Aragón	**38.20**	**j**	**30.83**	**ij**	**40.82**	**i**	**51.34**	**i**	**53.18**	**ij**

The mean was calculated taking into account the number of samples collected in each place. The mean with different letters in a column shows differences at a level of significance of 5% according to Duncan' s test. Letters a,b,c,d and e are used for comparison between districts; u,v,x and y for comparison between provinces; and letters i,j and k for comparison between Castilla-La Mancha, Arargón.

a* values, eight units, and b* values, twenty units lower. This means that the coordinate which increased most on grinding was b*, indicating the yellow component increased more than the red. This was confirmed by the values for the H* parameter which, on grinding, increased an average of 8 units. When saffron stigmas including the style were ground, yellowness increased, redness decreased and the samples showed greater luminosity or brighter colors.

There were significant differences in L* values only between Aragón saffron and powdered samples from India (35.90) and between filament samples from Aragón and Iran (22.20). The non-Spanish samples had lower L* values both in the filament and powdered form, making them darker in color than their Spanish counterparts (total Castilla-La Mancha and total Aragón).

The mean values of a*, were higher in powdered samples from Castilla-La Mancha than the Aragón ones, the highest being those obtained from Landete and in filaments from Sierra. There were significant differences in a* when the samples from Aragón were compared with those from several areas of Castilla-La

Table 3 - Mean chromatic parameters of saffron filament.

Area	L*		a*		b*		C*		H*	
SPANISH SAFFRON										
La Mancha I	26.14	b-d	26.44	c-e	23.87	cd	36.32	cd	41.16	b-e
Sierra	25.80	a-d	28.50	e	23.80	cd	37.34	d	39.81	b-d
Centro	25.70	a-d	26.96	c-e	22.62	b-d	35.28	cd	39.98	b-d
La Manchuela	25.06	a-c	27.99	de	22.56	b-d	36.17	cd	38.64	bc
Hellín	23.60	a-c	26.47	c-e	21.22	bc	33.79	a-d	38.70	bc
Province of Albacete	25.11	uv	27.32	vx	22.61	vx	35.68	x	39.34	vx
Alcázar de San Juan	29.36	d-f	23.24	a-c	24.80	cd	34.15	a-d	47.11	d-f
La Solana	27.43	c-e	24.62	b-e	23.34	b-d	34.25	a-d	43.76	c-f
Province of Ciudad Real	27.82	vx	24.35	uvx	23.63	x	34.23	vx	44.43	xy
Landete	25.06	a-c	26.59	c-e	23.42	b-d	35.53	cd	41.43	b-e
Cuenca	27.09	c-e	26.12	c-e	22.86	b-d	34.31	b-d	41.13	b-e
La Mancha II	26.25	-d	27.71	de	23.48	b-d	36.32	cd	39.95	b-d
Province of Cuenca	26.37	vx	26.99	vx	23.25	vx	35.49	x	40.59	vx
Toledo	32.08	f	20.69	a	25.64	d	33.45	abc	49.72	f
Province of Toledo	32.08	y	20.69	u	25.64	x	33.45	uvx	49.72	y
TOTAL Castilla-La Mancha	**26.58**	**ij**	**26.06**	**ij**	**23.26**	**jk**	**35.22**	**j**	**41.48**	**jk**
Río Jiloca	30.34	ef	21.93	ab	24.58	cd	33.01	a-c	48.13	ef
Muniesa	25.54	c-e	23.13	a-c	23.39	b-d	33.14	a-c	45.42	c-f
Province of Teruel	29.90	xy	22.07	u	24.44	x	33.02	uvx	47.82	y
TOTAL Aragón	**29.90**	**j**	**22.07**	**i**	**24.44**	**k**	**33.02**	**ij**	**47.82**	**1k**

The mean was calculated taking into account the number of samples collected in each place. The mean with different letters in a column shows differences at a level of significance of 5% according to Duncan' s test. Letters a,b,c,d and e are used for comparison between districts; u,v,x and y for comparison between provinces, and letters i,j and k for comparison between Castilla-La Mancha and Aragón.

Mancha. For example, the saffron samples from La Mancha I and Sierra had significantly higher values than those from Aragón in both the filament and powdered form. The same two growing districts in Albacete produced redder saffrons than the two districts from Aragón. The lowest a* values were obtained with saffron in the filament form from the Toledo province. In a previous study, ALONSO *et al.* (2000) observed that saffron from these same districts in Toledo produced a higher percentage of style than all the other Spanish growing districts. This suggested that the lower a* value was due to the increase in whiteness contributed by the styles. This is supported by the higher L* value of these samples. However, other studies have pointed to the technique of detaching the stigma in the Toledo province (ALONSO *et al.*, 1998b; SÁNCHEZ-FERNÁNDEZ, 1996). Among the non-Spanish samples, the highest mean a* values were obtained with Indian samples in the powdered form and with Iranian samples in the filament form.

Generally, there were no significant differences for the b* parameter between the powdered saffron samples, nor be-

tween the filament saffron. However, the highest b* values were obtained from powdered samples of Indian saffron (51.32), whereas in the filament form Toledo had the highest mean value and India (16.25) and Iran (20.20) had the lowest values. As in the case of parameter a*, the greater prevalence of the style in the saffrons from this growing area might explain this finding.

The highest C* values in Spanish saffron were obtained in Castilla-La Mancha samples, particularly those from the powdered saffron from the Mancha I growing area, which also had the highest mean values and showed significant differences from the Aragón samples. The highest mean C* value in samples in filament form was obtained from Sierra saffron in Castilla-La Mancha. Among the non-Spanish samples, the highest mean values of C* in powdered saffron were in the Indian samples (61.89), while the filament form from Iran (34.83) had the highest values, although these latter values were still below the Castilla-La Mancha mean.

The H* coordinate in powdered saffron exceeded 45º in all the samples, confirming the predominance of yellow over red. However, in the filament form the saffron from all the areas of the Albacete province, Cuenca province and other countries had H* values below 45º, indicating the predominance of the red component (+a*) over the yellow one (+b*) (CIE, 1976). Among the Spanish saffron, the mean H* values measured in Castilla-La Mancha samples, as powdered and filament forms, were below those found in samples from Aragón. Taking into account all the samples, the highest mean values for H* in powdered saffron were obtained in Italian (55.53) and Indian (54.79) samples, and as filaments, samples from Toledo were the highest.

Among the chromatic characteristics, C* and H* best represented the color perceived since they provide information on both pureness and hue of color. Accordingly, the samples from Castilla-La Mancha, particularly those from the Albacete and Cuenca provinces, had the purest color and reddest hue. It has always been accepted that the saffron from the above two provinces has the highest quality, partly due to the high stigma content and relatively small style.

As there are categories for coloring power, different categories are proposed, taking into account the colorimetric distance. The colorimetric distance was calculated in relation to a saffron sample from La Manchuela which had the highest coloring power (273) and its CIELab parameters were: 25.38 for L_o*, 29.08 a_o* and 21.64 for b_o*. The projection in the plane for a* and b* parameters is shown in Fig. 1 with the limits of each colorimetric category. Such categories were selected by a previous ANOVA treatment for saffron from the different provinces, resulting in 4 categories (Albacete and Cuenca, Ciudad Real, Toledo and Teruel). Such province division corresponds to the oldest saffron classification (MORALES, 1945), when saffron was classified visually. To establish the limits of the category, the mean value of each province was calculated and the medium point between them gave the category limits. This parameter is called the "colorimetric distance" (D). If D is less than 5.78, it is specified as category A; category B has a range of 5.78-8.27; category C is between 8.27-10.70 and D is greater than 10.70. CIELab categories (Table 1) for all samples coincided with the categories established by the ISO 3632 normative. Non-Spanish samples were used to prove the efficiency of the new categories. Only the sample from Italy did not coincide with the ISO category. Nevertheless only one sample was used and this is not representative of the whole country.

Discriminant analysis made it possi-

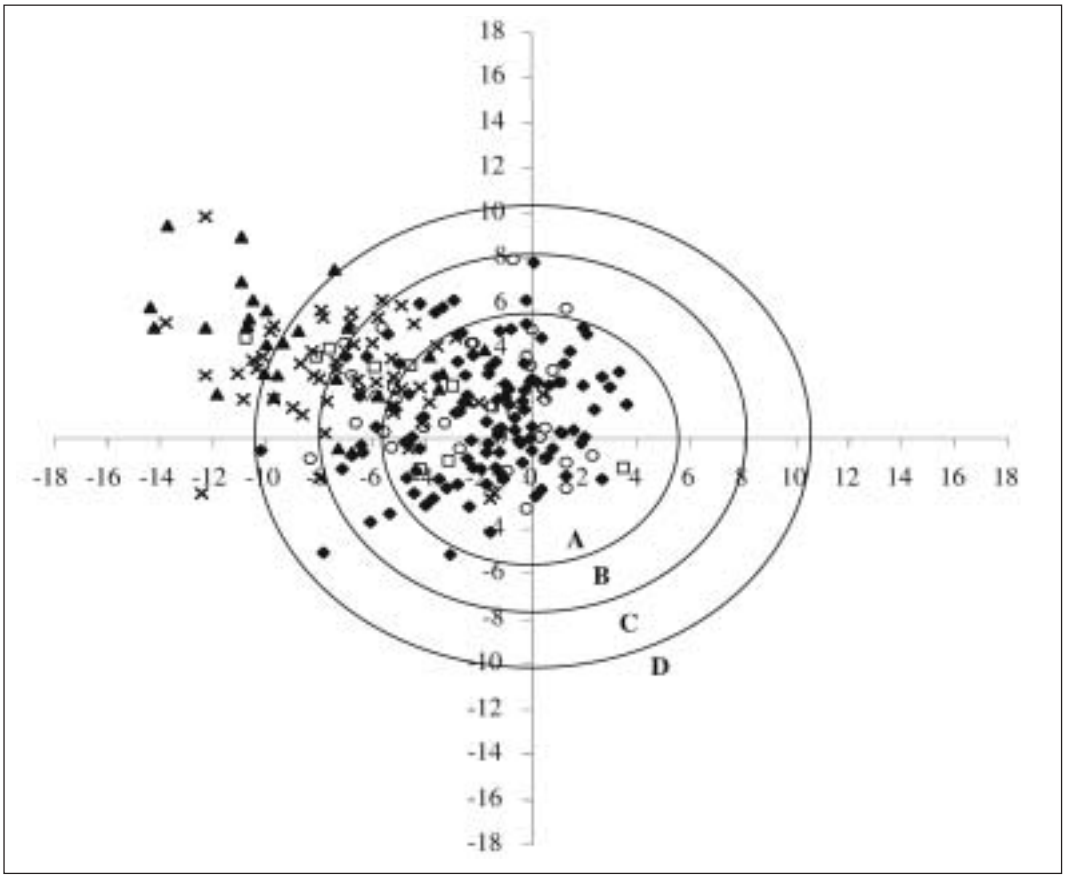

Fig. 1 - Projection of a* and b* parameters in the plane for all saffron samples (□ Province of Ciudad Real; ▲ Province of Toledo; X Province of Teruel; ○ Province of Cuenca; ◆ Province of Albacete; A, B,C and D: proposed categories).

ble to differentiate between saffron in the filament form and the areas of production, such as Castilla-La Mancha or Aragón, with 91% being correctly classified from Castilla-La Mancha and 71% from Aragón. The coefficients of the function were: -0.891 for a*, 0.806 for H*, 0.73 for L*, -0.638 for C* and 0.375 for b*.

CONCLUSIONS

The coordinates L*, a* and b* showed a relationship between the color of the samples, meaning that differences can be used to differentiate between areas of production. A linear correlation between the chromatic parameters and coloring power was obtained with the samples in the filament form. The range of variation in coloring power and chromatic parameters was established for saffron in the powdered and filament form. The chromatic parameters obtained by reflection might be considered a useful tool for saffron quality control and could perhaps be introduced into the corresponding normatives.

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PAPER

QUALITY IMPROVEMENT OF NATURALLY GREEN TABLE OLIVES BY CONTROLLING SOME PROCESSING PARAMETERS

CONTROLLO DI ALCUNI PARAMETRI DI PROCESSO
PER IL MIGLIORAMENTO DELLE OLIVE DA TAVOLA VERDI AL NATURALE

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ABSTRACT

The results of making some technological corrections, designed to avoid the main processing problems of naturally processed olives are reported. The following processing parameters were kept constant throughout time: salt (2 and 4%), pH (acidification with lactic acid to pH=4), fermenting temperature (25°C) and brine level. Results indicate that pH did not exceed the threshold of 4.5, while there was a weak free, combined and volatile acidity. Sugar and polyphenol diffusion into the brines was

RIASSUNTO

Vengono riportati i risultati di uno studio sull'efficacia di alcuni interventi correttivi con l'intento di cercare di superare i principali problemi durante la trasformazione di olive verdi al naturale. I parametri di processo delle salamoie sono stati tenuti costanti durante tutto il processo per ciò che riguarda: sale (2 e 4%), pH (acidificazione con acido lattico a pH=4), temperatura di fermentazione (25°C) e livello della salamoia. I risultati indicano che il pH non ha superato il valore soglia di 4,5, men-

- Key words: brines, naturally green olives, polyphenols, processing -

higher in the 2% brined olives than in the 4% ones. Gas pockets were not recorded, while a low incidence of irreversible shrivelling was noted only in the 4% brined olives. Panelists rated the olives as excellent for firmness and for residual bitter taste, and expressed a slight preference for 4% olives, because they were saltier.

tre si è avuto uno sviluppo modesto dell'acidità libera, combinata e volatile. Le olive al 2% hanno fatto registrare una maggiore diffusione di zuccheri e polifenoli nelle salamoie, rispetto a quelle al 4%. In entrambe le tesi non si è avuta presenza di "gas-pockets", mentre si è registrata una lieve incidenza di raggrinzimento irreversibile nella tesi al 4%. Gli assaggiatori hanno giudicato eccellenti entrambe le tesi sia per la consistenza, sia per la presenza di un sapore amaro residuo, anche se hanno leggermente preferito le olive trasformate al 4%, in quanto maggiormente salate.

INTRODUCTION

Naturally processed olives are one of the most popular table olive varieties in southern Italy. The olives are harvested at the green or black stage, sorted, size-graded and then placed in an 8 to 14% NaCl brine. This differs from Spanish-style processing, in which the bitterness is totally removed in a very short time (8-12 h) by hydrolysis with a dilute NaOH solution (lye) (BORBOLLA y ALCALÁ and REJANO NAVARRO 1981; FERNANDEZ DIEZ, 1971). In naturally green olive processing there is no debittering treatment. Hence, oleuropein, which is primarily responsible for the unacceptable bitter taste of unprocessed olives, is only partially removed by diffusion from the flesh to the brine or to a lesser extent, by acid hydrolysis (BRENES BALBUENA *et al.*, 1992) over a period of 6 months to one year.

Fermentation is also somewhat different in the two processes, as lactic acid bacteria develop in the brines of Spanish-style olives, while anaerobic yeasts predominate in the brines of natural-style olives (FARRIS *et al.*, 1989). There are also some sensorial differences, compared with the Spanish-style processing, as naturally processed olives retain some bitterness and develop a more acidic taste due to a higher acetic acid content.

The processing of naturally green olives is normally carried out by small-scale producers or, more rarely, on an industrial scale. After years of observation at the processing plants and in our laboratories, the following problems have been noted: a) the pH value of brines and olives is generally higher than 4.5. Since the product does not normally receive any sterilisation treatment, there could be serious safety risks if the sodium chloride concentration (≥7%) and pH values (≤4.5) are not respected. Moreover, high pH values at the beginning of the process favour the growth of Gram-negative bacteria (from contamination of industrial devices in contact with the brine and olives), which cause gas-pockets in the olives and putrid fermentation. b) The salt concentration is always so high at the beginning of the process that it causes shrivelling of the peel and inhibits the growth of lactic acid bacteria, which in turn, does not allow the pH to go below 4.5. Furthermore, the salt concentration is rarely checked during processing. c) Since evaporated brine is rarely replaced, the surface soon develops oxidative

yeasts and moulds. The yeasts consume lactic acid, which results in a higher brine pH, while the moulds could pose risks due to aflatoxin production, even though brines, in general, strongly inhibit mycotoxin-producing fungi (PASTER *et al.*, 1988; GOURAMA *et al.*, 1989). d) Since there is rarely temperature control during processing, severe loss can occur from gas-pockets due to yeasts, even if the pH and salt concentrations are controlled (personal observations). This is true especially when the brine is exposed to sunlight or high temperatures in concrete or fibre-glass tanks.

The lack of scientific data on naturally processed green olives has stimulated our department to conduct research on the technological processing, that could lead to improvement of the quality and safety of the product. Therefore, this study was carried out to determine the effect of: a) acidifying of brines; b) using brines at low salt concentration (<4%); c) controlling brine level; d) controlling temperature.

MATERIALS AND METHODS

Plant material

Olives were selected according to marketing (n. olives/kg, mean weight, % flesh and pit, flesh to pit mean ratio) and technological (percentage distribution in each calliper class) parameters, as well as the suitability of the cultivar for this processing method. For several decades only certain cultivars have been used for processing olives processed in the region of Sardinia; of theses "Tonda di Cagliari" olives were chosen for this investigation. This cultivar has a flesh to pit ratio of 3.65 (the minimum required is 3 for table olives) a mean weight of 4.6 g (216 olives per kg) and a good distribution in the various callipers (80% of the olives were in the 16-17 and 18-19 mm range).

Harvesting and sampling

Ripe green olives were hand-harvested during the first ten days of October in an irrigated grove in southern Sardinia and were immediately transported to the laboratory. Only olives free of blemishes, cuts and insect punctures were selected. The olives were then size-graded with laboratory callipers ranging from 16 to 21 mm in transversal diameter, and were equally divided into three 11 kg replications.

Processing

The olives were washed with tap water to remove dust, placed in 20 L sterilised plastic containers (three per trial) and brined with freshly prepared 2 and 4% (w/w) NaCl brine. The brine was acidified with lactic acid to pH 4.0. Processing was carried out at a temperature not exceeding 25°C. The brine concentration and pH were kept constant throughout the fermentation process. Corrections for NaCl concentration and acidity were made at 5, 9, 12, 16, 22, 30, 45 and 60 days and afterwards at monthly intervals. A perforated cap was used to submerge olives in the brine. Containers were not tightly sealed during the first 10 days, in order to allow the large amount of CO_2 evolving from fermentation and fruit respiration to escape. After this time, the containers were filled to the top with fresh brine and carefully closed, so that air could not enter (to avoid growth of oxidative yeasts and moulds on the brine surface). The brine level was adjusted whenever necessary. The measurements were discontinued at 210 days as most of the assessed parameters had reached a steady state. It must be noted that all four processing parameters (pH, NaCl, temperature, lactic acid) were modified simultaneously, because the results were inconsistent, if one parameter was changed at a time.

Determinations

The following determinations were carried out on the brine: pH with a glass electrode (Orion mod. 420, Beverly, USA), free (g of lactic acid per 100 mL of brine), combined (mEq/L) and volatile acidity (g of lactic acid per 100 mL of brine), and reducing sugars (g of glucose/100 mL of brine), according to GARRIDO FERNANDEZ *et al.* (1997). The free acidity value obtained was corrected by subtracting the initial and subsequent amounts of lactic acid added. Polyphenols were extracted according to BRENES *et al.* (1990) and determined spectrophotometrically at 760 nm (HP 8453, Palo Alto, California), after reaction with the Folin-Ciocalteu reagent (expressed as mg gallic acid/100 g of olive flesh). For pH and free acidity determinations were carried out at the time of each correction time and monthly for the others. The parameters pH, salt, reducing sugars and total polyphenols were determined on homogenised flesh. Sugars, were extracted by centrifuging twice ten grams of flesh with 40 mL of water at 6,000 rpm for 15 min. They were determined using the Fehling method on the supernatant. Polyphenols were extracted according to AMIOT *et al.* (1986) and determined as previously described for brines. Salt concentration was determined as reported previously (PIGA *et al.*, 2001).

Laboratory personnel (10 people) performed an informal taste test at 180 days of brining. They were asked to detect off-flavours and to indicate the trial they preferred. They also judged saltiness, consistency and crispness of the olives. The assessments were reported as written comments and not as ratings. The incidence of gas-pockets and shrivelling was calculated as the percentage of affected fruits per 1,000 olives from each container. Shrivelling was specified as either reversible or irreversible, by placing the olives in water and checking for irreversible (permanence) or reversible (disappearance) shrivelling after 24 hours.

When appropriate, data were subjected to analysis of variance, where brine concentration was the group variable, and means were separated by Duncan's Multiple Range Test at $P<0.01$ level. The tests were replicated three times over three consecutive years and no significant differences were found between the three years. Thus the data from only one year are presented as representative of the three-year study.

RESULTS AND DISCUSSION

Chemical changes in brines and olives

Processing green olives by the traditional method implies using a high salt concentration simply to avoid the growth of putrefactive microorganisms. This may inhibit lactic acid bacteria, cause shrivelling of the peel and limit diffusion of sugar from flesh to brine, thus slowing fermentation and subjecting the olive flesh to attacks by polygalacturonase-forming yeasts. Therefore, monitoring of the pH and salt concentration of the brines is of paramount importance from the technological and safety point of view.

In this study, diffusion of water-soluble compounds from the olives to the brine and salt uptake occurred, until equilibrium was reached. Microorganisms fermented reducing sugars, diffusing from olives, to other compounds (mainly lactic acid). Salt concentration and pH triggered the development of the different microorganisms (bacteria and yeasts). Data related to the pH and corrected free acidity values are reported in Figs. 1 and 2. The correction of pH with lactic acid was beneficial for maintaining safe values, with the exception of the first nine days, when the pH values of the brines reached 4.6, as a result of compounds diffusing from the olive flesh. The pH values ranged from 4 to 4.1 during the first four months and from 3.8 to 3.9 afterwards. The rise in pH during

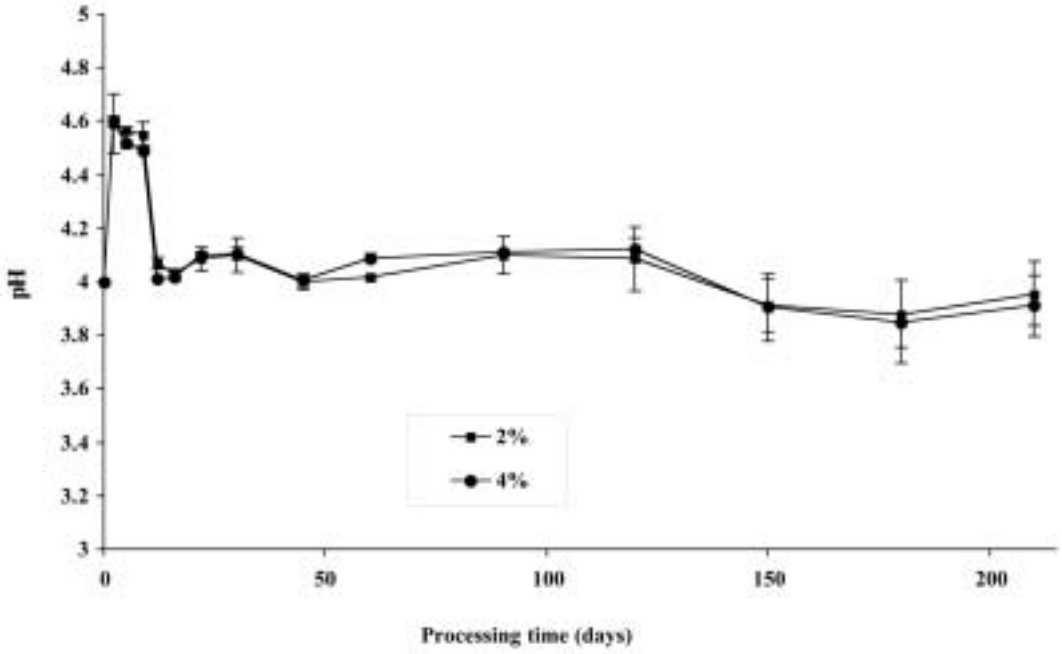

Fig. 1 - Change in the pH of brines of fermented "Tonda di Cagliari" table olives during 210 days of brining. Each value is the mean of nine determinations. Vertical bars indicate standard deviation.

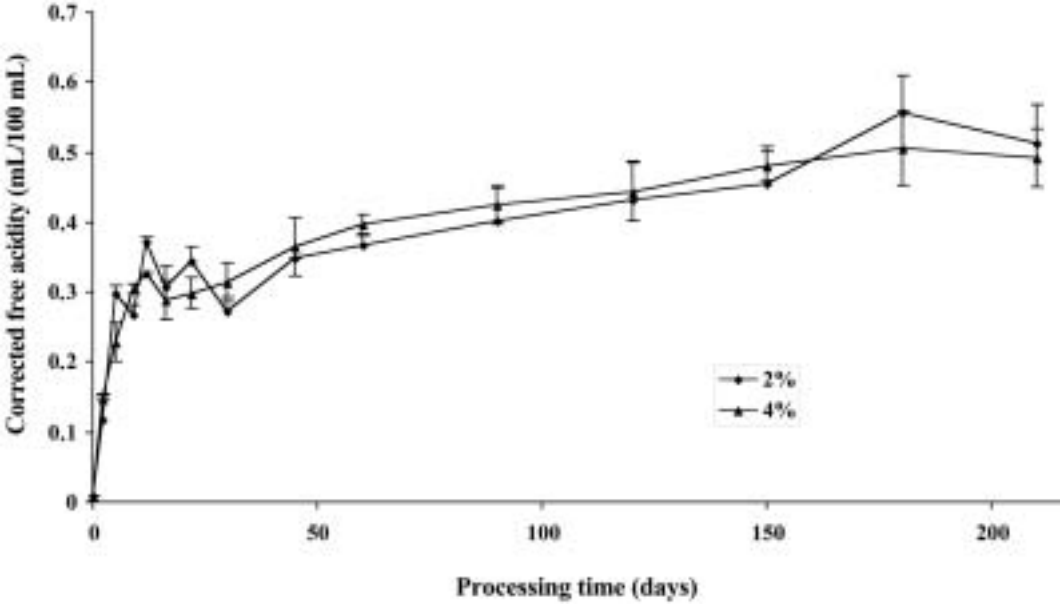

Fig. 2 - Change in free corrected acidity of brines during 210 days of fermentation of "Tonda di Cagliari" table olives. Each value is the mean of nine determinations. Vertical bars indicate standard deviation.

the first 120 days may be ascribed to the increase in combined acidity (Table 1), while the decrease was probably caused by the concomitant reduction in combined acidity and the increase in free acidity (Fig. 2). It should be pointed out that the simple addition of lactic acid, which did not exceed 4 mL/L of brine during the 210 days of processing, was sufficient to adjust the pH in the brines. Natural acidification in the brines, sufficient enough to save further pH corrections, did not occur. This was surely due to the microorganisms that were able to grow in the brines. Green olives, are rich in polyphenols, whose inhibitory effect on lactic acid bacteria (LAB) has been demonstrated (JUVEN and HENIS, 1970; RUIZ-BARBA *et al.*, 1990). Since diffusion of fermentable substrates in these untreated olives is very slow (as will be seen below), LAB do not find a favourable substrate on which to grow. Fermentation was therefore probably caused by fermentative yeasts, which are tolerant of high polyphenol levels (BALATSOURAS *et al.*, 1983), but produce small amounts of lactic acid, in contrast to LAB. Analysis of the data concerning corrected free acidity, further demonstrates this hypothesis (Fig. 2). In fact, the brines attained a weak acidity (not higher than 0.5 g of lactic acid per 100 mL of brine), which is evidence of fermentative yeast activity (BRENES *et al.*, 1986; FARRIS *et al.*, 1989; BALATSOURAS, 1990; MARQUINA *et al.*, 1992; BORCACKLI *et al.*, 1993). Since the focus of

Table 1 - Changes in combined acidity, volatile acidity, reducing sugars and polyphenols in brines at different salt concentrations of naturally processed green "Tonda di Cagliari" olives.

Salt concentration (%)	Sampling (days)	Combined acidity (mEq/L)	Volatile acidity (%)	Reducing sugars (%)	Polyphenols (mg/100 mL)
2	15	16.1±0.42[a]	0.018±0.001	<0.1	39.5±4.48
4		14.7±0.42	0.018±0.002	<0.1	39.4±1.18
2	30	22.0±1.69	0.027±0.001	<0.1	69.2±6.40
4		20.4±0.56	0.027±0.001	<0.1	69.0±1.97
2	60	25.5±0.49	0.0315±0.006	<0.1	96.8±8.11
4		22.6±1.69	0.036±0.001	<0.1	97.7±2.28
2	90	31.1±1.41	0.065±0.02	0.11±0.01	110.9±4.24
4		32.3±0.28	0.04±0.02	0.12±0.001	118.2±4.17
2	120	42.5±2.56	0.065±0.02	0.15±0.01	122.0±13.25
4		40.2±1.23	0.07±0.02	0.14±0.05	165.0±34.60
2	150	40.8±4.52	0.08±0.04	0.14±0.05	171.1±11.40
4		36.8±0.98	0.085±0.04	0.13±0.04	161.8±5.27
2	180	31.2±2.82	0.095±0.04	0.13±0.04	162.5±3.51
4		32.56±0.68	0.155±0.001	0.11±0.001	161.6±5.48
2	210	35.2±4.52	0.115±0.05	0.13±0.04	165.1±5.64
4		36.2±1.02	0.15±0.06	0.11±0.04	166.7±21.74

[a] Each value is the mean of nine determinations (three per each container) plus or minus standard deviation.

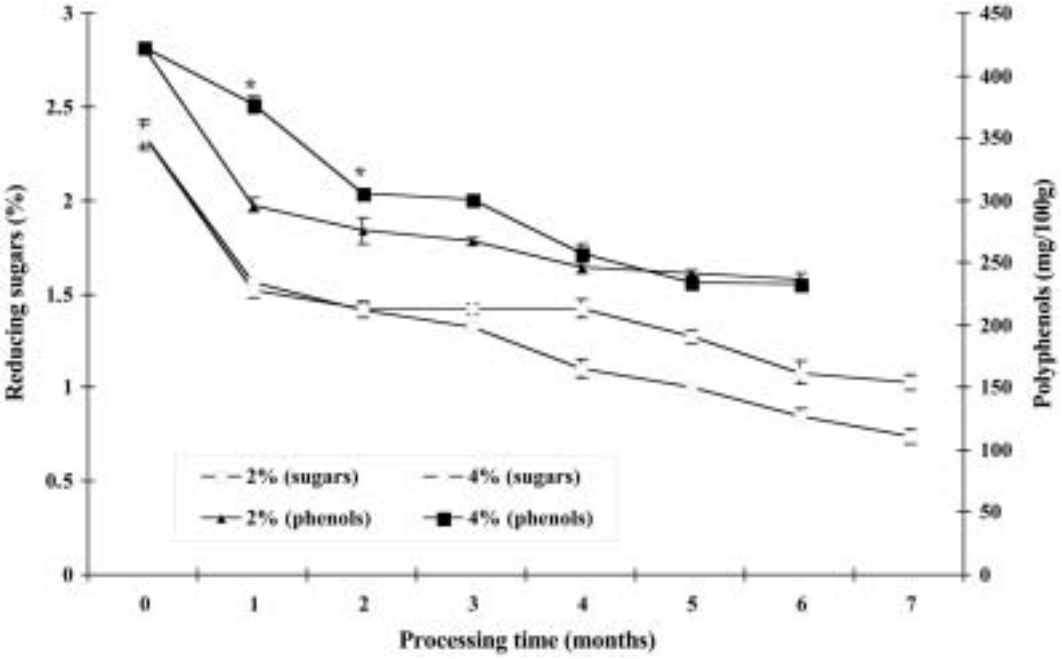

Fig. 3 - Changes in the reducing sugars and polyphenol content in the flesh of fermented "Tonda di Cagliari" table olives during 210 days of brining. Each value is the mean of nine determinations. Vertical bars indicate standard deviation. * Significantly different means within each period.

this study was mainly on technological aspects, microbiological analyses were not conducted, but are projected in future studies. The pH values and corrected free acidity showed no significant differences between the two trials.

As expected, the 2% brined olives exhibited a very slow salt uptake, while salt uptake was more pronounced in the 4% trial and equilibrium was never reached (data not shown). DRUSAS *et al.* (1988) found that the diffusion coefficients of untreated olives were about one fifth of those of alkali-treated ones. At the end of the process, the total amount of salt diffused inside the olive flesh was 0.33 and 1.71 g/100 g for the 2% and 4% trials, respectively. These values are much lower than those encountered with normal processing.

The combined and volatile acidity evolved as expected. The former was about 35 mEq/L at the end of the 210 days for the two trials, while the latter ranged from 0.09 to 0.15 g lactic acid per 100 mL of brine (Table 1). The combined acidity in this kind of process is derived from organic acids in the olives (mainly polyphenolic) as well as from the fermentation process, and reaches the values observed in our experiment and in turning colour and black olives (BORBOLLA y ALCALÁ *et al.*, 1971; FERNANDEZ DIEZ and GARRIDO FERNANDEZ, 1969; PIGA *et al.*, 2001). The volatile acidity was low (Table 1).

The sugar content in the brines was always very low during processing, less than 0.1% during the first two months, which indicates that fermentation proceeded very slowly. Fig. 3 shows that the reducing sugars in the olive flesh decreased. This indicates that fermentable substrates were used by microorganisms as soon as they diffused into the brine. This confirms the hy-

pothesis that reducing sugars were the limiting factor of fermentation. Diffusion of sugars depends on several factors (GARRIDO FERNANDEZ *et al.*, 1997). Skin permeability, salt concentration, temperature and olive-to-brine ratio probably affected the rate of the osmotic process. As expected, the amount of sugars in the flesh was significantly lower from the fourth month of fermentation in the 2% trial (GARRIDO FERNANDEZ *et al.*, 1997), compared to the 4% concentration. The 0.1–0.2% sugar content in the brine after 210 days of processing, and above all the large residual amount in the flesh, may pose problems in storage brines during marketing.

The polyphenol contents in the brine and flesh are shown in Table 1 and Fig. 3, respectively. An equilibrium in the polyphenol concentration between brines and flesh was reached at the fourth month for the 4% brines and at the fifth month for the 2% brines. Statistical analysis showed that the diffusion of polyphenols from the flesh was significantly higher in the 2% brines until the second month of processing, as previously observed by GARRIDO FERNANDEZ *at al.* (1997). In contrast, the same behaviour was not found in the brines. This was probably due to a slightly higher brine-to-olive ratio, which was the result of continuous brine replacement, which led to the dilution of polyphenols.

The main problem encountered by processors of this table olive variety is product loss due to gas pockets and shrivelling. Gas-pockets, or what the Spanish call "afarolado", occur as pressurised gas between the peel and the flesh and result in a transparent pocket. The data in Table 2 demonstrate that gas pocket development was completely inhibited by accurately controlling the temperature and pH of the brines. Shrivelling, was very low and totally reversible in olives brined at 2%, while a slight but significantly higher incidence of shrivelling was found in 4% brined olives. Processors may have up to 30% incidence of irreversible shrivelling when the same olive cultivar is brined at 8%. The results of fermentation with brines of less than 4% salt concentration would appear to be very beneficial for reducing shrivelling loss.

Table 2 - Incidence of gas pockets and shrivelling in "Tonda di Cagliari" olives brined with different salt concentrations after 210 days of fermentation.

Brine (%)	Gas pockets (%)	Peel shrivelling	
		reversible	irreversible[z]
2	0a[y]	0.5b	0b
4	0a	7.8a	3.5a

[z] Irreversible shrivelling was estimated by placing olives in water and checking for permanence of the shrivelling after 24 hours.
[y] Values followed by different letters are significantly different according to Duncan's multiple range test at $P \leq 0.01$.

Good results were also obtained in the sensorial evaluation, as the panelists did not report any off-flavour or off-odour and judged all olives to be excellent for firmness and residual bitter taste. They showed a slight preference for 4% olives, due to a more pronounced saltiness (data not shown).

CONCLUSIONS

Good results were obtained by correcting some process parameters (pH, salt, brine level and temperature) in the processing of naturally green table olives. In particular, safe pH values, a correct fermentation pattern and very good sensorial properties were obtained, with no microbial alterations.

Problems may arise when olives are transferred from the fermentation to the storage brines, where no adjust-

ment of pH is possible after packaging. Since there is a low combined acidity, brines have a weak buffering capacity, so the pH may exceed 4.5, the threshold for pathogen growth. Moreover, sugar residues in the flesh may be a source of microorganisms in storage brines. It is also necessary to shorten the processing time, the length of which is determined by loss of the bitter taste. Pasteurisation can solve the problem of brine stabilisation. Initial olive washing or the oleuropeinolytic bacteria can be used instead of chemicals for fast debittering. These alternatives may be too weak due to the slow diffusion of oleuropein in brines, which, in turn, may not be depleted with washing nor be available for bacterial (CIAFARDINI *et al.*, 1994) or yeast metabolism (BALLONI *et al.*, 1977).

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PAPER

IMPROVEMENT OF PEAR YOGHURT INGREDIENTS USING VACUUM INFUSION

MIGLIORAMENTO QUALITATIVO DI PERA IN PEZZI PER YOGURT MEDIANTE INFUSIONE SOTTO VUOTO

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ABSTRACT

Freezing ensures the long-term preservation of fruits destined for use in fermented milk products, but causes structural damage. Vacuum Infusion (VI), which consists of filling porous fractions of the fruit with an external solution by hydrodynamic transfer, could be used to improve fruit resistance to freezing injuries. VI was studied as a method to improve and maintain the texture of pear cubes also after their addition to yoghurt. Colour and sensory characteristics of both fruit and

RIASSUNTO

La tecnica di Vacuum Infusion (VI), che consiste nella rimozione dell'aria dai tessuti vegetali ed infusione di sostanze funzionali per trasferimento idrodinamico, è stata proposta per ridurre i danni strutturali a carico dei frutti, provocati dai processi di congelamento. Nel presente lavoro è stato studiato come un trattamento di vacuum infusion possa migliorare la consistenza di cubetti di pera da utilizzare come ingredienti per yogurt, e mantenere tale struttura anche dopo aggiunta allo yo-

- Key words: pear, vacuum impregnation, vacuum infusion, yoghurt -

yoghurt during shelf life were also studied. VI significantly improved the texture of pear cubes even during yoghurt storage. Objective data were confirmed by sensory analysis.

gurt. Analisi chimico-fisiche e sensoriali sono state condotte sia sul prodotto finale sia sulla componente frutta durante la shelf life. Il pre-trattamento di VI ha migliorato significativamente la consistenza della pera in pezzi per tutto il periodo di conservazione. I risultati delle analisi chimico-fisiche sono risultati in accordo con quelli dei test sensoriali.

INTRODUCTION

Among dairy products, fermented milk products continue to grow in popularity specifically for their health image, so the use of pieces of fruit in them has been proposed as an alternative to fruit puree. The principal technological problems related to the production process are the low compatibility between milk and fruit pieces and the high risk of microbiological contamination. Usually, fruit pieces have to be frozen at the time of harvesting, in order to be available all year round, and then, at the production stage, they have to be pasteurised in order to avoid contaminating the yoghurt. Freezing and heat treatment are the most crucial points of the process, causing textural damage mainly due to disruption of the cellular structure and loss of turgor pressure (ILKER and SZCZESNIAK, 1990).

Cryostabilization, proposed to preserve frozen fruit quality, consists in the use of cryoprotectants (low molecular weight solutes) or cryostabilizers (high molecular weight solutes) in the formulation to increase the glass transition temperature (Tg') of the maximally cryoconcentrated food liquid phase or to reduce the freezable water content (SLADE and LEVINE, 1991). Reduction of the freezable water content contributes to reducing the damage produced by ice crystals because of the reduction of their volume.

The controlled Vacuum Infusion (VI) process (FITO, 1994; FITO and CHIRALT, 1995) provides interesting prospects in developing pre-treatments to modify (in a short time) the initial composition of porous fruit, making it more resistant to damage caused by freezing-thawing processes. Controlled VI consists of filling porous fractions of the product with an external solution of a desired composition. This is carried out by applying vacuum pressure in the tank containing the products (immersed in the solution) for a short time, and afterwards restoring it to atmospheric pressure while the product remains immersed. The restoration to atmospheric pressure produces a new gradient of pressures that acts as a driving force and makes the solution partially fill the intracellular spaces. The impregnated sample volume fraction has been modelled on the basis of the "hydrodynamic mechanism" (FITO, 1994). Slight sample deformation can occur coupled with the liquid penetration depending on the pressure drop during the liquid in-flow and the stiffness of the solid matrix (FITO *et al.*, 1996). This treatment is directed towards preserving texture by minimising tissue softening due to freezing or pasteurisation processes (MARTINEZ-MONZO *et al.*, 1998; MUNTADA *et al.*, 1998).

In the framework of a demonstration project (FAIR, 2002), this study was carried out to investigate how VI treatment before fruit pasteurisation could improve

the texture of pear pieces and maintain them also after their addition to the yoghurt. The yoghurt made using VI fruits was compared with yoghurt made using the same pear pieces not VI treated (control). The influence of the VI procedure on the texture, colour and sensory characteristics of both fruit and yoghurt during shelf life was studied.

MATERIALS AND METHODS

Raw materials

Pears, cultivar "Abate Fetel", picked at fresh market maturity, were peeled and mechanically cut into 10 mm cubes. Chemical and physical characteristics of the fresh pears were: dry matter 17.5%, refractive index (°Bx) 15, pH 5.0, colour parameters: L* 43.2, a* 0.7, b* 6.7.

Vacuum Infusion

The principal elements of the pilot plant (Niro Soavi S.p.a., Via M. da Erba Edoary, 29/A,43100 Parma, Italy) are: a vacuum vessel, 50 L tank for product treatment, a 40 L agent tank for texture liquid recovery, a 40 L tank for calcium solution recovery and an electrical control and power panel for controlling, monitoring and recording processing parameters. The baskets with fruit pieces were loaded into the processing vacuum vessel and, when the required vacuum level was reached, the texture agent solution was pumped in, so as to completely cover the fruit pieces. Afterwards the atmospheric pressure was restored and the excess solution was transferred to the agent tank.

Vacuum infusion was performed in 2 cycles (MATRINGE *et al.*, 1999; SAUREL, 2001). In the first cycle 10 kg of pear cubes were infused using 25 L of texture agent solution: 25% sucrose (commercial sugar, Beghin Say, Paris, France), 5% alginate (SKW-Satialgine S20, SKW Biosystems, Carentan, France). In the second cycle the infused pear cubes were further infused with 25 L of calcium solution: 25% sucrose (commercial sugar, Beghin Say), 1% dehydrated food grade calcium chloride (Chemie-Plus, Basel, Switzerland). The operating parameters were as follows: vacuum level: 0.1 bar, time to reach the vacuum level: 1 min, time under vacuum after solution intake: 1 min, time to reach atmospheric pressure: 30 sec, temperature of samples: 25°C, temperature of the solutions: 40°C, time of rinsing by spraying with cold water (18°C) after the first and second vacuum infusion cycles: 15 sec.

Freezing

Freezing of the raw and vacuum infused pear cubes was carried out in an air-blast cell (5 kg capacity) operating at - 40°C air temperature and 4 m/sec air velocity. Frozen fruits were stored at -18°C.

Heat treatment

The frozen fruits were heat treated in batch, following an industrial (Kerry Aptunion, Quartier Salignan, 84405 Apt, France) formula to reach a final sugar concentration of 45°Bx: 50% frozen pear cubes (NT) or frozen VI pear cubes, 3% glucose syrup, 32.5% sucrose, water to 100%. The solution containing fruits was heated to 85°C in 15 min, then held at this temperature for 5 min. The solution was then rapidly cooled by immersing the container in an ice bath.

Yoghurt preparation

Heat treated pear preparations were added, under sterile conditions, at 15% content to 500 g of commercial plain yoghurt (Centrale del Latte di Milano, Milano, Italy). The fruit yoghurts obtained were stored at 4°C for 15 and 30 days.

Chemical and physical analyses

Chemical and physical analyses were carried out on the fruits after VI and heat treatment. Chemical, physical and sensory analyses were also carried out on the fruit and yoghurt after 15 and 30 days of storage at +4°C. The following parameters were monitored in duplicate: pH (AOAC 1980; FIL-IDF, 1991a), titratable acidity (AOAC 1980; FIL-IDF, 1991a), dry matter (AOAC 1980; FIL-IDF, 1991b), water activity (a_w), measured using a AQUA LAB CX2 (Decagon Devices, Inc., Pullman, Washington, USA). The refractive index (°Bx) was determined as the average of four measurements, using a CRFM81 refractometer (BS, Tunbridge Wells, Kent, UK). Colour parameters were measured by tristimulus colorimetry using a CR200 Minolta Meter colorimeter (Minolta Camera Co. Ltd., Osaka, Japan). Values reported for fruits are the average of 10 readings made on a double layer of pear cubes arranged on a transparent glass bottom of a 10 cm diameter black-walled cylinder.

Texture was determined with a model 4301 Instron (Instron, High Wycombe, UK), measuring the maximum force (kg) with a standard Kramer Shear Press Cell on 30 g of pear cubes. Data reported are the average of 6 determinations obtained using a crosshead speed of 10 cm/min. Yoghurt viscosity was determined in duplicate at 4°C by using a Haake VT 500, mod. MV3 viscometer (Haake Mess-Tecnik GmbHu. Co., Germany) and applying a speed rate of 80 1/s for 2 min. The shear stress at 0 min (τ_0) was calculated by using a logarithmic equation (SCHRAMM, 1989). The sugar content was determined in duplicate by HPLC under isocratic conditions according to FORNI *et al.* (1992). The extraction step on yoghurt samples was performed according to BOUZAS *et al.* (1991). A Milton Roy CM 4000 HPLC with the following characteristics was used: column: Aminex Carbohydrate HPX-87P 300*7,8 mm (Biorad Labs, Ltd., Richmond, CA, USA) with a pre-column (Biorad); temperature: 80°C; flow: 0,5 mL/min x 30 min; loop: 20 µL; detector: LDC analytical refractometer. Standard mixtures (from 0.1 to 2.0%) of sucrose, glucose, galactose, fructose and lactose were used to make calibrations.

Sensory analyses

The sensory evaluation was carried out through a multiple comparison test (LARMOND, 1977). On two consecutive days, a group of 9 trained panelists evaluated the sensory characteristics of fruit cube consistency in the yoghurt, and the density and colour of the yoghurt.

RESULTS AND DISCUSSION

As shown in Table 1 there were no significant differences in the chemical characteristics and colour parameters between the pear preparations before adding them to the yoghurt (NT, VI). The addition of the pear preparations to the yoghurt caused a slight increase in pH throughout the storage period and in acidity after 30 days. As expected, there was an increase in water activity and a decrease in both dry matter and °Brix values. The increase in acidity after 30 days of storage was higher in the not pretreated pear cubes (NT). This could be linked to a possible buffering action of the compounds used in the vacuum infusion treatment. After 15 and 30 days of storage there was a significant increase in all colour parameters in both NT and VI pear cubes, indicating an overall browning effect. No significant differences were noticed between colour parameters of NT and VI pear preparations except for a* values both at 15 and 30 days of storage, with the VI pears having the highest values.

As indicated in Table 2, the chemical and physical characteristics of the yo-

Table 1 - Chemical and physical characteristics of pear preparations before (NT, VI) and after addition to yoghurt at 15 (NTP15, VIP15) and 30 days (NTP30, VIP30) of storage at 4°C. Different letters indicate significant differences among samples (P≤5%).

PEAR	pH	Titratable acidity (meq/100 g)	Dry matter (%)	°Brix	Water activity (a_w)	Colour parameters		
						L*	a*	b*
NT	4.09a	4.39a	47.0a	44.33c	0.948a	33.35a	0.01c	0.85a
VI	4.09a	4.23a	47.9a	45.17c	0.949a	32.30a	0.01c	0.62a
NTP15	4.25b	4.67a	10.9b	12.20a	0.988b	59.14b	2.09b	6.37b
VIP15	4.31b	4.53a	10.2b	13.87b	0.989b	58.63b	2.60a	7.04b
NTP30	4.28b	6.06c	15.4c	11.94a	0.987b	59.35b	1.83b	7.66b
VIP30	4.26b	5.26b	14.4c	11.42a	0.989b	59.61b	2.69a	8.19b

Table 2 - Chemical and physical characteristics of yoghurt before (plain yoghurt) and after the addition of NT and VI pear preparations at 15 (NTY15, VIY15) and 30 days (NTY30, VIY30) of storage at 4°C. Different letters indicate significant differences among samples (P≤5%).

Yoghurt	pH	Titratable acidity (°SH/50mL)	Dry matter (%)	°Brix	Water activity (a_w)	Colour parameters			Viscosity
						L*	a*	b*	Shear stress (t=0)
plain yoghurt	3.97a	51.29c	12.6a	7.34a	0.984a	82.58c	-2.69a	5.69a	1.04c
NTY15	3.92a	46.35a	16.9b	12.02b	0.985a	80.68b	-2.37c	6.15 bc	0.41a
VIY15	3.88a	47.34b	16.2b	14.45d	0.982a	80.80b	-2.48b	5.97b	0.39a
NTY30	3.89a	48.95b	17.5b	12.89c	0.990a	80.10a	-2.38c	6.35c	0.74b
VIY30	3.88a	49.23b	17.7b	12.88c	0.990a	79.89a	-2.35c	6.19c	0.73b

ghurt, apart from pH and a_w values, were affected by the addition of both NT and VI pear preparations. Titratable acidity decreased while dry matter and °Brix values increased as a result of the exchanges between the fruit and yoghurt. These exchanges also caused a modification in the colour parameters: L* and a* values decreased, while b* values increased throughout storage, indicating a slight yellowing of the yoghurt. There were no significant differences in the chemical characteristics and colour parameters between yoghurts with NT and VI pear preparations. As for viscosity (shear stress) there were no significant differences between yoghurts with NT and VI cubes. After 15 days fruit yoghurts were less viscous than the plain yoghurt before the fruit addition. After 30 days, the viscosity increased but did not reach the same level of plain yoghurt.

As shown in Fig. 1, VI pre-treated pear cubes had higher texture values than NT pear cubes, up to 30 days of shelf life. The texture improvement could be due to greater cell wall integrity linked to the functional solutions used during VI impregnation, in accordance with literature data (MUNTADA *et al.*, 1998). The texture of VI pear cubes increased after their addition to the yoghurt and during storage. This could be due to an enhancement of cell cohesion, linked to the possible pen-

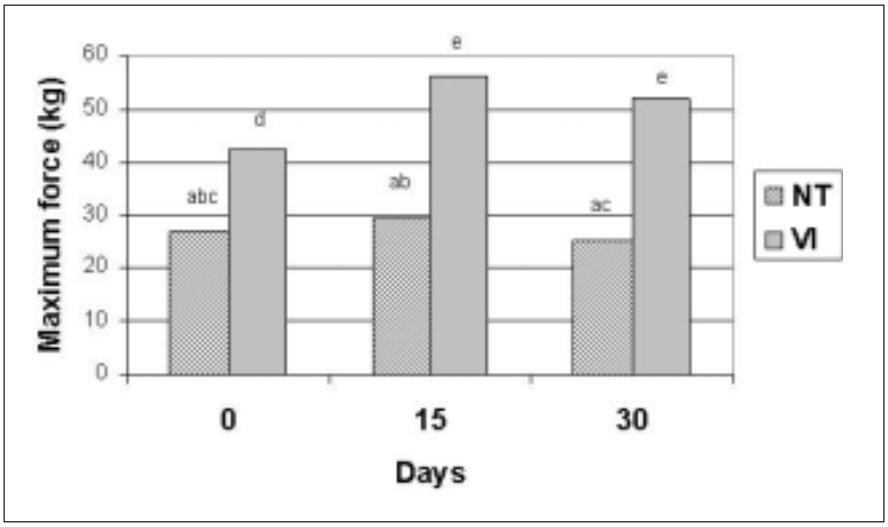

Fig. 1 - Maximum force (kg) values of NT and VI pear preparations before (0 days) and after addition to yoghurt at 15 and 30 days of storage. Bars with different letters indicate significant differences among samples (P≤5%).

etration of calcium lactate (MUNTADA *et al.*, 1998) in the fruit from the yoghurt.

As shown in Figs. 2 and 3, the concentration of lactose and galactose in yoghurt decreased after 15 days of storage due to being absorbed into the fruit pieces. These sugars were replaced by sucrose, glucose and fructose, absent in the commercial plain yoghurt used as control. This indicated a dynamic sugar exchange from yoghurt to fruit and vice versa. A further 15 days of storage did not affect sugar exchange between the fruit and yoghurt, indicating that an equilibrium had been reached.

Texture data of the fruit cubes removed from the yoghurt were confirmed by the sensory analysis (Fig. 4). The panel judged

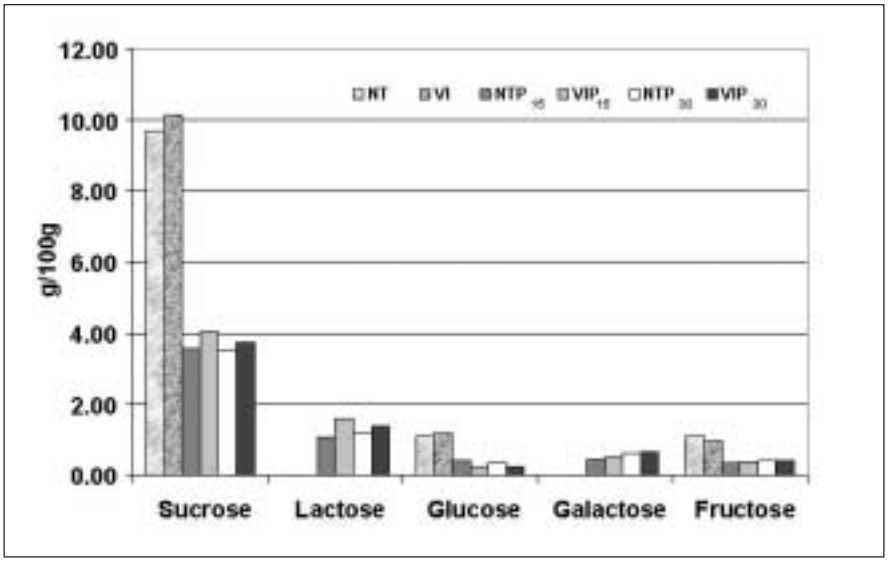

Fig. 2 - Sugar content of the pear preparations before (NT, VI) and after the addition to yoghurt at 15 (NTP15, VIP15) and 30 (NTP30, VIP30) days of storage at +4°C.

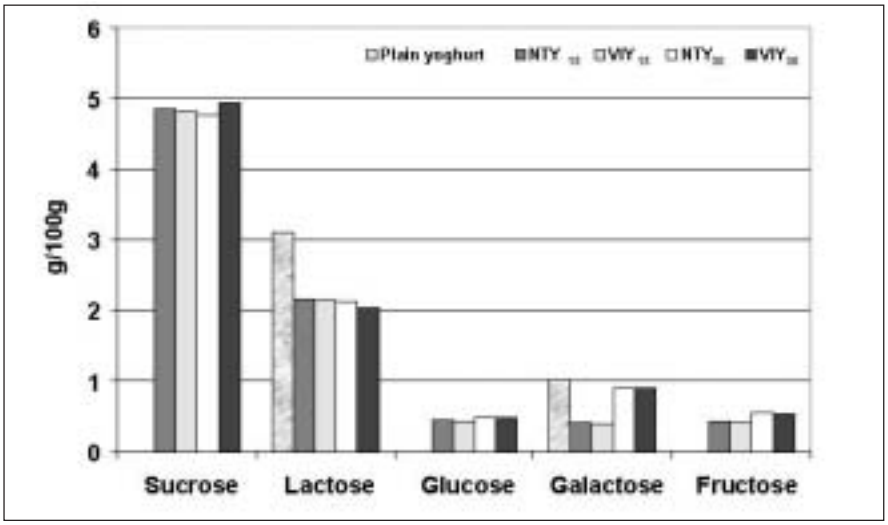

Fig. 3 - Sugar content of yoghurt before (plain yoghurt) and after the addition of NT and VI pear preparations at 15 (NTY15, VIY15) and 30 (NTY30, VIY30) days of storage at +4°C.

the consistency of the VI pear cubes higher than that of the NT ones, throughout the storage period, while the panelists did not notice any significant difference in the density and colour characteristics between the NT and VI yoghurt. The only exception was for NT yoghurt density which was judged higher than that of VI yoghurt at 15 days of storage.

The best correlation (R^2 = 0.99) between sensory and objective indexes was found by plotting consistency data provided by the panelists against the textural data of fruit cubes, showing the actual improvement in pear texture when the VI technology was applied. To evaluate the influence of the improvement in fruit texture due to VI pre-treatment, on

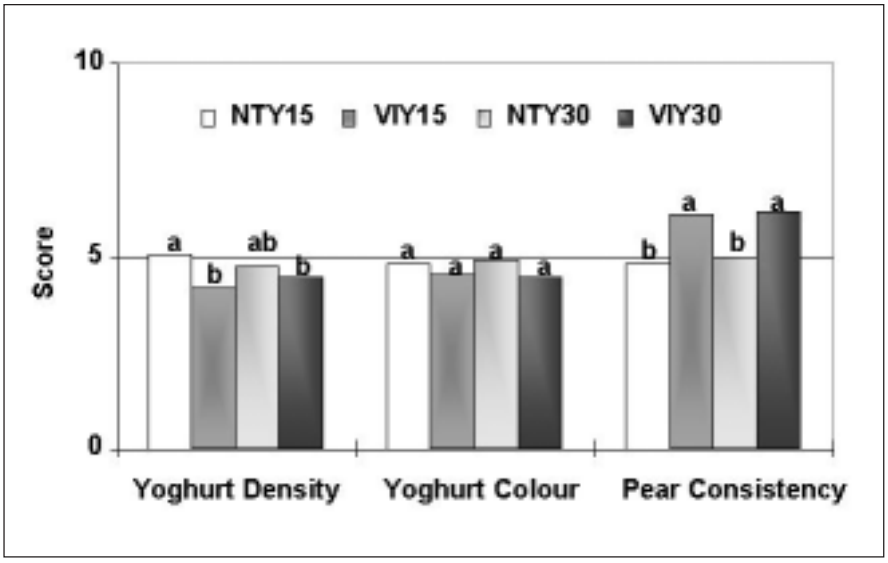

Fig. 4 - Sensory analysis scores of NT and VI pear consistency in yoghurt, and of colour and density of yoghurt at 15 (NT15, VI15) and 30 (NT30, VI30) days of storage at +4°C. Bars with different letters indicate significant differences among samples (P≤5%).

the acceptability of the final product, the panel was also asked to classify the NT and VI pear yoghurt samples based on their personal preferences. Only 14% of the panelists did not like the VI pear yoghurt, 61% preferred it and 25% did not detect any difference between the two pear yoghurts.

CONCLUSIONS

The use of Vacuum Infusion improved and maintained pear cube texture when the fruit was incorporated in the yoghurt as an ingredient and kept up to 30 days of storage. Sensory results confirmed objective data, showing that the added VI pear cubes were characterised by a better consistency than the NT pear cubes. Furthermore, the panel found the VI pear yoghurt more acceptable because of the better fruit consistency.

These results suggest interesting opportunities for the application of VI technology in preparing "new" dairy food ingredients, able to improve not only the quality of the preparations, but also to have a positive effect on the quality characteristics of the final product. This technology now needs to be tested on the pre-industrial scale.

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PAPER

EFFECT OF SOY, MAIZE AND EXTRUDED MAIZE FLOUR ADDITION ON PHYSICAL AND SENSORY CHARACTERISTICS OF PASTA

EFFETTO DELL'AGGIUNTA DI FARINE DI SOIA, MAIS E MAIS ESTRUSO SULLE CARATTERISTICHE FISICHE E SENSORIALI DELLA PASTA ALIMENTARE

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ABSTRACT

The influence of the addition of maize, extruded maize and soy flour and soy/maize flour blends on the chemical and sensory properties of pasta were evaluated. The pasta samples (noodle) were produced by substituting 5, 10, 15, 20, and 25% of soft wheat flour with soy, maize, extruded maize flour and soy/maize flour blends. The pasta was dried by high (HT) temperature drying cycles. The protein and dry gluten content and colour were determined. The following parameters of cooked pasta were deter-

RIASSUNTO

Il presente lavoro studia l'effetto dell'aggiunta di farine di soia, mais, mais estruso e miscele di farina di soia e mais sulle caratteristiche fisiche e sensoriali della pasta alimentare. I campioni di pasta corta sono stati ottenuti sostituendo 5, 10, 15, 20 e 25% di farina di grano tenero con farine di soia, mais, mais estruso e miscele di farina di soia e mais. La pasta è stata essiccata con cicli ad alta temperatura (HT). Sono stati determinati il contenuto in proteine, glutine ed il colore insieme ad alcune proprietà

- Key words: extruded maize / wheat flour blends; maize / wheat flour blends; pasta cooking quality; soy / wheat flour blends -

mined: optimum cooking time (min), volume increase (%) and cooking loss (%). Sensory evaluations were carried out by a group of seven assessors. Pasta samples supplemented with maize and soy flour were evaluated on a scale of 1-5 for four quality parameters: odour, external appearance, flavour and mouthfeel. Based on these results, flours of maize, extruded maize and soy could be used in pasta formulations for many developing countries. The results showed that the highest quality pasta was made from wheat flour with the addition of 25% maize and the pasta produced with the addition of 20% extruded maize flour.

della pasta cotta come il tempo ottimale di cottura, l'aumento percentuale di volume e la perdita percentuale di cottura. La valutazione sensoriale della pasta è stata valutata utilizzando un gruppo di sette degustatori. I campioni di pasta aggiunti di farina di mais e soia sono stati valutati all'interno di una scala 1-5 per i seguenti parametri qualitativi: odore, apparenza esterna, sapore e consistenza. Sulla base dei risultati sperimentali sembra possibile utilizzare le aggiunte di farina di mais, mais estruso e soia nelle preparazioni di paste alimentari destinate ai paesi emergenti. I risultati ottenuti dimostrano che le paste arricchite di migliore qualità sono quelle ottenute con l'aggiunta al grano tenero del 25% di mais o con il 20% di farina di mais estruso.

INTRODUCTION

The use of pasta in the diet will surely increase due to all the tendencies to use healthy food, macrobiotic food and various types of dietetic foods (reduced amount of fats, products without NaCl, increased amount of protein, etc.). In Croatia, pasta is not adequately present in the diet and its annual consumption has been continuously decreasing since 1985. The reasons vary from consumer habits to the reduced assortment of products.

Durum wheat semolina is considered the best raw material for pasta production because of the functional characteristics of its proteins and the high pigment content. Pasta made from durum semolina usually has a good texture, resists surface disintegration and retains a firm structure when cooked. However, not all durum wheat semolina produces pasta of good cooking quality. Many variables are involved in pasta manufacturing, and their role is not completely understood (D'EGIDIO *et al.*, 1990).

The limited availability and the high cost of durum wheat, compared with other cereals, imply that the use of this cereal is of limited interest for many countries. The Croatian market is particularly characterized by egg pasta produced from common wheat flour, which accounts for 80% of the total pasta consumption, whereas in other E.C. countries this accounts for no more than 5-10% of the total pasta market (SEIBEL, 1990). The production of pasta products made from various domestic raw materials, such as maize and soy flours could be of economic significance for many developing countries. Despite the considerable production of maize and soy flours, they are insufficiently used in the human diet in Croatia. Maize is the predominant cereal crop in Croatia, but it is used mainly for animal feed. Recently, more attention has been given to increasing the production of maize hybrids for human food in Croatia.

Maize flour has been proposed for pasta production as untreated and heat-

treated flour with or without soy flour (MOLINA *et al.*, 1975, 1976, 1982; TAHA *et al.*, 1992; KOLEVA JOTOVA and SEIBEL, 1992; WU *et al.*, 1987; BUCK *et al.*, 1987). KESHINRO *et al.* (1993) reported changes in the nutrient composition during the preparation of Nigerian maize products.

Extruded maize flour has been used to improve the structure of pasta. It is known that addition of pregelatinized flours results in better cooking quality (PAGANI, 1986). Soy flour has also been used to produce high-protein pasta products (LAIGNELET *et al.*, 1976; TAHA *et al.*, 1992a,b). It is well known that soy bean protein and fiber have been shown to lower blood cholesterol and alter the distribution of plasma lipid fractions (ERDMAN and FORDYCE, 1989).

In order to produce economic, nutritious and organoleptically satisfactory pasta, the influence of the use of maize, extruded maize and soy flours and maize/soy flour blends, as well as their effect on the chemical and sensory properties of the pasta, were examined.

MATERIALS AND METHODS

Sample processing

Soft wheat flour was obtained from an industrial blend of wheat harvested in the year 2000 and is the raw material normally used in commercial pasta production in Croatia. Defatted soy, maize and extruded maize flour were purchased from a local market. The content of added maize, extruded maize and soy flour, and soy/maize flour blends was 5, 10, 15, 20 and 25%.

Laboratory tests

Chemical analyses of wheat, maize and soy flour were determined using Standard Methods (ICC 1995), for moisture (Method 110/1), ash (Method 104/1), protein (Method 105/2), total dietary fibre (Method 156) and total fat (Method 136). To determine wet gluten, 10 g of flour and 5 mL of distilled water were mixed by hand for about 2 min. The dough was then washed with a solution of 2% NaCl buffered at pH 6.8. The resulting gluten was worked between the fingers until it became tacky and was weighed (Method 106/1).

Pasta production

Pasta (noodle) was produced by mixing 1,000 g of wheat flour or blends of wheat/soy, wheat/maize, wheat/extruded maize and wheat/soy/maize flour in a minipress (Braibanti, Italy), in the pasta factory in Osijek, Croatia. Deionized water was slowly added to give a final moisture content of 32.2%. The mixing lasted 15 min. The resulting dough was extruded to give pasta strands with a length of 15 cm, a breadth of 1 cm and thickness of 2 mm. The pasta was dried in a laboratory drier (Instrumentaria, Croatia) to 13.0% moisture using a two-stage drying cycle. In the first stage, the cabinet temperature was raised from 25° to 55°C during the first hour and held at 55°C for 2 h. In the second stage, the cabinet temperature was raised to 70°C and lowered to 40°C for a total drying time of 8 h. Relative humidity was lowered from 85 to 30% (Fig. 1). The moisture content of the dried pasta ranged from 12.6 to 13.5%. Samples were stored at 20°C before analyses. All pasta samples were produced in duplicate.

Colour measurement

Pasta colour was evaluated by measuring L*, a*, b* parameters by means of a reflectance colorimeter (CR 300 Chroma-metter, Minolta, Japan) on fresh pasta shaped into square forms (10 g), size 7x7 cm, with thickness of 2 mm. Values are the means of seven determinations.

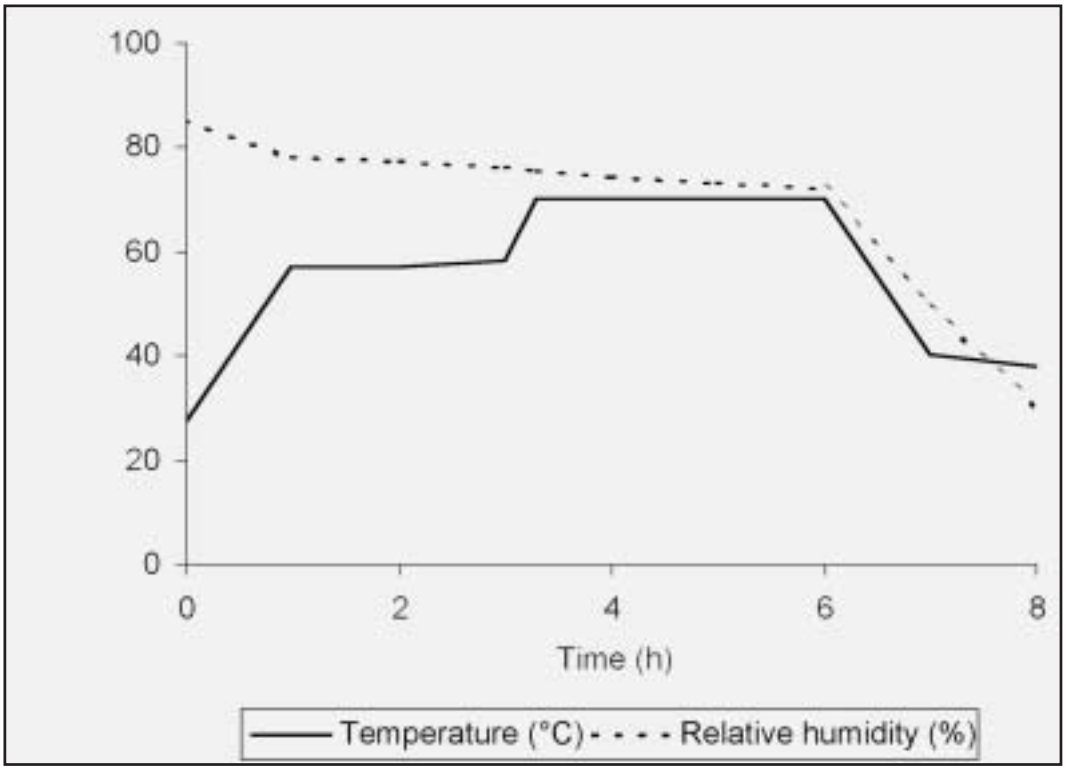

Fig. 1 - Pasta drying profile humidity (%) and temperature (°C) vs. time.

Sensory analysis

The following parameters of cooked pasta were determined: optimum cooking time (min), volume increase (%), cooking loss (%), according to CROATIAN OFFICIAL METHODS (1991). A standard cooking method was used: 100 g of pasta (strands of 15 cm length, breadth of 1 cm and of 2 mm thickness) was cooked in 1 L of salted (5 g/L NaCl) boiling tap water to optimum time, which was defined as the time associated with the disappearance of the white core in the center of the pasta. Pasta volume increase was calculated as the ratio of the volume of cooked pasta to that of raw pasta. Cooking loss was defined as the mass of solids lost in the cooking water during boiling. Sensory assessment was made by a trained panel of seven assessors. Panelists were selected in a preliminary session and were experienced with product and terminology. Pasta samples supplemented with maize and soy flour were evaluated on a scale of 1-5 for four quality parameters: odour, external appearance, flavour and mouthfeel, according to Table 1.

The data are the averages of at least two determinations and the mean standard deviation (SD). The differences between the control sample and samples with supplements were determined by the t-test.

RESULTS AND DISCUSSION

Ash, protein, fat and total dietary fiber contents of wheat, maize and soy flours are listed in Table 2. The highest content of protein (47.6%), ash (5.52%),

Table 1 - Scoring sheet for cooked pasta samples.

Parameter	Score				
	5	4	3	2	1
Odour	Odour characteristic of pasta	Weak odour of pasta, less odour of the raw material (flour, eggs)	Very weak odour, strong odour of the raw material	Weak sour odour, strong odour of additives (eggs, etc.)	Very strange odour not characteristic of cooked pasta
Appearance (stickiness and resilience)	Pasta is fully resilient, voluminous	Pasta is mainly resilient	Pasta is partially sticky	Pasta is very sticky	Pasta is completely sticky without volume
Flavour	Pasta is of satisfactory full characteristic	Pasta is not aromatic enough	Pasta has weak taste	Pasta has very weak taste	The flavour is not characteristic of cooked pasta
Mouthfeel	Pasta is consistent and firm but neither too firm nor too sticky	Pasta is consistent enough and a little sticky	Pasta is soft and sticky	Pasta is much softer and very sticky	Pasta is very soft and very sticky

Table 2 - Chemical composition (% on dry weight basis) of wheat, maize and soy flours.

Component	Wheat flour	Maize flour	Soy flour
Ash	0.47	0.44	5.52
Protein	9.8*	5.4**	47.6**
Fat	0.7	2.9	3.2
Total dietary fiber	0.7	5.4	8.7
	*N x 5.7	**N x 6.25	***N x 5.71

fat (3.2%) and total dietary fiber (8.7%) were found in the soy flour. Total dietary fiber and fat content were higher in the maize and soy flours than in the wheat flour, whereas the protein content of the maize flour was lower than that of the wheat flour.

The data regarding the protein and gluten content of wheat flour and combinations of supplemented maize, extruded maize and soy flours are given in Table 3. The supplementation of maize and extruded maize flour resulted in a decrease in protein. Supplementation of the soy and soy/maize blends resulted in an increase in protein (9.8 to 18.4% and 9.8 to 13.9%, respectively).

All wheat flour – maize, extruded maize and soy flour blends showed a lower amount of wet gluten. Many research-

Table 3 - Protein (N X 5.7) and dry gluten content (% on dry weight basis) of flour samples supplemented with soy and maize flours.

Component	Maize flour level (%)					
Protein content	0	5	10	15	20	25
	9.8±0.32	9.6±0.21	9.4±0.28	9.3±0.35	9.1±0.32	8.6±0.39
Dry gluten content	8.2±0.21	7.6±0.46	7.4±0.23	7.4±0.31	7.2±0.31	6.9±0.21
	Soy flour level (%)					
Protein content	0	5	10	15	20	25
	9.8±0.32	11.8±0.25	13.4±0.31	15.9±0.25	16.9±0.32	18.4±0.28
Dry gluten content	8.2±0.21	7.7±0.23	7.6±0.32	6.6± 0.34	5.7±0.28	5.3±0.27
	Extruded maize level (%)					
Protein content	0	5	10	15	20	25
	9.8±0.32	9.6±0.31	9.4±0.40	9.3±0.28	8.9±.28	8.8± 0.31
Dry gluten content	8.2±0.21	7.3±0.32	6.6±0.39	5.6±0.32	5.1±0.32	4.8±0.31
	Soy / maize flour blends level (%)					
Protein content	0	2.5/25	5.0/50	7.5/75	10.0/10.0	12.5/2.5
	9.8±0.32	10.8±0.25	11.4±0.28	12.4±0.28	13.2± 0.21	13.9±0.24
Dry gluten content	8.2±0.27	8.1±0.31	7.8±0.34	7.3±0.34	6.8± 0.35	6.2±0.43

$\overline{X}$ ± SD Arithmetic mean and standard deviation.

ers (MATSUO and IRVINE, 1970; MATSUO *et al.*, 1972; Walsh and Gilless, 1971; DEXTER and MATSUO, 1979; GRZYBOWSKI and DONNELLY, 1979) have established that the content and composition of proteins and gluten strength, in particular, are important for the cooking quality of pasta. Low gluten content has a negative effect on the technological and cooking qualities. Widespread use of high temperature (HT) and ultra-high temperature (UHT) drying in the pasta industry has generally improved pasta quality. Pasta of accteptable quality can be produced from raw materials of secondary quality because gluten strength and protein content become less critical when HT or UHT drying is used (YUE *et al.*, 1999).

Colour values

Pasta colour is an important parameter of pasta quality. Consumers prefer bright yellow translucent pasta products. The yellow colour is due to the presence of xanthophyll and other related compounds in the endosperm (LEPAGE and SIMS, 1968). The L*, a* and b* values of pasta samples are presented in Table 4. The L* values of pasta supplemented with soy flour decreased (from 78.57 to 71.12 and 78.57 to 73.16, respectively), indicating a significant increase ($P<0.05$) in the dark-grey colour. This colour was especially evident with the samples containing 25% soy flour. The samples of maize and extruded maize flour were bright yellow (higher b* values and lower a* values). This was due to the higher carotene content of maize flour compared to that of wheat flour. The pasta supplemented with extruded maize flour showed the lowest colour loss after cooking.

Cooking properties

The most reliable test for pasta cooking quality still remains sensory evaluation, because it gives an overall assessment of its characteristics, even if subject to individual bias (CUBADDA 1988; MATSUO 1988). Cooked pasta must be

Table 4 - Colour indices of pasta with soy and maize flour addition.

Parameter		Maize flour level (%)					
		0	5	10	15	20	25
$\bar{x}$	L*	78.57	79.73	78.32	77.96	79.33	80.90
	a*	-2.12	-2.32	-2.89	-2.66	-3.21	-3.60
	b*	14.11	17.49	19.67	20.57	23.10	24.46
SD	L*	0.37	0.98	0.45	1.82	0.54	0.19
	a*	0.10	0.08	0.09	0.09	0.06	0.08
	b*	0.13	0.29	0.38	0.38	0.29	0.43
		Soy flour level (%)					
		0	5	10	15	20	25
$\bar{x}$	L*	78.57	75.98	76.09	73.14	71.06	71.12
	a*	-2.12	-0.60	0.54	1.68	2.53	3.50
	b*	14.11	19.41	22.97	25.57	26.71	29.36
SD	L*	0.37	0.68	0.58	1,13	0,92	0.77
	a*	0.10	0.05	0.10	0.17	0.07	0.05
	b*	0.13	0.42	0.19	1.46	1.73	0.22
		Extruded maize level (%)					
		0	5	10	15	20	25
$\bar{x}$	L*	78.57	78.22	78.75	77.04	78.70	77.95
	a*	-2.12	-3.12	-3.68	-4.10	-3.91	-3.66
	b*	14.11	20.07	25.46	28.21	30.94	32.31
SD	L*	0.37	1.60	1.00	0.42	0.15	3.89
	a*	0.10	0.14	0.17	0.09	0.06	0.39
	b*	0.13	0.41	0.32	0.36	0.18	1.73
		Soy/maize flour level (%)					
		0	2.5 / 2.5	5.0 / 5.0	7.5 / 7.5	10.0/10.0	12.5/125
$\bar{x}$	L*	78.57	70.60	71.63	72.41	73.50	73.16
	a*	-2.12	-1.39	-0.70	0.15	0.78	1.35
	b*	14.11	17.56	20.38	22.92	24.53	26.25
SD	L*	0.37	0.76	0.72	1.02	0.46	0.29
	a*	0.10	0.01	0.05	0.11	0.01	0.05
	b*	0.13	0.23	0.15	0.24	0.18	0.10

*Arithmetic mean and standard deviation of seven determinations.

firm, resilient and nonsticky for maximum consumer acceptance. The volume increase of cooked pasta caused by cooking water absorption was generally higher for the samples with maize flour and the soy/maize blend. On the contrary, pasta samples containing soy and extruded maize flour showed lower swelling values than control samples (Table 5). BUCK *et al.* (1987) also reported that pasta containing soy had a lower cooked weight than the pasta with maize gluten meal. The results of cooking time showed that the optimum time increased for the flour blends with extruded maize flour. These increases were not statistically significant. Cooking time might play an important role in determining stickiness of cooked spaghetti, since increasing cooking time results in reduced firmness

Table 5 - Quality parameters of cooked pasta.

Sample	Level of supplement (%)	Volume increase (cm^3)	Cooking time (min)	Cooking loss (%)
Wheat flour	0	190	7.30	11.35
Maize flour	5	185	7.48	10.90
	10	175	7.30	9.52
	15	195	7.21	9.32
	20	200	7.20	10.34
	25	205	7.35	9.57
Soy flour	5	175	7.23	11.88
	10	178	7.42	13.20
	15	185	7.30	14.08
	20	170	7.21	13.84
	25	180	7.31	13.54
Extruded maize	5	180	7.80	10.72
	10	170	8.00	10.90
	15	150	8.50	11.85
	20	160	8.30	11.40
	25	155	8.35	12.30
Soy / maize flour	25/25	210	7.59	10.93
	50/50	200	7.6 0	9.90
	75/75	200	7.80	11.40
	100/100	210	7.80	12.80
	125/125	205	7.50	12.21

Arithmetic mean of three determinations.

and resilience of cooked spaghetti (DEXTER and MATSUO, 1977; GRZYBOWSKI and DONNELLY, 1979). According to Menger (1980), taste panel results showed that cooked pasta tends to become sticky as cooking time increases. In contrast, DEXTER *et al.* (1983) found that all samples, tested instrumentally, exhibited a significant ($P \leq 0.01$) tendency to decrease in stickiness with increased cooking time. Among the blends, cooking loss ranged from 9.32% for pasta containing 15% maize flour to 14.08% for pasta containing 15% soy flour. Cooking loss is undesirable and according to WU *et al.* (1987), it should not exceed 10% of the dry weight. The highest cooking loss occurred in the samples with soy flour addition. BUCK *et al.* (1987) also reported a high cooking loss for soy flour blends.

Sensory evaluation

Mean scores of the sensory parameters: odour, appearance, flavour, mouthfeel and total quality score are shown in Table 6. The results of the sensory evaluation indicated that the addition of maize and soy flour was preferred to the control samples (without maize and soy addition). Among the blends, the samples processed from flour with the addition of 25% maize and 20% extruded maize flour had the highest total sensory score (4.6), while the control sample had the lowest score (3.9). Pasta containing soy flour had lower scores for odour (3.0 for sample with 25% soy and soy/maize flour addition). Several panel members commented that the pasta with soy flour addition had an objectionable odour.

Table 6 - Total quality scores of pasta samples from the blends of maize and soy flours.

Sample	Level of supplement (%)	Odour	External appearance	Flavour	Mouth feel	Total quality score
Wheat flour	0	4.5 ± 0.35[a]	3.0 ± 0.35	4.0 ± 0.35	4.3 ± 0.27	3.9 ± 0.15
Maize flour	5	4.7 ± 0.27	3.3 ± 0.27	4.3 ± 0.27	4.5 ± 0.35	4.1 ± 0.26
	10	4.7 ± 0.27	3.5 ± 0.50	4.6 ± 0.42*	4.6 ± 0.42	4.3 ± 0.34*
	15	4.8 ± 0.27	3.7 ± 0.27*	4.8 ± 0.27*	4.7 ± 0.27*	4.4 ± 0.19*
	20	4.9 ± 0.22	3.9 ± 022*	4.9 ± 0.22*	4.8 ± 0.27*	4.6 ± 0.22*
	25	4.9 ± 0.22	4.0 ± 0.35*	4.9 ± 0.22*	4.9 ± 0.2*	4.6 ± 0.25*
Soy flour	5	4.5 ± 0.35	40 ± 0.50*	4.6 ± 0.22*	4.6 ± 0.22	4.4 ± 0.14*
	10	4.0 ± 0.35	4.0 ± 0.50*	4.7 ± 0.27*	4.7 ± 0.27*	4.3 ± 0.28*
	15	3.5 ± 0.35*	4.0 ± 0.35*	4.7 ± 0.27*	4.7 ± 0.45	4.3 ± 0.21*
	20	3.3 ± 0.27*	4.2 ± 027*	4.5 ± 0.50	4.6 ± 0.42	4.2 ± 0.32
	25	3.0 ± 0.35*	4.3 ± 0.45*	4.0 ± 0.50	4.5 ± 0.35	4.0 ± 0.21
Extruded maize	5	4.5 ± 0.35	3.7 ± 027*	4.0 ± 0.35	4.6 ± 0.42	4.2 ± 0.26
	10	46 ± 0.22	4.0 ± 0.50*	4.1 ± 0.22	4.7 ± 0.27*	4.3 ± 0.29*
	15	4.7 ± 0.27	4.0 ± 0.50*	4.3 ± 0.27	4.7 ± 0.27*	4.4 ± 0.28*
	20	4.5 ± 0.35	4.5 ± 0.50*	4.5 ± 0.50	4.8 ± 0.27*	4.6 ± 0.21*
	25	4.3 ± 0.27	4.5 ± 0.35*	4.5 ± 0.35	4.8 ± 0.27*	4.5 ± 0.19*
Soy / maize flour	25/25	4.5 ± 0.50	3.5 ± 0.35	4.5 ± 0.35	4.5 ± 0.35	4.2 ± 0.22*
	50/50	4.0 ± 0.35	3.6 ± 0.22*	4.6 ± 0.22*	4.6 ± 0.42	4.2 ± 0.24*
	75/75	3.5 ± 0.35*	3.8 ± 027*	4.8 ± 0.27*	4.7 ± 0.27*	4.2 ± 0.24*
	100/100	3.3 ± 0.27*	4.0 ± 0.35*	4.8 ± 0.27*	4.8 ± 0.27*	4.3 ± 0.22*
	125/125	3.0 ± 0.35*	4.0 ± 0.35*	4.6 ± 0.42*	4.8 ± 0.27*	4.2 ± 0.24

[a] Mean value ± standard deviation.
*Samples significantly different (P<0.05) from the control sample (without supplement).

CONCLUSIONS

Based on these results, flours of maize, extruded maize and soy could be used in pasta formulations in many developing countries. An improved yellow coloured pasta product can be processed by blending maize flour with common wheat flour up to 25 and 20% extruded maize flour without addition of egg.

Addition of soy flour in pasta production improves the quantity and nutritional quality of protein, since cereal proteins are deficient in lysine and tryptophan. Improved nutritional quality of products justifies the use of soy flour in pasta production. However, a high level of soy flour has a negative effect on pasta colour and odour. Coloured pasta products can be processed by blending maize with soy flours up to 20% without deterious effects on sensory quality.

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SHORT COMMUNICATION

EFFECTS OF STARTER CULTURE ON THE CHARACTERISTICS OF TYBO CHEESE

EFFETTO DI DIVERSE COLTURE MICROBICHE SULLE CARATTERISTICHE DI FORMAGGIO TYBO

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ABSTRACT

Cheeses prepared with selected mixed starter cultures of *L. bulgaricus* and *S. thermophilus* (LBB+CP2 or LBP+CP2) and those prepared with a single strain of lactobacilli cultures (LBB or LBP), produced with and without sodium formate were compared with respect to organoleptic and microbiological characteristics. During ripening the following analyses were carried out: pH, bacterial counts, proteolysis, organic acid con-

RIASSUNTO

Sono state confrontate le caratteristiche organolettiche e microbiche di formaggi preparati sia con colture miste e selezionate di *L. bulgaricus* e *S. thermophilus* (LBB+CP2 e LBP+CP2) che con colture pure degli stessi microrganismi, con e senza formiato di sodio. Per ogni formaggio sono stati analizzati: pH, conta microbica, attività proteolitica, acidi organici, umidità, analisi sensoriale e strutturale. Le

- Key words: cheese ripening, mixed starter cultures, semi-hard cheese, sensorial analysis, single strain culture, sodium formate -

tent, moisture content, texture and sensorial analysis. Textural properties and proteolysis in cheeses prepared with (*L. bulgaricus* + *S. thermophilus*, LBB+CP2) were similar to those found for cheeses prepared with LBB + sodium formate. The assay of sensorial acceptability indicated that the organoleptic properties of cheeses made with LBB + sodium formate were better than those of cheeses produced with LBB and comparable to those prepared with a mixed starter culture. These results suggest that formate could replace streptococci in cheese production.

proprietà strutturali e l'attività proteolitica del formaggio preparato con LBB + CP2 (*L. bulgaricus* + *S. thermophilus*) erano simili a quelle del formaggio preparato con (*L. Bulgaricus*), LBB + formiato di sodio. Questi risultati mostrano che le caratteristiche organolettiche del formaggio aggiunto di formiato erano migliori di quelle dei formaggi ottenuti con streptococchi e paragonabili a quelli preparati con colture miste. Questi risultati indicano come il formiato potrebbe sostituire l'azione degli streptococchi nella preparazione del formaggio.

INTRODUCTION

Lactic acid bacteria play important roles in the production and preservation of foodstuffs, especially in the dairy industry. Mixed "starter cultures" containing selected strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are generally used in the production of yogurt, soft- and semi-hard cheese. Both species have a synergistic relationship. Proteolytic enzymes produced by *L. delbrueckii* subsp. *bulgaricus* degrade casein, releasing low-molecular-weight peptides and amino acids. These molecules have been identified as growth factors for *S. thermophilus* (MOREIRA *et al.*, 2000). The growth of lactobacilli is stimulated by carbon dioxide and formic acid produced by the streptococci.

Cheese ripening is characterized by a series of complex physical, chemical and microbiological changes that affect the principal components of the cheese (BEVILACQUA, 1997). Texture is one of the features that determines not only the type of cheese but also its quality and acceptability (ATTAIE *et al.*, 1996). Variations in texture may reflect structural changes during ripening. Texture depends on moisture content, pH and degree of casein proteolysis (FENELON *et al.*, 2000; VICENTE *et al.*, 2000; ADAMOPOULOS *et al.*, 2001). Considering the organoleptic properties of cheese, hardness is one of the most important traits of texture regarding consumer preference. Hardness can be defined as the force required to penetrate a cheese sample with the teeth; it can be quantified by an instrumental assay (uniaxial compression test) as the maximum force employed to penetrate the sample. Values obtained for this parameter by both instrumental and sensorial analysis show good correlation if experimental conditions are appropriately chosen (BEVILACQUA, 1997).

MOREIRA *et al.* (2001) reported that a soft cheese prepared with *L. bulgaricus* (LBB) plus 40 mg of sodium formate per kg of milk had soluble nitrogen values, meltability and textural properties that were similar to those of cheese prepared with a mixed-starter culture (LBB+CP2). These results suggested that formate could replace streptococci in cheese production. An assessment of sensory acceptability indicated that formate could supplement single strain cultures and be used instead of streptococci, since the organoleptic properties were similar for both types of cheese.

The strains for mixed-starter cultures were selected on the basis of the results

obtained from a screening of strains found in milk (MOREIRA *et al.*, 1999). An important synergistic effect was observed between strains regarding the rheological properties of the coagulum, good syneresis properties and stimulated streptococci growth produced by lactobacilli (MOREIRA *et al.*, 2000). For the aforementioned reasons, the selected strains of cheese starters were evaluated and the characteristics of the resulting semi-hard cheeses prepared using mixed-starter cultures or single-strain cultures were compared. The effects of sodium formate on the production of a semi-hard, Tybo-type cheese were also investigated.

Tybo is a semi-hard, Argentinean cheese that accounts for 38% of the total cheese production in Argentina (350,000 tonnes produced in 1999). According to Argentinean Food Standards, Tybo is a half-fat product. The curd is scalded and washed, molded, compressed, salted and packaged in a flexible plastic film with controlled gas permeability (BERTOLA *et al.* 1996).

The purpose of this work was to analyze the effects of using sodium formate (SF) in the production of Argentinean Tybo cheese throughout the ripening process. Semi-hard cheeses, prepared with selected mixed-starter cultures, were compared with those made with single-strain cultures with respect to the organoleptic and microbiological characteristics.

MATERIALS AND METHODS

Bacterial strains and growth conditions

L. delbrueckii subsp. *bulgaricus* CIDCA 331 (LBB) and CIDCA 332 (LBP) and *S. thermophilus* CIDCA 321 (CP2) were isolated and identified in our laboratory (MOREIRA *et al.*, 2001). Strains were maintained at -80°C in milk. Stock cultures were propagated in UHT (ultra high temperature) skim milk (12% milk solids, 2.8-2.9% fat and 3.3% protein) (Parmalat S.A, Buenos Aires (1649), Argentina) at 37°C for 18 h and then sub-cultured in milk at 37°C until pH 5 (for 6 to 8 h, to obtain an active inoculum).

Starter preparation

Five different starter cultures were used during the cheese-producing processes.

Starter 1: milk free of sodium formate (SF) was inoculated with an active single-strain culture of *L. bulgaricus* CIDCA 331 (LBB).

Starter 2: milk free of SF was inoculated with an active single-strain culture of *L. bulgaricus*, CIDCA 332 (LBP).

Starter 3: milk plus SF solution, with a final concentration of 40 $mg.kg^{-1}$, was inoculated with an active single-strain culture of *L. bulgaricus* CIDCA 331 (LBB plus SF).

Starter 4: milk free of SF was inoculated with an active mixed-starter culture of *L. bulgaricus* CIDCA 331+ *S. thermophilus* CIDCA 321, (LBB+CP2) in a 1:1 ratio.

Starter 5: milk free of SF was inoculated with an active mixed-starter culture of *L. bulgaricus* CIDCA 332+ *S. thermophilus* CIDCA 321, (LBP+CP2) in a 1:1 ratio.

Starters were incubated at 37°C to reach a final pH of 5.

The initial counts (cfu / mL) of starter cultures added to the milk to start the production were: single-strain cultures, starter 1: LBB, $35x10^6$; starter 2: LBP, $55x10^6$; starter 3: LBB + sodium formate, $40x10^6$ and mixed-starter cultures, starter 4: LBB + CP2, $70x10^6$ lactobacilli / $110x10^6$ streptococci and starter 5: LBP + CP2, $60x10^6$ lactobacilli / $100x10^6$ streptococci.

Cheese production

Tybo Argentinean cheese (MARSILI *et al.*, 1981; AOAC, 1984) was prepared with pasteurized milk (whole milk with

3.3-3.6% fat and 17°D). While the vat was being filled with milk at 34°-35°C, the following were added: $CaCl_2$ (200 g per 1000 L), the different starters being investigated (1%) and cheese color (60-80 mL per 1000 L, yellow color, single strength, Marschall Products, Rhone-Poulenc, Inc., Madison, WI). Then, 300 mL of rennet (Marzyme Supreme, single strength, Marschall Products, Rhone-Poulenc, Inc., Madison, WI) were added and mixed for 2 or 3 min. The coagulum was cut into 1-cm cubes, cooked at 40°-42°C and washed with water at 43°C (100 mL water/L cheese milk, stirred for 15-20 min). The whey was separated from the curds in draining vessels and the curds were pressed (2 kg/cm^2) for 20-30 min, turned, and then put into moulds and pressed (10-15 kg/cm^2) for 3 h. The blocks of curd (3.0-3.5 kg) were soaked in brine (20 g/100 mL, 8-10°C, pH 5.2) for 24 h. Cheeses were packaged in a low-permeability film (LOVAC OT 60-Q bags, Envaril, Chivilcoy, Argentina) and ripened at 10°C for 60 days.

Two batches of cheese were produced with each of the five starters, in two independent experiments. Each batch yielded sixteen to twenty cheese loaves and two loaves from each batch were used to study the evolution of ripening. The sample at day 0 was taken when the cheeses were packaged.

In the cheese produced with SF, the SF was added to the pasteurized milk to reach a final concentration of 40 mg.kg^{-1} and immediately after, the single-strain culture was added. For the production of Tybo cheese prepared with starter 1 (LBB), starter 2 (LBP), starter 4 (LBB+CP2) and starter 5 (LBP+CP2) sodium formate was not added to the milk.

Ripening parameters (0, 30 and 60 days): pH was determined at 25°C using a Cole-Parmer (Jenco Electronics Ltd, Model 1671, Taiwan) combined glass-calomel microelectrode.

Bacterial counts: Counts of aerobic mesophilic bacteria were made on plate count agar (PCA) medium, lactic acid bacteria were counted on the differential medium of LEE *et al.* (1974), moulds and yeasts were counted on YGC medium agar (containing yeast extract, glucose and chloramfenicol).

Proteolysis: Cheese samples were diluted 1/10 (w/v) in tryptone 0.1% (w/v) (MOR-MUR *et al.*, 1982). TCA-soluble nitrogen in cheese was determined according to the method of CITTI (MOREIRA *et al.*, 2001). Proteins were precipitated with TCA at a final protein concentration of 8.0 g/100 mL, followed by centrifugation at 7,000 x g 15 min. The TCA soluble-N was determined in the supernatant using Folin's reagent and expressed as mg Tyr/100 mL. Absorbance at 650 nm was determined using a Shimadzu (Kyoto, Japan) Model 1601 PC UV double-beam spectrophotometer.

Moisture content: was determined as the water loss from a sample placed in a vacuum oven at 80°C until constant weight was achieved. Results are expressed as g water /100 g cheese.

Hardness: Cylindrical cheese samples (1.5 cm in diameter and 1.8 cm thick) were taken from the inside of each cheese using a cork borer-type cutter. The cheese cylinders were compressed to 80% of their original thickness using a 3.5 cm diameter plate at a crosshead speed of 10 cm/min in an Instron Universal Testing Machine (1132 model, Instron Corp., Canton, MA, USA) with a compression cell of 50 kg. Tests were run at 20°C. The uniaxial compression test was performed and the peak height was considered as the hardness value and is expressed in Newtons, N (BEVILACQUA, 1997). Each test was repeated at least six times for each of the two cheese loaves from the same batch, and the mean values are reported.

Sensorial analysis: The following sensorial acceptability assay was carried out: after 60 days of storage, the cheeses were evaluated by a consumer panel of 40 volunteers (20 males and 20 fe-

males). Each block of cheese was cut into pieces of 1.5 cm which were then placed on plates labelled with three digits, and held at room temperature (22°C) for 1 h before being presented to the panelists. Sensory attributes (colour, taste, overall preference and texture) of the cheese were scored on a nine-point hedonic scale (9=like extremely to 1= dislike extremely).

Determination of organic acids in cheese: 7 g from each of the cheese samples were added to 50 mL of 0.009N H_2SO_4 (mobile phase), homogenized, extracted for 1 h with agitation on a shaker (model 75, Burrel Scientific, Pittsburgh, PA, U.S.A) and centrifuged at 7,000 x g for 5 min. The supernatant was filtered once through filter paper and twice through a 0.45 µm membrane filter (Sartorius SM 11606). The filtrate (0.01 mL) was injected into the HPLC column (CALIFANO and BEVILACQUA, 2000). Duplicate analyses were performed on all samples.

HPLC Analysis: A Waters liquid chromatograph (Waters Associates, Milford, MA), equipped with a model 717 autosampler, a model 600 controller, a photo- diode array UV Vis detector (model 996). The UV detector was set at 214 nm.

The analytical method was that described by BOUZAS *et al.* (1991). A cation exchange (Aminex HPX 87 H) column was used (Bio-Rad Laboratories, Richmond, CA), where the operating conditions were: mobile phase, 0.009N H_2SO_4, filtered through 0.2 µm membrane filters (Sartorius SM 11606) and degassed by sonication under vacuum; flow-rate, 0.7 mL/min; column temperature, 65°C.

Statistical analysis

Experimental data were statistically analysed using Systat-Software (Systat Version 5-0, Systat, Inc. USA). The LSD analysis (Least - Significant- Difference Test) was performed at $\alpha<0.05$.

RESULTS AND CONCLUSIONS

Semi-hard, Tybo-type cheese was produced using single strain and mixed-starter cultures as described in Materials and Methods. As shown in Table 1, the cheeses produced using single strain cultures, and mixed-starter cultures or LBB plus 40 $mg.kg^{-1}$ of sodium formate reached the desired pH values (5.7-5.8) at the end of ripening. The initial content of lactic acid ranged from 1,300 to 1,700 $mg.kg^{-1}$ in all cases, with the exception of the cheese produced with the single strain LBB culture, which had a much lower value (130 $mg.kg^{-1}$). After 30 days of ripening, the lactic acid content in cheeses made with mixed-starter cultures was higher with respect to those produced with single strain cultures.

Initially, acetic acid was not detectable in any cheese. After 30 and 60 days of ripening the acetic acid content was similar for all the cheeses, with the exception of the cheeses produced with LBB. The only difference recorded in the total bacterial counts between the cheeses was found in the cheeses made with LBB at 30 and 60 days of ripening. After 60 days of ripening, lactic acid bacteria counts were 10^7 cfu/g for the cheeses prepared with single strain cultures (LBP, LBB or LBB + formate). In cheeses prepared with mixed starter cultures (LBB+CP2 or LBP+CP2), streptococci were one logarithm unit higher than lactobacilli, both at 30 and 60 days of ripening. It was observed that the cheeses prepared with mixed-starter cultures (LBB+CP2) or single strain cultures plus SF (LBB + formate) had lower counts (total bacteria, fungi and yeast, data not shown) than the cheeses prepared with a single strain culture (LBB) (Table 1).

There were significant differences ($\alpha<0.05$) in moisture content between the cheeses prepared with single strain cultures (LBB and LBP), and mixed-starter

Table 1 - Properties of semi-hard, Tybo-type cheese during ripening.

Variables	Ripening time, d	Cheese prepared with starters				
		LBB	LBB+ formate	LBB+CP2	LBP	LBP+CP2
pH	0	6.48±0.02a	6.10±0.01a	6.15±0.03a	5.97±0.12a	6.06±0.08a
	30	5.94±0.01a	5.99±0.07a	5.90±0.02a	5.67±0.15a	5.83±0.04a
	60	5.88±0.00a	5.82±0.11a	5.88±0.01a	5.69±0.12a	5.73±0.02a
Lactic acid	0	129.3±2.8a	1,330±2.5b	1,606±10.0b	1,327.8±72.2b	1,652.5±43.5b
($mg.kg^{-1}$)	30	1,366.4±100a	1,500±60.0a	1,827.5±6.5b	1,465.4±313a	1,873.7±71.0b
	60	1,961.5±38.5a	1,620±62.0b	1,744.5±214b	2,193.6±18.4a	1,805±50.0b
Acetic acid	0	0.00a	0.00a	0.00a	0.00a	0.00a
($mg.kg^{-1}$)	30	89.7±7.3a	30.0±1.2b	25.9±1.55b	31.3±10.0b	20.0±2.0b
	60	111.0±5.0a	19.6±6.6b	24.0±5.0b	26.2±3.5b	17.5±8.5b
Total counts	0	18.0±1.0	25.0±10.0	100.0±5.0	10.0±2.0	250.0±10.0
(10^6cfu/g)	30	540.0±10.0	130.0±30.0	100.0±20.0	64.0±15.0	70.0±10.0
	60	550.0±50.0a	3.0±1.70b	11.0±7.0b	3.6±0.1b	9.0±1.0b
Lactobacilli	0	17.0±3.0	35.0±15.0	8.0±0.70	20.0±10.0	15±0.70
counts	30	25.0±5.0	10.0±2.0	9.0±0.55	40.0±10.0	6.0±0.60
(10^6cfu/g)	60	24.0±4.0a	18.0±8.5a	3.0±0.35b	16.0±3.7a	2.0±0.35b
Streptococci	0	—	—	400±12.0	—	400±21.0
counts	30	—	—	100±9.19	—	150±15.0
(10^6cfu/g)	60	—	—	120±10.0c	—	120±3.5c

(LBB, LBB+SF and LBP) single strain cultures of *L. bulgaricus.*
(LBB+CP2 and LBP+CP2) mixed-starter cultures of *L. bulgaricus* + *S. thermophilus*.
pH values, as well as viable counts and organic acid production correspond to the average of duplicates obtained for each of the two cheese loaves from the same production.
a, b and c: Numbers with the same letters are not significantly different ($\alpha<0.05$), in the same row.
Each production of the cheeses was repeated twice, in two independent experiments.

cultures and LBB plus 40 $mg.kg^{-1}$ of sodium formate at the end of ripening (Table 2).

Regarding proteolysis, TCA-soluble nitrogen values were significantly different ($\alpha<0.05$) in cheeses prepared with LBB + CP2, LBP + CP2 or LBB + SF compared with their corresponding controls (LBB or LBP), after 60 days of ripening. The levels of soluble nitrogen in the cheeses prepared with LBB + SF were similar to those in cheeses prepared with mixed-starter cultures (LBB+CP2). Textural properties were significantly different between cheeses prepared with mixed-starter cultures (LBB+CP2 and LBP+CP2) or LBB + SF and the controls (LBB and LBP) ($\alpha<0.05$). Textural properties and proteolysis in cheeses prepared with LBB + CP2 were similar to those for cheeses prepared with LBB + SF. These results suggest that formate could replace streptococci during cheese production. There was a good correlation between increased TCA-soluble nitrogen during ripening and decrease in hardness.

In terms of overall preference, consumers noted differences between cheeses prepared with a single strain culture, mixed-starter cultures and cheeses prepared with LBB + SF. Similar differences were noted with regard to color, taste and texture (Table 3).

The cheeses made with mixed-starter cultures (LBB+CP2 or LBP+CP2) or a single strain culture (LBP and LBB + SF)

Table 2 - Moisture content, hardness and proteolysis during cheese ripening.

Variables	Ripening time, d	Cheese prepared with starters				
		LBB	LBB+ formate	LBB+CP2	LBP	LBP+CP2
Moisture content	0	52.02±1.46a	46.20±1.80b	49.05±1.05b	53.67±1.22a	47.57±0.62b
$g._{water}$ /100g_{cheese}	30	50.30±0.50a	45.63±0.71b	45.21±1.24b	51.42±0.13a	46.35±1.14b
	60	49.54±0.56a	37.37±0.77b	40.17±0.82b	45.23±0.66a	39.75±1.55b
Hardness (N)	0	10.7±0.7a	45.9±1.9b	27.8±0.1b	16.8±1.4a	28.5±1.0b
	30	10.6±0.6a	33.7±5.1b	24.3±3.1b	11.4±0.9a	25.8±2.0b
	60	6.9±1.1a	17.8±3.3b	17.7±0.6b	5.8±0.25a	11.4±0.8b
TCA-soluble	0	0.83±0.27a	0.75±0.12a	0.13±0.01a	0.19±0.02a	0.24±0.06a
Nitrogen	30	4.69±1.16a	6.16±0.02b	8.77±0.44b	3.96±0.43a	5.87±0.05b
(mg tyr/100 mL)	60	6.68±0.01a	9.15±1.04b	9.42±0.40b	5.32±0.57a	12.10±1.00b

Hardness and moisture values correspond to an average of six replicates obtained for each of the two cheese loaves from the same production.
Values of TCA-soluble nitrogen correspond to an average of four replicates obtained for each of the two cheese loaves from the same production.
a, b and c: Numbers with the same letters are not significantly different ($\alpha<0.05$), in the same row.
Each production of the cheeses was repeated twice, in two independent experiments.

Table 3 - Sensorial acceptability (after 60 days of ripening).

Attribute	Cheese samples				
	LBB	LBB+formate	LBB+CP2	LBP	LBP+CP2
Overall preference	2.08±1.03a	5.54±0.26b	6.59±0.21c	5.16±0.22b	6.54±0.10c
Color	2.05±1.09a	5.55±0.33b	6.55±0.34c	5.06±0.26b	6.52±0.30c
Taste	2.06±1.10a	5.47±0.29b	6.41±0.47c	4.95±0.34b	6.37±0.38c
Texture	2.07±1.10a	5.46±0.26b	6.31±0.63c	4.89±0.21b	6.28±0.62c
GA (1)	2.06±1.08a	5.59±0.28b	6.46±0.11c	5.00±0.25b	6.40±0.35c

Values correspond to an average of forty data entries (non-trained panel composed of 40 people) obtained for two cheese loaves for each of the different cheeses.
Sensory attributes of the cheeses were scored on a nine-point hedonic scale.
a, b and c: Numbers with the same letters are not significantly different ($\alpha<0.05$).
(1) GA (General Acceptability, average of overall preference, color, taste and texture values).

were accepted by panelists during the sensorial assay (General Acceptability ≥5), with the exception of cheeses made with LBB.

The results of the sensorial acceptability evaluation indicate that formate could supplement a single strain culture and be used instead of streptococci, since the organoleptic properties were better than the control cheese (LBB) and comparable to the cheese prepared with a mixed starter (LBB+CP2).

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SHORT COMMUNICATION

PRELIMINARY STUDY ON THE MICROBIOLOGICAL QUALITY OF OSTRICH (*STRUTHIO CAMELUS*) CARCASSES DRESSED IN SMALL ITALIAN ABATTOIRS

STUDIO PRELIMINARE SULLE CARATTERISTICHE MICROBIOLOGICHE DI CARCASSE DI STRUZZO (*STRUTHIO CAMELUS*) PRODOTTE IN MACELLI ITALIANI DI PICCOLE DIMENSIONI

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ABSTRACT

The total aerobic plate count (APC) and *Enterobacteriaceae* were evaluated on the surface of ostrich carcasses dressed in three different medium/small-sized abattoirs, where other domestic and/or farmed wild mammals are also slaughtered. Very similar slaughtering procedures are carried out in these plants, but differences exist in skinning methods and final carcass washing. Samples were taken immediately after skinning and at the end of carcass dressing. The mean values of

RIASSUNTO

È stata valutata la carica microbica aerobia totale e la presenza di *Enterobacteriaceae* sulla superficie di carcasse di struzzo prodotte in tre diversi impianti di macellazione di medie-piccole dimensioni, che macellano anche altre specie domestiche e/o selvatiche allevate. In questi stabilimenti sono adottati procedimenti di macellazione molto simili, ma che differiscono per alcuni aspetti relativi alla spellatura ed al lavaggio finale delle carcasse. I campioni sono stati prelevati subito dopo la spellatura ed alla fine del-

- Key words: Abattoir, carcass, contamination, hygiene, meat, microbiology, ostrich -

the microbial load were relatively low in all carcasses at each of the two processing steps. The skinning procedure assisted by mechanical air inflation did not negatively affect the surface microbial load. In two plants where final external carcass washing is not a practice, there was a slightly higher APC value at the end of carcass dressing with respect to the value obtained immediately after skinning, thus showing a positive effect of the final carcass washing procedure. In order to evaluate the hygienic standard of the slaughter-line, the microbiological parameters specified by Commission Decision 2001/471/EC for bovine, ovine, and swine carcasses also seem to be applicable for ostrich, but more extensive research is needed.

le operazioni di preparazione delle carcasse. In tutte le carcasse sono stati riscontrati valori medi delle cariche microbiche relativamente bassi in ciascuna delle due fasi considerate. L'insufflazione meccanica di aria per agevolare la spellatura non ha provocato alcun effetto negativo sulla carica microbica superficiale. In due impianti, nei quali non viene effettuato il lavaggio finale esterno della carcassa, si è registrato un lieve incremento di carica batterica alla fine del processo di lavorazione rispetto a subito dopo la spellatura e questo indicherebbe un effetto positivo del lavaggio finale delle carcasse. Infine, i parametri microbiologici indicati dalla Decisione 2001/471/CE per le carcasse di animali da macello (bovini, ovini e suini) allo scopo di valutare lo standard igienico della linea di macellazione sembrano applicabili anche alle carcasse di struzzo, ma ulteriori e più ampie ricerche sono necessarie.

INTRODUCTION

The increased consumption of ostrich meat in Italy in the last decade has been an incentive for farmers to raise ostriches (BURLINI, 1996; PIRANI, 2000). However, until few years ago producers could not slaughter these animals because of the lack of legislation governing the slaughtering procedure (BURLINI, 1996; AVELLINI and REA, 1998). In 1998, the Italian Ministry of Health (C.M.S., 1998) gave producers permission to slaughter ostriches either in specific plants or in abattoirs for other animal species (domestic or farmed wild mammals), provided that they were equipped with a suitable slaughter-line. In many cases ostriches are slaughtered at abattoirs for large mammals (cattle and sheep), especially in central Italy where the number and frequency of birds delivered is usually small. The Commission Decision 2001/471/EC (C.D., 2001) specified microbiological parameters for carcasses of slaughter animals in order to monitor the hygienic standard of the abattoir. The aim of this study was to gather preliminary data on the microbiological characteristics of ostrich carcasses dressed in Italian abattoirs and assess whether the parameters laid down by the Commission Decision for large domestic mammals can also be adopted to evaluate the hygienic standard of an ostrich slaughter-line.

MATERIALS AND METHODS

Carcasses of 31 ostriches slaughtered in three different medium/small-sized abattoirs located in central Italy were investigated. Animals (domestic and/or

farmed wild animals) other than ostriches are slaughtered in these abattoirs on different days. One day a week is usually reserved for ostrich slaughtering and not more than 10/12 birds per day are processed. The slaughtering procedures are substantially similar in all the plants. Ostriches are leg hung immediately after stunning until the skinning process is completed, then they are wing hung for further dressing. The differences regarding the de-feathering and skinning methods, and final carcass washing are reported in Table 1. Mechanical subcutaneous air inflation was experimentally adopted only in abattoir C to facilitate the skinning process. The skin was mechanically inflated with filtered air by means of a needle which was manually inserted at the tip of the right wing.

Samples were collected in each abattoir immediately after skinning and at the end of the carcass dressing process, before chilling. Ten carcasses each were sampled in abattoirs A and B, and 11 in abattoir C. Internal and external surfaces of the right thigh were sampled. At each sampling site an area of 20 cm^2 was swabbed (wet and dry cotton wool swabs) in each sampling site. Both the cotton wool swab sticks used for each sampling site were put in a sterile tube containing 10 mL of 0.1% peptone + 0.85% NaCl diluent and kept refrigerated until analyses (within two hours after sampling). Serial decimal dilutions were prepared and poured onto agar plates. Plate Count Agar (PCA, Oxoid, Milano, Italy) was used for total aerobic plate count (APC) and Violet Red Bile (VRBG, Oxoid, Milano, Italy) for *Enterobacteriaceae*. Plates for APC were incubated at 32°C for 48 h, while plates for *Enterobacteriaceae* were incubated at 37°C for 24 h.

The microbiological analyses were performed according to the procedures and methods specified by Commission Decision 2001/471/EC (C.D., 2001).

The raw microbiological data were transformed to $\log_{10}$ cfu/cm^2 and then the statistical analyses were performed. Mean $\log_{10}$ values and standard deviations of APC were calculated and a comparison between the two different dressing steps in each abattoir was performed by using a paired *t* – test (StatView programme, S.A.S., 1997). A value of -0.5 $\log_{10}$ cfu/cm^2 was assumed for all samples in which there was less than 1 cfu/cm^2 in APC.

RESULTS AND DISCUSSION

Samples for the microbiological analyses were collected from the internal and external surfaces of the right thigh, considering that legs are the most valuable part of the ostrich carcass. Moreover, they seem to be particularly exposed to contamination due to the opening cuts and detaching of the skin when they are leg hung and then during evisceration and further handling when they are wing

Table 1 - Carcass dressing procedures used in the abattoirs.

	Pre de-feathering shower	De-feathering	Mechanical air inflation before skinning	Final external carcass washing
Abattoir A	No	manually	No	Yes
Abattoir B	Yes	manually	No	No
Abattoir C	No	manually	Yes	No*

* Only the internal surface (thoraco-abdominal cavity) of the carcasses was washed.

hung. The results of preliminary trials (SEVERINI, 2001) on a few birds slaughtered in one of the abattoirs showed that the external and internal sites of the thigh and the brisket had similar microbiological profiles. In some countries legs are detached immediately after skinning to reduce the risk of being contaminated in the subsequent dressing process (PALEARI *et al.*, 1995). Table 2 shows the mean values of the aerobic plate count (APC) of the carcass surfaces sampled in the three abattoirs. *Enterobacteriaceae* were detected at levels between 0.52 and 1.30 $\log_{10}$ cfu/cm^2 in only four carcasses after skinning (1 in abattoir A and 3 in abattoir C) and in two carcasses at the end of the dressing process (one in each abattoir B and C).

There was a relatively low microbiological contamination in all the carcasses at each of the two steps of the carcass dressing process. Even if reliable statistical analysis cannot be performed due to the limited number of carcasses and the high standard deviation, the data indicate that the differences in the slaughtering-dressing processes did not greatly affect the microbiological quality of the carcass surface. Indeed, the mean APC values recorded immediately after skinning were low and similar, even though a pre de-feathering shower was implemented in abattoir B and mechanical air inflation to assist skinning was adopted in abattoir C. Regarding the use of air inflation to assist skin removal, there were no negative effects on the microbial load. At present, this method is neither considered nor forbidden for ostrich in the EU legislation. This method has already been used on ostrich (MORRIS *et al.*, 1995; PALEARI *et al.*, 1995). Its use on lambs weighing less than 15 kg is permitted in the European Union and no proof of negative consequences has been reported (SEVERINI *et al.*, 2000; E.C., 2001). Further research is still needed to evaluate the advantages and disadvantages of adopting this technology to ostrich and to evaluate the consequences on the microbial load of the carcass surface. Regarding carcass washing, significantly higher ($P<0.05$) APC values at the end of the dressing process with respect to immediately after skinning were recorded for the carcasses of abattoirs B and C, but not for those of abattoir A, where final washing was adopted. The effect of fleece and skin dirtiness on carcass microbiological quality has been reported for slaughter animals, but opinions regarding the efficacy of various methods to reduce skin and carcass contamination are controversial (ELLERBROEK *et al.*, 1993; BISS *et al.*, 1996; SOFOS and SMITH, 1998; E.C., 2001). In this study the pre de-feathering shower did not affect the carcass microbial load but the final carcass washing could have had a positive effect, although the sample numbers are insufficient at this stage to determine a definitive effect. The good microbiologi-

Table 2 - Mean (standard deviation) aerobic plate count (APC) of ostrich carcasses immediately after skinning and at the end of the carcass dressing process ($\log_{10}$ cfu/cm^2).

	Number of sampled carcasses	Immediately after skinning	At the end of carcass dressing
Abattoir A	10	1.34 (1.47)	1.60 (1.05)
Abattoir B	10	0.84 (0.68)a	2.22 (1.02)b
Abattoir C	11	0.98 (0.77)a	1.60 (0.78)b

Different letters in the same row indicate significant difference ($P<0.05$).

cal quality recorded in all the carcasses was probably due to the good hygienic standard of these medium/small-sized establishments and the care that went into processing a limited number of birds per day.

Very few data are available in the literature concerning the microbiological quality of ostrich carcasses and meat, making it difficult to compare these results with others. LEY *et al.* (2001) refer only to pathogenic bacteria, OTREMBA *et al.* (1999) examined leg muscles after cutting and packaging, and other authors (HARRIS *et al.*, 1994; TULLIO *et al.*, 1997; GILL *et al.*, 2000) have considered different sampling sites and/or adopted different methods than those chosen in this study.

In order to evaluate whether the microbiological parameters specified by Commission Decision 2001/471/EC (C.D., 2001) for evaluating the hygienic standard of slaughter-lines for large domestic mammals could also be applied to ostrich, the raw values of the aerobic plate count and *Enterobacteriaceae* recorded at the end of the dressing process were multiplied by a factor of five. The Commission Decision specifies values for samples taken by the destructive method (excision of 20 cm^2 pieces of tissue with a maximum thickness of 5 mm) and stresses that swab sampling removes only a part (often 20% or less) of the total flora present on the meat surface. Therefore, the results of the swab test must be adjusted in order to correctly correlate them with the destructive method. The 5:1 ratio was tentatively applied to meet the recommendations of the Italian Ministry of Health (NOTA MINISTERO DELLA SALUTE, 2002), even though the ratio between the swabbing and excision methods is controversial (GILL and JONES, 2000). Our results show that 84% of the calculated APC values (26 carcasses) fell within the "*acceptable range*" for cattle and sheep (<3.5 $\log_{10}$ cfu/cm^2) and the remaining 16% (5 carcasses) within the "*marginal range*" (3.5-5.0 $\log_{10}$ cfu/cm^2), but 97% (30 carcasses) fell within the "*acceptable range*" for pigs (<4.0 $\log_{10}$ cfu/cm^2) and no carcass was within the unacceptable range (>5.0 $\log_{10}$ cfu/cm^2). The values of *Enterobacteriaceae* recorded in two carcasses (1.83 and 1.88 $\log_{10}$ cfu/cm^2, respectively) were within the "*marginal range*" for cattle/sheep (1.5-2.5 $\log_{10}$ cfu/cm^2) and within the "*acceptable range*" for pigs (<2.0 $\log_{10}$ cfu/cm^2). Overall, the ostrich carcasses had a good microbiological quality, even considering the converted values.

In conclusion, the microbiological criteria indicated in the Commission Decision could be adopted for evaluating the standard of slaughter-lines for ostrich carcasses when the birds are slaughtered and dressed in abattoirs for large mammals. However, further research is needed on carcass sampling methods and their effectiveness, especially when unusual animal species like ostrich are considered.

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AFLATOXIN M_1 OCCURRENCE IN MILK SAMPLES DESTINED FOR PARMIGIANO REGGIANO CHEESE PRODUCTION

PRESENZA DI AFLATOSSINA M_1 IN CAMPIONI DI LATTE DESTINATI ALLA PRODUZIONE DEL FORMAGGIO PARMIGIANO REGGIANO

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ABSTRACT

The occurrence of aflatoxin M_1 in the milk produced in an area of the Emilia region was surveyed from 1993 to 1999. A total of 332 samples were collected from dairy farms delivering milk to factories for Parmigiano Reggiano cheese production. Aflatoxin M_1 was detected in 95.5% of the samples. Twenty-eight (8.4%) exceeded 50 ng/kg, the limit set by the Commission of the European Communities (CEC) in 1998, and 4 exceeded 100 ng/kg. The most contaminated samples were collected (23 out

RIASSUNTO

Negli anni 1993-1999, è stata condotta una indagine sulla contaminazione da aflatossina M_1 del latte prodotto in alcune zone delle provincie di Reggio Emilia e Modena; sono stati raccolti complessivamente 332 campioni, da allevamenti conferenti il latte a caseifici che lo destinavano alla produzione di Parmigiano Reggiano. L'AFM_1 è risultata presente nella quasi totalità dei campioni (95.5%); in 28 (8.4%) di questi il livello superava i 50 ng/kg, limite fissato dalla CEE nel 1998, ma solo in

- Key words: aflatoxin M_1, immunoaffinity, milk, Parmigiano Reggiano, survey -

of 28) in the first two years of sampling, whereas in the later years there was a general trend towards lower aflatoxin M_1 levels.

4 la contaminazione eccedeva i 100 ng/kg. I campioni che superavano il limite vigente sono stati in gran parte (23 su 28) raccolti nei primi due anni di campionamento, mentre negli anni successivi risulta evidente una tendenza generale verso minori livelli di contaminazione da AFM_1.

INTRODUCTION

Lactating dairy cows fed rations contaminated by aflatoxin B_1 (AFB_1) excrete the 4-hydroxylated metabolite aflatoxin M_1 (AFM_1) into the milk. This molecule has shown acute toxic effects similar to AFB_1 in ducklings and rats, but is less carcinogenic by one or two orders of magnitude than AFB_1 in trout, rats and in genotoxicity studies (VAN EGMOND, 1989).

A Tolerable Daily Intake of 0.2 ng/kg b.w. for AFM_1 was calculated by KUIPER-GOODMAN (1990) and this toxin has been categorised by the International Agency for Research on Cancer (IARC) as a class 2B, possible human carcinogen (IARC, 1993). In the assessment of cancer risk, it must be considered that milk is a major constituent of the diet of infants and that young animals have been found to be more susceptible to AFB_1 than adults (VAN EGMOND, 1989). Therefore the presence of AFM_1 in milk and milk products is considered to be undesirable.

Due to this, the Commission of the European Communities (CEC, 1998, 2001) has fixed a limit for AFM_1 in milk and milk products (50 ng/kg for milk and a variable limit for milk products, depending on the concentration caused by the drying process or processing into cheese), which went into effect on 1 January 1999. In this Regulation the Commission stated that "even if AFM_1 is regarded as a less dangerous genotoxic carcinogenic substance than AFB_1, it is necessary to prevent the presence thereof in milk and milk products intended for human consumption and for young children in particular". The Commission has also set a limit for AFB_1 of 5 µg/kg for supplementary feedstuffs intended for lactating dairy cattle (CEC, 1991, 2002). However this tolerance level might not be able to be observed, because in order to produce milk with less than 50 ng AFM_1 per kg, the average daily individual intake in a herd should be limited to 40 µg AFB_1 per cow (VELDMAN *et al.*, 1992). In Italy, the Ministry of Health has furthermore set a limit for AFM_1 of 10 ng/kg for infant foods (MINISTERO DELLA SANITA', 1999).

The occurrence of AFM_1 in milk and milk products world-wide and in Italy was reviewed by PIVA *et al.* (1987), GALVANO *et al.* (1996, 1998, 2001) and PALERMO *et al.* (2001). In Italy, the presence of AFB_1 in feeds is chiefly a problem associated with the use of raw materials like cottonseed, coconut meals and corn by-products imported from countries with a warm and humid climate. The AFB_1 contamination of home-grown products is less frequent and usually low, due to both climatic conditions and better agronomic, harvesting and storage techniques.

Parmigiano Reggiano and Grana Padano are two semi-fat long ripened cheeses manufactured in the Po valley using a similar technique. Both are classified as PDO (Protected Denomination of Origin) and constitute a fundamental element for the agricultural economy of the area (ROSA, 1995). In 1998 the pro-

duction was, respectively, 110,128 and 137,478 t of which about 13% was exported (ASSOLATTE, 1999).

The two producer Consortia are continually committed to guaranteeing various aspects of the cheese quality. One of the objectives is to limit as much as possible the AFM_1 contamination of the milk destined for cheese manufacture. For Parmesan-like cheese, a 5.8-fold increase in AFM_1 concentration, as compared to the milk of origin, has been reported (BRACKETT and MARTH, 1982). The AFM_1 contamination of these cheeses was surveyed by BARBIERI *et al.* (1994) who analysed 200 Parmigiano Reggiano cheese samples and found only 18 positive for AFM_1, with a maximum value of 190 ng/kg; PIETRI *et al.* (1997) analysed 223 Grana Padano samples, most of which (91%) were in the range of 5-100 ng/kg, 15 (6.7%) were in the range 100-250 ng/kg and only one exceeded 250 ng/kg.

Parmigiano Reggiano is produced in small cheese factories in a narrow area of four provinces in the Emilia region: Parma, Reggio, Modena, part of Bologna and part of Mantua in Lombardy. About 90% of the milk produced in this area is destined for cheese manufacturing (ASSOLATTE, 1999). Parmigiano Reggiano has a high market image and top quality is guaranteed by a "production code" issued by the Consortium which, among other things, prohibits the use of silage in the feeding of milking cows; therefore in this area the ration is made up of concentrates (mainly industrially produced) and hay in the winter and of concentrates, hay and fresh forage in the summer. In order to prevent AFM_1 contamination of the milk and consequently of the cheese, the "production code" prohibits the feeding of groundnut, coconut, cottonseed and palm-kernel meals and sets a limit of 3 µg/kg of AFB_1 in the concentrates instead of 5 µg/kg.

Since the early 1990s, voluntary test systems carried out in co-operation between dairy federations and feed manufacturers have been operating in some European countries to ensure that aflatoxin levels in feed and milk are kept as low as possible. (ANONYMOUS 1992, 1997, 1999; BLÜTHGEN and HEESCHEN, 1995; DRAGACCI and FREMY, 1993; SPAHR *et al.*, 2000). The results of this action have been so impressive that milk produced in some regions could be declared of "baby-food quality" (AFM_1 < 10 ng/kg).

The aim of this study is to report the results of a survey carried out between 1993-1999 on the occurrence of AFM_1 in the milk produced in some areas of the provinces of Reggio Emilia and Modena and destined for Parmigiano Reggiano cheese manufacturing.

MATERIALS AND METHODS

Samples

Between the years 1993-1999 (except 1997), a total of 332 milk samples were collected from dairy farms delivering their milk to cheese factories located in the provinces of Reggio and Modena. The milk samples were frozen on collection and kept at -20°C until the time of analysis.

Analysis for aflatoxin M_1

AFM_1 in milk was extracted using an immunoaffinity column (Aflatoxin Easi-extract, Rhône Diagnostics Technologies, Glasgow, UK) according to the method reported by MORTIMER *et al.* (1987). Briefly, 50 g of de-fatted milk was applied to the column which had been previously washed with 20 mL of a phosphate-buffered saline solution (pH 7.4). Then the column was washed with 20 mL of water, and the AFM_1 was slowly eluted with 2 mL of methanol into a glass vial. The final extract was evaporated under a stream of nitrogen and re-dis-

solved in acetonitrile:water (25:75, 1 mL). The extract was filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45 µm) before HPLC analysis.

The HPLC system consisted of a Perkin Elmer 200 (Perkin Elmer, Norwalk, Connecticut, USA), equipped with an ISS 200 sampling system and a Jasco FP-920 (Jasco Corporation, Tokyo, Japan) fluorescence detector set at 365 nm excitation and 440 nm emission. The system was governed by Turbochrom PC software (Perkin Elmer). A Superspher 100 RP-18 column (Merck, Darmstadt, Germany, 4 µm particle size, 125x4 mm I.D.) was employed at ambient temperature, with a mobile phase of water:acetonitrile (75:25) at 1.0 mL/min. Amounts of standard AFM_1 (Sigma-Aldrich, St. Louis, Missouri, USA) between 1 and 70 pg were injected.

The standard stock solution was checked for AFM_1 concentration according to A.O.A.C. method 970.44 (A.O.A.C., 1995) and stored at -20°C when not in use. Quantification was made on the basis of peak areas using the Turbochrom PC software.

Statistics

The normal distribution of AFM_1 content was checked by using the "Proc. Univariate Normal" (SAS Institute, Inc., 1999, Release 8). Since the mycotoxin content was not always normally distributed, the statistical significance of differences between years was examined by a two sample t-test, equivalent to a Wilcoxon rank sum test, using the t-approximation for the level of significance.

RESULTS AND DISCUSSION

The recovery of the method for five replicate samples of skimmed milk spiked at 50 ng/kg averaged 92.0% with a standard deviation of 3.7%. The competency of the laboratory was regularly checked by participation in a national proficiency test (Progetto Trieste c/o Tecna S.r.l.). The limit of detection of the method was 1 ng/kg at a signal-to-noise ratio of 3:1. The results were not corrected for recovery.

The data concerning the AFM_1 concentration of the milk samples collected during the survey are shown in Table 1. AFM_1 was detected in the majority of samples (95.5%). Of the 332 samples analysed, 28 (8.4%) exceeded the 50 ng/kg, limit set by the Commission of the European Communities. Samples with the highest AFM_1 concentration (23 out of 28) were generally collected during the first two years of the survey. The highest percentage of samples exceeding 50 ng/kg (32.5%) occurred in 1993. In 1994 this percentage was lower (16.3%), nevertheless in that year two samples were found with a level greatly exceeding 100 ng/kg (406 and 270 ng/kg). Another highly contaminated sample (397 ng/kg) was found in 1998.

Considering the annual means for AFM_1, a general trend towards lower levels is evident. In fact, the average levels from 1995 onwards were significantly lower with respect to 1993 and 1994. The only partial exception was 1998, when 4 samples exceeded the present legal limit (50 ng/kg).

In a survey carried out on 214 milk samples collected in the same area in the period January 1991- February 1992 (BAGNI *et al.* 1993), the mean AFM_1 concentration was slightly lower than the concentration found in the first two years of the present sampling (24 *vs* 38 ng/kg); the highest amount found was also less (101 *vs* 406 ng/kg, respectively). In the present survey, the concentrations appear to be higher and less homogeneous, however a general trend for lower AFM_1 levels is evident in the later years.

The action of providing information by technicians from the Consortium and Breeder Associations to cheese and milk producers as a consequence of the milk

Table 1 - Aflatoxin M_1 occurrence in milk samples destined for Parmigiano Reggiano cheese production.

Year of sampling		Number of samples with aflatoxin M_1 in range (ng/kg)					Total number analysed	Average concentration (mean±s.d., ng/kg)
		<1	1-10	11-50	51-100	>100		
1993	Number	0	11	18	14	0	43	37±29[d]
	Percentage	0	25.6	41.9	32.5	0	100	
1994	Number	0	13	33	7	2	55	38±63[d]
	Percentage	0	23.7	60.0	12.7	3.6	100	
1995	Number	12	49	2	0	0	63	5±3[a]
	Percentage	19.0	77.8	3.2	0	0	100	
1996	Number	3	26	15	1	0	45	11±11[b]
	Percentage	6.7	57.8	33.3	2.2	0	100	
1998	Number	0	30	32	3	1	66	22±49[c]
	Percentage	0	45.5	48.5	4.5	1.5	100	
1999	Number	0	39	21	0	0	60	10±8[b]
	Percentage	0	65.0	35.0	0	0	100	
Total	Number	15	168	121	25	3	332	18±38
	Percentage	4.5	50.6	36.5	7.5	0.9	100	

Values within a column with different superscripts ([a, b, c, d]) differ at P<0.05.

contamination observed on some farms resulted in a remarkable reduction of the AFM_1 content in the following years. This resulted in mean levels approaching those obtained in other European countries. It is noteworthy that in 1999, the year that the legal limit (50 ng/kg) entered into force, no sample exceeded the limit.

In conclusion, the presence of AFB_1 in feeds for dairy cows and of AFM_1 in milk destined for Parmigiano Reggiano cheese manufacturing must be further monitored. Due to the small size of the cheese factories, the probability that in some periods whole cheeses with AFM_1 levels exceeding 200-250 ng/kg could be produced is low but not negligible.

ACKNOWLEDGEMENTS

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FLAIR-FLOW EUROPE

TRACEABILITY OF FISH

FFE 557/02/SME59

A new and very practical traceability system for captured and farmed fish has been developed by a group of European scientists and industries in the frame of a project financed by the EU. This new system can help fulfil the requirements both from consumers and from new EU regulations and directives, and is also being proposed for a new CEN standard (European Committee for Standardisation, isss@cenorm.be).

The need for this kind of system in the food and feed chains is becoming increasingly important. The root causes of many recent food safety problems have been found in the primary production sector, although the problems are manifested at the other end of the food chain, in the products sold to consumers. Consequently, consumers and food businesses, in particular large retailers and those producing branded goods, are increasingly demanding product traceability to assure their standards and protect their businesses.

As a consequence, the EU adopted a new regulation, the "General Principles and Requirements of Food Law", which will take effect on 1st January 2005, and which the national states must adopt before 1st January 2007. By traceability the EU means "the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into food or feed, through all stages of production, processing and distribution" (articles 3, 18 and 19).

The working groups in this project extended traceability proposals for fish and fish products to cover the vessels, vessel landing businesses and auctions, processors, transporters and stores, traders and wholesalers, retailers and caterers. They also worked out electronic systems to handle the information needed. The information to secure traceability of packs, boxes, pallets or the entire catch is divided into:

- "shall" covering the fundamental traceability information, in particular the handling operator identity and the physical movements;
- "should", information relevant to safety, quality and labelling;

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- “may”, covering information such as methods, ethical aspects or GMP and HACCP data.

A related EU-funded Concerted Action dealing with fish quality labelling and monitoring and coordinated by Dr Joop Luten from the Dutch Institute for Fisheries Research (RIVO), held its final conference last April. For further details on this Action (FAIR-98-4174), you may visit the website at http://www.fqlm.nl/.

Project N.: QLK1-2000-00164 (Tracefish) www.tracefish.org

Contact Details: Mr Petter Olsen, Seafood and Industrial Processing Dept., Fiskeriforskning, Muninbakken 9-13, Breivika, N-9291 Tromsø, Norway, Tel. +47 77 629231; Fax +47 77 629100, e-mail: petter.olsen@fiskforsk.norut.no, URL: http://www.fiskforsk.norut.no/IndexE.htm.

NEW DEVELOPMENTS IN MEASURING EXPOSURE

FFE 561/02/HP60

A project is now underway which will facilitate the assessment of exposure to a range of food chemicals, including food additives, pesticides and nutrients. This new technology is intended for use by regulatory authorities, industries, nutritionists and researchers.

There is a growing demand for guarantees of the safety of food for the purpose of protecting consumer health. It is, therefore, extremely important that risk assessors and regulatory authorities have data and tools that allow insights into all aspects related to the safety of the food supply; including exposure to, for example, pesticide residues, micronutrients and mycotoxins.

The project, called “MonteCarlo” will use powerful mathematical modelling techniques to analyse and assess exposure so that risk (or potential benefit, in the case of assessing nutrient intakes) to health may be evaluated more accurately.

At a simple level, the procedure for estimating exposure to a food chemical (e.g. an additive) is to multiply food intake by the concentration of the chemical in food. If foods containing the chemical of interest are under-reported, then the exposure of the chemical from those foods, and therefore the risk, may be underestimated.

A first objective is to conduct a multi-centre study, using existing national data, to explore the influence of input distributions on model output for the key components of a stochastic model of food chemical intake. These components are food intake, chemical occurrence, chemical concentration, market share, brand loyalty, and correlated foods.

A second objective is to generate databases of true intakes of:

(1) food additives, based on brand-level food consumption and ingredient composition;

(2) pesticide residues, based on duplicate diets, and

(3) nutrients, based on biomarker studies.

The final objective is to use the true databases to validate the probabilistic models and the associated computer code. The project website provides more information on other objectives and expected outcomes.

Project Reference: QLK1-1999-00155 (MonteCarlo) http://www.tchpc.tcd.ie/projects/montecarlo/

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VITAMINS A AND E FOR THE ELDERLY

FFE 562/02/CG58

As elderly population of Europe increases, scientists are attempting to reveal the secret of ageing and to relieve its "symptoms".

Fat-soluble vitamins A and E and carotenoids are at the centre of a project supported by the European Commission with the objective of providing sound scientific evidence about the role of these vitamins during human ageing.

Ageing is often associated with the so-called degenerative diseases, comprising certain cancers, neurological disorders and heart and eye diseases. Most of them implicate dysfunction of the defensive mechanisms of the body, the immune and the antioxidant system. A healthy diet can improve the function of the immune system and the antioxidant defence, and fat-soluble vitamins, including vitamin A, E and carotenoids, are known to play an important role in this sense.

In the project, geographical comparisons are being performed: researchers recruited 300 volunteers in three European countries, and grouped them according to their different dietary habits and life style. The target is to detect differences between countries in the relationship between vitamins/carotenoids and ageing.

Good dietary sources of vitamin E are plant oils and products containing them, like margarine and mayonnaise, with fruit, berries and vegetables also containing some quantities of it. The recommended daily intake is fulfilled for example with one tablespoon of sunflower oil or two tablespoons of rapeseed oil or with small sized coffee cups of nuts. Good sources of vitamin A are liver and dairy products. As for carotenoids, strong-coloured vegetables like carrots, broccoli or tomatoes contain it in abundance. There is no risk of suffering a carotenoid overdose.

Project N.: QLK1-1999-00830 (Vitage)

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NEW BIOPOLYMERS FOR FOOD PACKAGING

FFE 566/02/SME62

Hard cheeses packed in new biopolymers which will give them an extended shelf-life may be on the shelves in the future. Substituting fossil plastic materials by renewable biopolymers may benefit the environment and at the same time improve the utilization of agricultural by-products.

The new biopolymers may be based on proteins like casein, on carbohydrates like starch, cellulose or chitosan, on lipids, and also on polymers from surplus monomers produced in agriculture such as polylactate (PLA), and finally, on bacterial produced polymers from microorganisms grown on waste, like poly 3-hydroxy-butyrate (PHB).

Participants in a new QLK project are dealing with these perspectives for the packaging of hard cheeses: they are developing a new proactive packaging material based on PLA, and are incorporating oxygen scavengers and preservatives encapsulated in cyclodextrins to reduce cheese oxidation (development of rancid taste) and surface growth of moulds. They expect to increase the hard cheese shelf-life from 2-3 months to 9 months, and to produce in pilot scale the new and competitive packaging materials. They also aim to demonstrate, through an environmental impact assessment, the economical and environmental benefits.

Some of the participants were also previously engaged in a completed FAIR Concerted Action, which reviewed the production and application of biobased packaging materials for the food industry. Results from the project are published on http://www.mli.kvl.dk/foodchem/special/biopack/Foodbiopack.pdf.

Project N: QLK5-2000-00799 (Biopack) http://www.biopack.org/

Project Co-ordinator: Prof Grete Bertelsen, Centre for Advanced Food Studies (LMC-KVL), The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark, Tel. +45 3528 3212, Fax +45 3528 3190, E-mail: grb@kvl.dk

First circular
INTERNATIONAL CONGRESS
FLOUR-BREAD '03
4th Croatian Congress
of Cereal Technologists
November 19-22, 2003
Grand Hotel Adriatic,
Opatjia, Croatia

Programme

The international Congress will last 3 full days and include plenary lectures, short oral presentations, posters and exhibition of laboratory and process equipment, cereals industry products and publications.

The topics of the Congress are:

1. Cereal quality;
2. New approaches to analytical and rheological methods;
3. The process of flour production;
4. Baking;
5. Breakfast cereals and snacks;
6. Nutritional aspects of cereal;
7. Extrusion and pasta production;
8. Cookies and waffle production.

The official languages of the Congress will be English and Croatian. Simultaneous translation will be provided.

Call for papers

Contributions for oral and poster presentation are being invited. Send preferably by e-mail before 1 September 2003, one page abstract of not more than 200 words, indicating if you prefer an oral or poster presentation sent to Congress Secretariat.

Web Site

Full information of the "Flour-Bread 03" Congress can also be found on the web site of the Faculty of Food Technology, Osijek: http://www.ptfos.hr/brasno-kruh/.

Deadlines

July 15, 2003 - Registration.

September 1, 2003 - Abstract submission.

October 1, 2003 - Deadline for early registration.

October 1, 2003 - Acceptance notification for authors.

Congress Secretariat

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Symposium Announcement

27TH INTERNATIONAL SYMPOSIUM ON CAPILLARY CHROMATOGRAPHY
May 31 - June 4, 2004
Palazzo dei Congressi,
Riva del Garda, Italy

Information:

Central organisation:

Prof. P. Sandra

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Local organisation

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International Association for Food Protection

ANNOUNCES TWO WORKSHOPS AT IAFP 2003

August 8-9, 2003 - New Orleans, USA

The International Association for Food Protection announces two workshops to be held August 8-9 in conjunction with IAFP 2003 at the Hilton New Orleans Riverside in New Orleans, Louisiana.

Workshop I - Assuring Confidence in Laboratory Data

This workshop will present principals for understanding and implementing microbial control in a food production environment by providing skills to address limitations in your current laboratory testing and documentation. Participants will learn, in an interactive environment, how to perform effectively sound food and environmental sampling and microbial testing that can be implemented into their standard operating procedures and will conform to today's QA and ISO requirements. Participants will also review and discuss material from practical case studies and present their findings to the group in an informal presentation that will facilitate open discussion.

Workshop II - A Hands-on Course in Quantitative Microbial Risk Assessment

This workshop will cover fitting data to statistical distributions, creating and using predictive models in risk assessment, developing a process risk model, using sensitivity analysis, and testing proposed mitigations to reduce risk.

Over the course of the workshop, the participants will build an actual working quantitative microbial risk assessment in Excel using BestFit and @Risk software. Participants will build, run, interpret, and determine the impact of various changes to the model. Two-way risk model will be run to show the value of separating variability and uncertainty in quantitative risk assessment. Participants will also learn to determine whether additional data, better process control or a redesigned process will produce the greatest reduction in risk.

Detailed descriptions and registration information regarding these workshops may be obtained by visiting our Web site at www.foodprotection.org.

INTERNATIONAL FOOD SAFETY ICONS NOW AVAILABLE

The International Food Safety Icons are now available from the International Association for Food Protection for use in safe food handling. The Icons are simple pictorial representations of important food safety tasks that can be recognized and understood regardless of a person's native language.

Uses of the icons include, but are not limited to: food safety training materials, signs or reminders at food and beverage workstations, food preparation and storage equipment and on food packaging. The Icon series includes the critical concepts and contributing factors of foodborne disease: (1) refrigeration / cold holding; (2) handwashing; (3) cooking; (4) hot holding: (5) cooling; (6) wash, rinse, and sanitize; (7) cross contamination; (8) no bare hand contact: (9) temperature danger zone; (10) do not work if ill; and (11) potentially hazardous food.

Guidelines for use, descriptions of each Icon and the Icons themselves are available from the IAFP Web site at www.foodprotection.org. High quality images are available on a CD at $25.

GUIDE FOR AUTHORS

ITALIAN JOURNAL OF FOOD SCIENCE - IJFS

1. Manuscript Preparation

(1) Manuscripts must be typed, double-spaced and **two** copies submitted along **with** the computer disk. There should be liberal margins on top, bottom and sides (2.5 cm). English is the official language. Authors who are not fluent in written English should seek help from a fluent person before the final version is typed. The Assistant Editor reserves the right to make literary corrections and to make suggestions to improve brevity, but **the paper should be revised by a native English speaker** before submission.

The paper must also be submitted on a Macintosh or Windows floppy disk. Indicate which word processor was used to generate the file and save the file also in format "Text only", DCA-RTF or ASCII, if you do not have programs for Macintosh; graphics, pictures and diagrams must be saved in TIF, JPEG, EPS, CGM or PICT formats (not included in MsWord documents).

(2) Every paper should be divided under the following headings in this order:

Title. Informative of the content of the article (<50 characters + spaces). Author(s). Initials and Surname, omit professional and official titles. The Institute and address where the research was carried out and the current address of each author should be given as a footnote on the title page.

Abstract. Clearly state the objective of the study, give a concise description of experiment(s), observations, results and conclusions. No references should be cited. DO NOT EXCEED 100 WORDS. An abstract and title in Italian must also be included.

Keywords. Up to six words, in alphabetical order, which describe the document must be given to aid data retrieval and indexing.

Introduction. Review pertinent previous work and cite appropriate references. State the purpose of the investigation.

Materials and Methods. Indicate apparatus, instruments, reagents, etc., giving sufficient detail to allow the work to be repeated.

Results and Conclusions. Results and Conclusions may be presented together or separately. Concisely present results using tables and figures to help justify conclusions (do not present the same information in both forms). Use statistical analysis when appropriate. Unsupported hypotheses should be avoided. Conclusions should point out the significance of the findings and, if possible, relate the new findings to some problem in Food Science and Technology.

Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses, or other services performed for a fee. Financial support, thanks for assistance, article number or thesis fulfillment may be included.

Units. A list of units particular to the paper may be included.

References. References should be arranged alphabetically, and for the same author should be arranged consecutively by year, typed double-spaced. Each individual citation should begin flush left (no indentation). Refer to attached examples taken from "Style Guide for Research Papers" by the Institute of Food Technologists (Chicago - Illinois - USA). Literature citations in the text should be referred to by name and year in parentheses (only the initials in capital letters). If there are more than two authors, mention the first author and add et al.

(3) Lines on all pages, including those pages for "References" and figure legends, must be numbered (by pen) in the left margin, beginning with number one at the top of the page.

(4) Tables should be as few and as simple as possible and include only essential data. Each table must be on a separate sheet and saved on floppy disk, and have an Arabic number, e.g. Table 4 NOT Tab. 4. Legends must be self-explanatory and on a separate sheet. Use lower-case letters for footnotes in tables and explain below the table in the order in which they appear in the table.

(5) Figures must be drawn on separate sheets of paper and saved on floppy disk in TIF, JPEG, EPS, CGM or PICT formats. They should be drawn so that on 50% reduction, lines, figures and symbols will be clearly legible and not overcrowded. A photocopy of how the figure should appear must be included. Photographs must be unmounted, glossy prints or slides. All figures must be given Arabic numbers, e.g. Fig. 3, in the text and in the final copy only on the back where the title of the paper, the senior author's surname and the top of the illustration must also be marked; for reviewing procedures, do not include this information in the first submitted copies. Legends for figures must be self-explanatory and should be typed on a separate sheet under "Legends to Figures".

(6) Standard Usage, Abbreviations and Units. The Concise Oxford and Webster's English Dictionaries are the references for spelling and hyphenation. Statistics and measurements should always be given in figures, e.g. 10 min, except when the number begins a sentence. When the number does not refer to a unit of measurement it is spelled out unless it is 100 or greater. Abbreviations should be used sparingly, only when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. International Standard abbreviations should generally be used except where they conflict with current practice or are confusing. For example, 3 mm rather than 3x10-3m. Abbreviations should be defined the first time that they are used in the text and they should be used consistently thereafter. Temperatures should be expressed in the Celsius (centigrade) scale. Chemical formulae and solutions must specify the form used, e.g. anhydrous or hydrated, and the concentration must be in clearly defined units. Common species names should be followed by the Latin binomial (italics) at the first mention. For subsequent use, the generic name should be contracted to a single letter if it is unambiguous.

2. Review Policy

Scientific contributions in one of the following forms may be submitted:

Opinions and Reviews - Papers may be sent directly to the Editor-in-Chief who will decide upon publication or articles will be requested directly from the authors by the Editor-in-Chief.

Short Communications and Surveys - They do not need to have the formal organization of a research paper; they will receive priority in publication;

Papers - The paper must follow manuscript preparation.

Short Communications, Surveys and Papers will be subjected to critical review by the referees. Upon receiving papers from authors, the Advisory Board with the Editor-in-Chief will select papers in relationship to innovation and originality and send copies to the referees. A letter stating that the paper has been accepted for refereeing will be sent to the authors. Papers needing revision will be returned to the author, and the author must return the revised manuscript to the Editor-in-Chief within 1 month, otherwise the paper will be considered as withdrawn. Papers not suitable for publication will be returned to the author with a statement of reasons for rejection.

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Referees may not be from the same institution as the author. Referees should make their comments and questions in detail and return the paper to the Editor-in-Chief as soon as possible, usually within 4 weeks. The identity and the report of the referees are made known to the Editor-in-Chief, but only the anonymous report is routinely sent to the author. If all referees recommend acceptance or rejection, the decision stands. If the opinions of the referees tie, the Editor-in-Chief has the freedom to decide upon acceptance or rejection of the paper. Manuscripts will be edited in the order received and accepted papers will be published as closely as possible in this order. A letter announcing the issue of publication will be sent to the author after the manuscript has been accepted by the Editor-in-Chief. Each paper is accepted with the understanding that it is the sole document under active consideration for publication covering the work reported (it has NOT been previously published, accepted or submitted for publication elsewhere). Upon acceptance of the paper for publication, the author agrees to pay the page charges as published on the first page of each issue. Authors take full responsibility for all opinions stated in their papers and published in this journal.

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Papers for publication and communications regarding editorial matters should be sent to:
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REFERENCE EXAMPLES

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is NOT to be included in the reference list.

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